ROLE OF GLUTAMATE DEHYDROGENASE IN AMMONIA MANAGEMENT IN A FRESHWATER AIR-BREATHING TELEOST, *Heteropneustes fossilis*

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Ph. D. (B.H.U.)

January 28, 1991

Certified that the thesis entitled " **ROLE OF GLUTAMATE DEHYDROGENASE IN AMMONIA MANAGEMENT IN A FRESHWATER AIR-BREATHING TELEOST, *HETEROPNEUSTES FOSSILIS*** ", submitted by Miss Jharna rani Das for the degree of **DOCTOR OF PHILOSOPHY** in **ZOOLOGY** of North Eastern Hill University, Shillong embodies the record of original investigations carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph. D. Degree. This work has not been submitted for any degree of any University.

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SHILLONG

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LIST OF ABBREVIATIONS

Ab - Antibody
ADP - Adenosine 5'-diphosphate
Ag - Antigen
Ala - Alanine
ALT - Alanine transaminas
AMP - Adenosine 5'-monophosphate
ARG - Arginase
ASL - Arginino succinase
Asp - Aspartate
ASS - Arginino succinate synthetase
ATP - Adenosine 5'-triphosphate
α - Alpha
BLD - Below limit of detection
BSA - Bovine serum albumin
°C - Degree centigrade
C - Competitive inhibitor
Cm - Centimetre
CPS - Carbamyl phosphate synthetase
Cys - Cysteine
Cyt. oxi- Cytochrome oxidase
DEAE - Diethylaminoethyl
D - Dextro rotatory
E.C. - Enzyme Comission
EDTA - Ethylenediaminetetra-acetate
ETS - Electron transport system
FAA - Free amino acid
FCA - Complete freund's adjuvant
Fig - Figure
g gram
Xg - Centrifugal force
GDH - Glutamate dehydrogenase
GDP - Guanosine 5'-diphosphate
Glu - Glutamate
GOT - Glutamate oxaloacetate transaminase
GOGAT - Glutamate synthase
GS - Glutamine synthetase
GTP - Guanosine 5'-triphosphate
γ - Gamma
Hrs Hours
IMP - Inosine monophosphate
Ka - Activation constant
Kav - Distribution coefficient
KG - Ketoglutarate
Ki - Inhibition constant
Km - Michaelis-Menten's constant of enzyme for particular substrate
l - Litre

λ - Lemda (wave length)
L - Levorotatory
Lys - Lysine
LDH - Lactate dehydrogenase
M - Molar
mA - Milliampers
µl - Microlitre
µM - Micromolar
µmole - Micromole
mg - Miligram
ml - Millilitre
mM - Millimolar
min - Minute
Mw - Molecular weight
nm - Nenometer
NAD^+ - Nicotinamide adenine dinucleotide
NADH - Nicotinamide adenine dinucleotide, reduced form
$NADP^+$ - Nicotinamide adenine dinucleotide phosphate
NADPH - Nicotimanide adenine dinucleotide phosphate, reduced form
NBT - Nitroblue tetrazolium
NC - Non-competitive inhibitor
ND - Not determined
N.S. - Non-significant
O.D. - Optical density
OTC - Ornithine transcarbamylase
o-u - Ornithine urea
ω - Omega
p - Probability (Level of significance)
PAGE - Polyacrylamide gel electrophoresis
PCA - Perchloric acid
PMS - Phenazine methosulphate
Pi - Inorganic phosphate
% - Percentage
Rm - Relative mobility
S.A. - Specific activity
S.D. - Standard deviation
SDS - Sodium dodecyl sulphate
SDS-PAGE - Polyacrylamide gel electrophoresis in the presence of SDS
Sec - Second
T.A. - Total activity
TCA - Trichloreacetic acid
TEMED - N,N,N',N'-tetramethylethylenediamine
Tris - Tris(hjydroxy methyl)aminomethane
Try - Tryptophan
Tyr - Tyrosine
UC - Un-competitive inhibitor
uv - Ultra violet

v	- volume
v/v	- volume/volume
Ve	- Elution volume
Vmax	- Maximum rate
Vo	- Void volume
Vt	- Total volume of the packed gel
w/v	- weight/volume

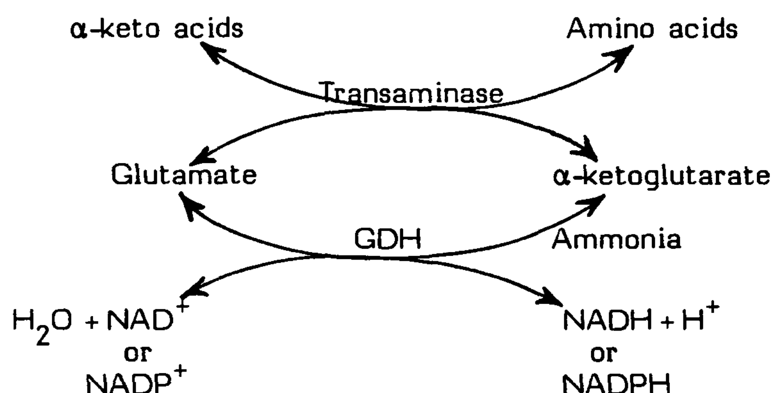
INTRODUCTION

Ammonia plays a central role in nitrogen metabolism in living organisms. It is both a product of catabolism and a precursor for the synthesis of many nitrogen containing biomolecules. It is, however, extremely toxic to animals if allowed to accumulate even at a very low concentration *in vivo*. Ammonia production, therefore, must be balanced by its excretion and/or conversion to some other less toxic compounds such as glutamate, glutamine, urea, uric acid etc. for temporary storage *in vivo* (Forster & Goldstein, 1969; Campbell *et al.*, 1972; Campbell, 1973; Watts & Watts, 1974; Hoar, 1984; Nener, 1988; Powers-Lee & Meister, 1988).

Ammoniogenesis

Ammonia production in animals has been shown by transamination and deamination of amino acids, amides, amines, purines, pyrimidines, nucleosides, nucleotides and hexosamines (Cohen & Brown, 1960; Meister, 1965; Campbell, 1973; Bishop, 1976; Kormanik & Cameron, 1981a,b; Cameron & Heisler, 1985; Evans, 1985; Evans & Cameron, 1986; Randall & Wright, 1987; Powers-Lee & Meister, 1988). The major pathway of ammoniogenesis in animals has been transdeamination of various amino acids (Forster & Goldstein, 1969; Krebs *et al.*, 1978; Walton & Cowey, 1982; Campbell *et al.*, 1983; Lehninger, 1987; Turner & Lushbangh, 1988; Prentø, 1989) besides deamination (Watts & Watts, 1974). Liver has been suggested as the primary site for ammonia production even though the necessary enzymes have also been located in some other tissues such as the kidney, gills and skeletal muscle (Goldstein & Forster,

1961; McBean et al., 1966; Walton & Cowey, 1977, 1982). Substantial amount of hepatic ammonia in fish arises from the oxidative deamination of glutamate catalyzed by glutamate dehydrogenase (GDH) (Pequin, 1962, 1967; Pequin & Serfaty, 1963, 1968; Janssens & Cohen, 1968; Forster & Goldstein, 1969; Wilson, 1973a; Vellas & Serfaty, 1974; van Waarde & Kesbeke, 1981a,b; Campbell et al., 1983; Casey et al., 1983; Chew & Ip, 1987). Glutamate is produced by transamination reaction between α-ketoglutarate and most amino acids released by hydrolysis of proteins (Hird & Marginson, 1966; Watts & Watts, 1974; Lehninger, 1987; Powers-Lee & Meister, 1988). The number, specificity and sub-cellular localization of various transaminases are still not fully understood. However, it has been clear since Schoenheimer (1942) that most amino acids can undergo reversible transamination. Campbell (1973) suggested that serine and threonine generally are not transaminated in animal tissues. These two hydroxy-amino acids are deaminated by specific dehydratases because of their initial action of removing water. Glutamate is also derived directly from the hydrolysis of protein and in the degradation of glutamine, proline and histidine (Salvatore et al., 1965; Janicki & Lingis, 1970). The overall reaction of liberation of ammonia from amino acids via glutamate, as presented below, is known as transdeamination (Braunstein, 1939).



Large amount of ammonia is also produced by deamination reactions (Meister, 1965). Ammonia may be cleaved from the amide group of glutamine, asparagine or the keto analogues of glutamine and asparagine catalyzed by the enzymes glutaminase, asparaginase and ω-amidase respectively. Glutamine and asparagine residues within proteins are also subjected to deamination. The utilization of glutamine via glutaminase (either phosphate dependent glutaminase-I; or phosphate independent glutaminase-II) to produce glutamate is a major source of ammonia in animals (van Slyke et al., 1943; Preuss, 1971; Curthoys & Lowry, 1973; Kalra & Bronsan, 1973, 1974; Joseph & McGivan, 1978; Haussinger & Sies, 1979; Campbell et al., 1983; Jahoor et al., 1988).

A flavin-dependent enzyme catalyzing oxidative deamination reaction, D-amino acid oxidase, acts on a number of D-amino acids and on the nonchiral amino acid glycine (Meister, 1965). This oxidase serves to degrade D-amino acids derived from exogenous sources, such as the diet and bacterial cell walls. Amino oxidase(s) deaminate a number of mono- and di-amines including epinephrine, norepinephrine and serotonin.

Deamination of nucleotides and their derivatives through the action of deaminases also produce substantial amount of ammonia *in vivo*. AMP-deaminase has been shown to be a key enzyme for ammoniogenesis (Braunstein, 1957; Lowenstein, 1972; McGivan & Chappell, 1975; Krebs et al., 1978). Its importance in ammonia production has been reported in teleosts in different tissues, (Makarewicz & Zydowo, 1962; Makarewicz, 1963, 1969; Dingle & Hines, 1967; Purzycka-Preis & Zydowo, 1969; Walton & Cowey, 1977; Chandrasena & Hird, 1978; Driedzic & Hochachka, 1978; Payan, 1978; Leray et al., 1979; van Waarde, 1981, 1983; van Waarde & Kesbeke, 1981a,b; van Waarde et al.,

1982; van Waarde & Dewilde- van Berge Hennegouwen, 1982). The quantitative importance of muscle ammoniogenesis to total ammonia excretion has been dependent on the activity level of the fish-increasing with increasing workload (Suyama et al., 1960; Fraser et al., 1966; Driedzic & Hochachka, 1976). The capacity for anaerobic ammonia production varies among different species (van den Thillart & Kesbeke, 1978; van den Thillart et al., 1980). Under aerobic conditions, most of the ammonia is produced in the liver of resting fish. However, during anoxia liver ammonia production is replaced by muscle proteolysis (Mathur, 1967; van Waarde et al., 1982; van Waarde & Dewilde-van Berge Hennegouwen, 1982). During exhaustive exercise deamination of adenylates in fish muscle becomes a major source of ammonia production (van den Thillart & Kesbeke, 1978) with most of the ammonia being utilized rather than excreted during the recovery period. van den Thillart et al. (1980) reported decrease in muscle adenylate pool and increase in IMP and NH_4^+. They suggested that the accumulated IMP was subsequently utilized in AMP synthesis in muscle.

Toxicity of ammonia:

Metabolic ammonia is released either in the form of ammonium ion (NH_4^+) or ammonia (NH_3). The later gets rapidly protonated at physiological pH (7 to 7.4) and approximately 99% of molecular ammonia in vivo exists in the protonated from (NH_4^+). The proportion of un-ionized ammonia increases with increasing pH and temperature (Emerson et al., 1975; Thurston et al., 1981; Hermanutz et al., 1987). Accumulation of ammonia is highly toxic to animals (Campbell, 1973; Evans & Cameron, 1986; Randall &Wright, 1987; Cooper & Plum, 1987; Nener, 1988). The deleterious effects of ammonia include, decrease in pH of body fluid and oxygen carrying capacity of haemoglobin (Sousa & Meade, 1977), and increased oxygen consumption, respiratory rate,

rate of heart beat (Smart, 1978) and urine output (Lloyd & Orn, 1969) in fish. It draws α-ketoglutarate from the TCA cycle as well as NADH available for oxidative metabolism (Campbell, 1973). Ammonia also interferes with the transport of various ions across membranes (Pressman, 1970; Campbell, 1973) affecting membrane potential and the excitability of neurons (Cooper & Plum, 1987). Consistent with such effects are the observed symptoms of hyper-ammonia in whole animals which include slowing of electroencephalograpm(EEG), seizures and coma (Cooper & Plum, 1987; Mialon et al., 1990) besides other neurological problems (Banister et al., 1976; Raabe & Lin, 1983, 1984, 1985; Raabe, 1989). Acute toxicity of un-ionized ammonia to mysids and larval inland silversides was influenced by pH and salinity in a species specific manner (Miller et al., 1990). Ammonia was most toxic at pH-7.0 and less toxic at pH-8.0 and 9.0 for mysids. In contrast, its toxicity to inland silversides was greatest at pH-7.0 and 9.0, and lowest at pH-8.0.

Utilization and detoxification of ammonia:

Ammonia has many advantages as an end product of nitrogen metabolism (Campbell, 1973). The conversion of protein nitrogen to ammonia operates primarily by deamination of glutamate via GDH to α-ketoglutarate and reduced pyridine nucleotide (NADH/NADPH). Both the products can be utilized for energy production through the TCA cycle or the electron transport system (ETS) respectively (Hochachka & Somero, 1973; Hillar, 1974; Smith et al., 1975; Eisenberg et al., 1976; Bassman & Pal, 1976; Bidigare & King, 1981; Campbell et al., 1983; Batrel & Gal, 1984; Teller, 1987). Owing to the relatively small molecular size, high solubility in water as free base and higher partition co-efficient, molecular ammonia (NH_3) is highly permeable through biological membranes compared to ammonium ion (NH_4^+) (Forster & Goldstein, 1969;

Castell & Moore, 1971; Klocke et al., 1972; Bown et al., 1975; Boron, 1980; Lockwood et al., 1980). NH_4^+ requires ion carriers for transport (Randall & Wright, 1987; Wright et al., 1988, 1989). The active transport of NH_4^+ is linked to the energetically favourable exchange of Na^+ across many cell membranes in freshwater fishes (Maetz & Garcia, 1964; Maetz, 1972; Kinsella & Aronson 1981; Wright & Wood, 1985; Evans & Cameron, 1986; Heming et al., 1986; Evans & More, 1988). Absorption of Na^+ has been critically important in maintaining salt and water balance in freshwater teleosts. Hence, in freshwater fishes the exchange of NH_4^+ for Na^+ serves the dual purpose of elimination of the nitrogenous waste product (NH_4^+) and absorption of Na^+ from the external freshwater environment.

Aquatic organisms rapidly dispose off ammonia to their ambient medium by diffusion across the gills and through body surface immediately after its formation to avoid its accumulation *in vivo* (Smith, 1929; Wood, 1958; Fromm & Gillette, 1968; Goldstein 1972; Vellas & Serfaty, 1974; Payan & Matty, 1975; Morii et al., 1978). Terrestrial organisms are unable to remove ammonia rapidly by diffusion due to limited availability of water. They manage this problem by converting toxic ammonia to urea. In those animals where conservation of metabolic water becomes highly essential due to unavailability of water in their environment ammonia was converted to insoluble uric acid (Campbell et al., 1972;Schmidt-Nielsen, 1972; Campbell, 1973; Hochachka & Somero, 1973; Nener, 1988; Powers-Lee & Meister, 1988). The animals have been classified depending on their major nitrogenous waste product into three following groups.

1. Ammoniotelic: Animals with ammonia as the major excretory product as in most of the aquatic animals where ammonia diffuses out into the available water in the ambient environment.

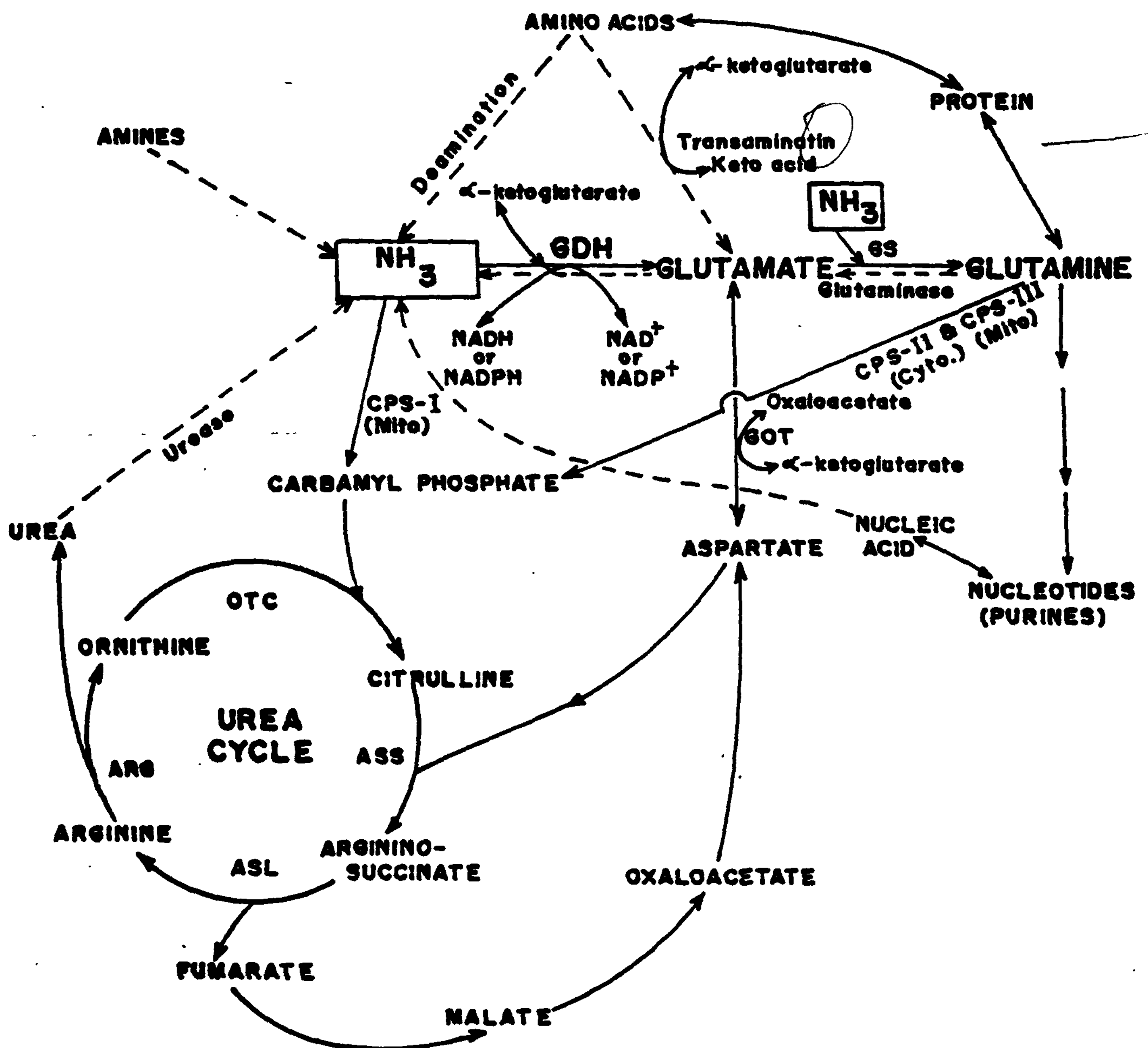
2. Ureotelic: Animals with urea as the major excretory product. Mammals and amphibians where water availability is limited, remove soluble urea in concentrated form in urine.

3. Uricotelic: Animals with insoluble uric acid as the major excretory product. Some insects, reptiles and birds where water availability is very much restricted ammonia is converted to insoluble uric acid to conserve metabolic water.

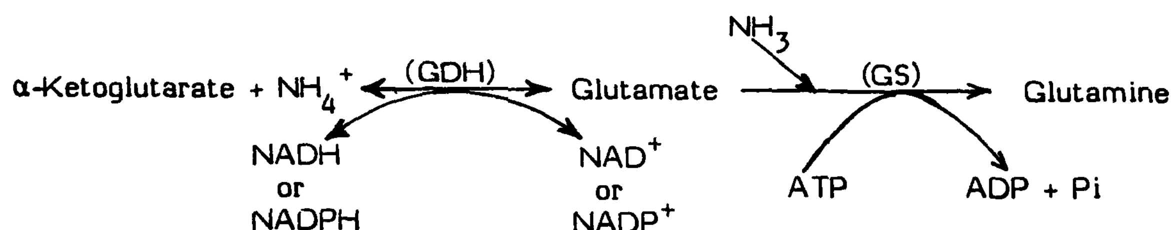
All the animals do not fall neatly into one category or another since some exhibit mixed patterns of nitrogen excretion. However, a particular type of nitrogenous waste predominates in a specific group of animals primarily depending on their environment. Amphibians can live both on land as well as in water. Their primary excretory product varies between ammonia and urea. They are ammoniotelic in water and ureotelic on land. The tadpole is ammoniotelic during early stages and ureotelic during later stages of development. Gordon (1970) suggested that nitrogen excretory pattern has been one of the most sensitive physiological processes to respond effectively to environmental variations.

Ammonia is also utilized as a precursor for the synthesis of many nitrogenous biomolecules *in vivo*. Thus, ammonia plays a central role in nitrogen metabolism even though it is extremely toxic to animals at very low concentrations.

The following chart can indicate the general pathways of synthesis (broken arrows) and utilization (solid arrows) of ammonia in animals.



GDH catalyzes the first step in ammonia utilization for biosynthesis by converting ammonia and α-ketoglutarate to glutamate besides playing a crucial role in ammonia detoxification at cellular level. Glutamate can accept another molecule of ammonia to form glutamine in the presence of the enzyme glutamine synthetase (GS) (Campbell, 1973; Smith et al.; 1975; McGivan & Chappell, 1975; Frieden, 1976; Krebs et al., 1978; Iwata et al., 1981; Benjamin, 1983; Duffy et al., 1983; Iwata & Kakuta, 1983; Schmidt & Schmidt, 1983; Berl & Clark, 1983; Cooper et al., 1985, Moyes et al., 1985; Iwata, 1988).



Glutamate dehydrogenase (GDH) is an allosteric enzyme very widely distributed in micro-organisms, plants and animals (Goldin & Frieden, 1971; Hillar, 1974; Smith et al., 1975; Eisenberg et al., 1976; Frieden, 1976; Storey et al., 1978a,b; Male & Storey, 1983; Fisher et al., 1986; Schmidt & Schmidt, 1988; Aguirre et al., 1989; Cioni & Strambini, 1989; Miret-Duvaux et al., 1990; Syed et al., 1990). The enzyme catalyzes the reversible reaction of oxidative deamination of glutamate to α-ketoglutarate and ammonia and the reductive amination of α-ketoglutarate with ammonia to glutamate in presence of pyrimidine nucleotide (NAD^+ or $NADP^+$) as oxidoreductive coenzyme. GDH may function as a reductive or oxidative enzyme depending on the substrate being utilized (Chamalaun & Tager, 1970; Goldin & Frieden, 1971; McGivan & Chappell, 1975; Tischler et al., 1977). However, in some cases GDH has greater affinity for either glutamate, α-ketoglutarate or ammonia

which influence the direction of the reaction. The enzyme is classified into three types on the basis of the coenzymes specificity as follows:

1. E.C. 1.4.1.2 - L-glutamate : NAD^+-oxidoreductase (deaminating)
2. E.C. 1.4.1.3 - L-glutamate: $NAD(P)^+$-oxidoreductase (deaminating)
3. E.C. 1.4.1.4 - L-glutamate: $NADP^+$-oxidoreductase (deaminating)

It has been suggested that NAD^+ dependent GDH plays a catabolic role in degradation of glutamate and formation of ammonia whereas $NADP^+$ dependent GDH serves as an anabolic function in synthesis of glutamate and utilization of ammonia (Holzer & Sneider, 1957; Sanwal & Lata, 1961; Lejohn et al., 1968; Ferguson & Sims, 1971; Tyler, 1978; Smith, 1980) in an organism.

1. NAD^+ dependent GDH (E.C. 1.4.1.2) activity predominantly occur in micro-organisms, plants and euryhaline invertebrates. It has been considered to be physiologically significant in glutamate catabolism with special reference to energy production and ammonia excretion (Campbell, 1973). The oxidative deamination function (NAD^+ dependent) was recognized in micro-organisms (Degani et al., 1974; Veronese et al., 1974a; Austen et al., 1977a,b,c; Hemmings 1978, 1980; Uno et al., 1984; van Lacre, 1988; Sállal & Nimer, 1990), plants (Hartmann et al., 1973; Nauen & Hartmann, 1980; Nagel & Hartmann, 1980; Ehmake et al., 1984), crustacea (Bidigare & King, 1981; Batrel & Regnault, 1985; King et al., 1985, 1987; Park et al., 1986; Regnault, 1989), molluscs (Storey et al., 1978b; Hayashi, 1987; Hoeger et al., 1987), annelids (Batrel & Gal, 1984), insects (Mills & Cochran, 1963; Bursell, 1975), in parasitic protozoa (Martin et al., 1976; Singh & Mohan Rao, 1983) and tapeworm (Mustafa et al., 1978).

2. NAD(P)$^+$ dependent GDH (E.C. 1.4.1.3) has been mainly reported in vertebrates catalyzing reversible reaction (Goldin & Frieden, 1971; Hillar, 1974; Smith et al., 1975; McGivan & Chappell, 1975; Eisenberg et al., 1976; Walton & Cowey, 1982; van Waarde, 1983; Plaitakis et al., 1984;Fisher, 1985; Chew & Ip, 1987; Randall & Wright, 1987; Aubby et al., 1988; Iwata, 1988; Schmidt & Schmidt, 1988; Cioni & Strambini, 1989; Lark & Colman, 1990). It has also been reported in some invertebrates (Reiss et al., 1977; Regnault & Batrel, 1987), micro-organisms and plants (Ehmke & Hartmann, 1978; Maulick & Ghosh, 1986; Yamamoto et al., 1987a,b; Saito et al., 1988).

3. NADP$^+$ dependent GDH (E.C. 1.4.1.4) - The reductive function (NADP$^+$ dependent) GDH was reported in micro-organisms helping in nitrogen fixation to synthesize glutamate (Blumenthal & Smith, 1975a,b; Neumann et al., 1976; Smith, 1980; Hernandez et al., 1983; Smits et al., 1984; Parker et al., 1985; Botton et al., 1987; Bascomb et al., 1987; Sokolov & Trotsenko, 1988; Bansal et al., 1989; Opden Camp et al., 1989). It has also been found in some protozoa (Shermann et al., 1971; Juan et al., 1978; Turner & Lushbangh, 1988). Many micro-organisms have been reported to possess both NAD$^+$ linked and NADP$^+$ linked GDH activity (Kato et al., 1962; Kapoor & Grover, 1970; Krämer, 1970; Kinghorn & Pateman, 1973; ldeToma & Laugidge, 1974; Peters & Sypherd, 1979; Janssen et al., 1980, Osmani & Scurtten, 1983; Kumar & Nicholas, 1984; Turner et al., 1986; Bischoff & Garrawy, 1987; Dudler et al., 1987).

GDH also plays a crucial role in animal tissues linking nitrogen and carbohydrate metabolic pathways (Frieden, 1971; Campbell, 1973; Smith et al., 1975; Dennis & Clark, 1977; Nicholas,1984; Schmidt & Schmidt, 1988).

It incorporates nitrogen in form of amino group in glutamate, neutralizing toxic ammonia *in vivo* which is also a common metabolite in a large number of reactions (Meister, 1965; Lehninger, 1987; Powers-Lee & Meister, 1988). The stored amino group is released as ammonia by GDH to meet the demand of either nitrogen, α-ketoglutarate or NADH/NADPH.

It has been known that incorporation of ammonia to glutamate also involved a combined system of glutamine synthetase (GS; E.C. 6.3.1.2) and glutamate synthase (GOGAT; E.C. 2.6.1.53). The L-glutamine obtained from GS activity transfers the second amino group to 2-oxoglutarate to form two molecules of glutamate in presence of GOGAT in higher plants (Miflin & Lee, 1976, 1977, 1980); algae (Cullimore & Smis, 1981a; Fayyaz-Chaudhary *et al.*, 1984, 1985) and bacteria (Meers *et al.*, 1970). When ammonia is available in excess, GDH serves as the main enzyme for ammonia assimilation in algae (Kates & Jones, 1964; Shatilov & Kretovich, 1977; Molin *et al.*, 1981; Tischner 1984; Bascomb *et al.*, 1986, 1987) and bacteria (Yamamoto *et al.*, 1984, 1987a,b), although activities of GS and GOGAT can be detectable. It has been reported that ammonia assimilation proceeds via GDH in several strains of *Bacillus* where GS and GOGAT activities could not be detected (Kimura *et al.*, 1977; Hemmila & Mäntsäla, 1978; White, 1979; van der Drift *et al.*, 1986; Opden Camp *et al.*, 1989).

GDH thus plays a role in variety of metabolic processes and many theories about its functions have been postulated. Braunstein and Kritzmann (1937) suggested that deamination of amino acids in which α-ketoglutarate acts as an acceptor for α-amino group by transamination produce glutamate. GDH functions in deamination in the resulting glutamate to ammonia. This general

process called transdeamination was later supported by others (Pequin & Serfaty, 1963; Janssens, 1964; Forster & Goldstein, 1969; Campbell, 1973; Wilson, 1973a; Walton & Cowey, 1977, 1982; Casey et al., 1983). On the other hand, Cohen (1966) proposed that in ureotelic animals GDH functions as an accessory enzyme for the urea cycle. It captures free ammonia which is subsequently converted to the second nitrogen-donor for urea synthesis in addition to ammonia i.e. aspartate by transamination between glutamate and oxaloacetate. This was later found in most ureotelic animals (Balinsky et al., 1970; Chamalaun & Tager, 1970; Krebs, 1976; Rognstand, 1977; Krebs et al., 1978). However, the importance of GDH in making ammonia available for ureogenesis has not been accepted by McGivan's group (McGivan et al., 1973, 1974; McGivan & Chappell, 1975; Chappell et al., 1976) and Jahoor et al., (1988).

Different theories of the metabolic role of GDH hinge on whether the enzyme works in the direction of glutamate oxidation, glutamate synthesis or in either directions. Thermodynamically the enzyme reaction favours reductive amination of α-ketoglutarate rather than oxidative deamination of glutamate. However, an equilibrium is considered to arise *in vivo* (Krebs & Veech, 1969) due to factors such as relative levels of nucleotides and removal of reaction products which favours glutamate oxidation.

Purification, molecular characterization and kinetics of GDH:

Glutamate dehydrogenases have been extracted and purified from a wide variety of sources such as bacteria, fungi, plants and numerous animal tissues. The enzymes differed in terms of their kinetics, metabolic and molecular properties.

GDH is a polymeric enzyme composed of four to eight subunits. Bovine liver enzyme is a hexamer with molecular weight 3,10,000-3,50,000 and subunit molecular weight of 53,000-57,000. Each subunit consists of 500 amino acid residues (Moon et al., 1972; Moon & Smith 1973; Eisenberg et al., 1976; Julliard & Smith, 1979). The molecular weight of rat liver GDH was 3,50,000 ± 20,000 and composed of six to eight polypetide chains, each of which had a molecular weight of 48,000 ± 5,000 (King & Frieden, 1970). The molecular weight and subunit pattern of frog, dogfish and tuna liver GDH were similar to that of the bovine liver GDH (Wiggert & Cohen, 1965; Corman et al., 1967; Veronese et al., 1976). Veronese et al. (1976) reported closer similarity in amino acid composition of GDH between mammals and tuna (teleost) than between mammals and dogfish (Chondricthyes). The $NADP^+$ dependent GDH of Neurospora crassa is also a hexamer with molecular weight 2,88,000 and subunit molecular weight of 48,800. Each subunit consists of 452 amino acid residues (Blumenthal & Smith 1973, 1975a,b; Wootton et al., 1974). The NAD^+ dependent GDH from micro-organisms composed of four subunits with molecular weight ranging between 48,500-1,12,000 (Lejohn et al., 1968; Veronese et al., 1974a,b; Haberland et al., 1980).

GDH from micro-organisms and plants are specific either for NAD^+ or $NADP^+$ for its activity. They are not markedly affected by purine nucleotides (Hooper et al., 1967; Lejohn & McCrea, 1968; Phibbs & Bernlohr, 1971; Bonete et al., 1986). On the other hand, GDH from animal sources can utilize both NAD(H) and NADP(H) and are strongly and specifically affected by purine nucleotides. In general, guanosine nucleotides strongly inhibit the enzyme, inosine nucleotide inhibit to a lesser extent and adenosine nucleotides (with exception of ATP) activate the reaction. The mammalian

hexameric GDH (Mw. 3,10,000-3,50,000) generally undergo a reversible polymerization to form polymers with molecular weight as high as 20,00,000 (Fisher et al., 1962; Eisenberg & Tomkins, 1968). In contrast to the mammalian enzymes which used NAD^+ or $NADP^+$ equally well, all the fish enzymes were more active with NAD^+ rather than $NADP^+$, and generally do not undergo a reversible polymerization reaction (Frieden, 1965; Goldin & Frieden, 1971; Hillar, 1974; Smith et al., 1975; Eisenberg et al., 1976; Schmidt & Schmidt, 1988).

The Km values differed markedly depending on the condition of the reaction and the sources. The vertebrate GDH, in general, showed lowest Km values for NADH and hignest for NH_4^+. On the other hand, in the microorganisms the GDH had Km value higher for glutamate than NH_4^+ (Frieden, 1965; Goldin & Frieden, 1971; Hillar 1974; Smith et al., 1975; Yamamoto et al., 1984; Saito et al., 1988; Opden Camp et al., 1989).

The facts mentioned above indicate that the structure, kinetics and regulations of GDH varies widely in different organisms primarily to meet the physiological demands. The role of GDH in glutamate production and, in the process, accelerating urea production have been shown in ureotelic animals. In ureo-osmotic marine fishes, GDH helps in production of glutamate which in turn synthesizes other free amino acids besides helping in urea synthesis for osmo-regulation. However, the freshwater teleosts which excrete ammonia by diffusion to ambient water medium, the role of GDH has been shown to be mainly in the process of ammoniogenesis. Some reports are also available to show induction of GDH for ammonia detoxification. Thus, GDH plays the role both in ammoniogenesis and in ammonia detoxification depending on the physiological need.

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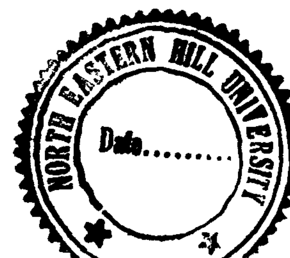


Freshwater teleosts were reported to be ammoniotelic and did not possess a complete ornithine-urea cycle (Brown & Cohen, 1960; Huggins et al., 1969; Wilson, 1973b).

However, reports from this laboratory have shown the presence of a complete o-u cycle in four out of five species of freshwater air-breathing teleosts (Saha & Ratha, 1987, 1989). Three species showed higher activity of all the enzymes in both liver and kidney. These fishes survived outside water for different periods of time varying from 8-12 hrs to 90-100 hrs (Saha & Ratha, 1989). The physiological level and rate of excretion of urea, and the activity of urea cycle enzymes were higher in the species capable of longer stay outside water. A direct correlation between ureogenesis and capability of water deprivation was indicated in these species. One of these species, Heteropneustes fossilis, showed a greater tolerance for ambient ammonia. Exposure to higher ambient ammonia caused accumulation of ammonia, the induction of the activity of o-u cycle enzymes, and accumulation and enhanced excretion of urea (Saha & Ratha, 1986; 1990). Induced ureogenesis under water deprivation and hyper-osmotic stress has also been reported (Saha, 1986). Chakravorty et al., (1989) reported a unique pattern of tissue distribution and sub-cellular localization of glutamine synthetase (GS) in H. fossilis which resembled those of uricotelic birds and reptiles and ureo-osmotic elasmobranchs. The activity of GS was induced and the kinetic of purified GS supported its active involvement in ammonia utilization/detoxification in H. fossilis during physiological ammonia load (Chakravorty, J. personal communication).

These findings suggest that the nitrogen metabolism and management of higher concentrations of ammonia *in vivo* are different in the ureogenic freshwater air-breathing teleosts than in the purely aquatic species. The o-u cycle may not be the only pathway to detoxify ammonia *in vivo*. GDH alongwith GS could play an important role in ammonia detoxification in this fish. Considering the importance of GDH in production, storage and detoxification of ammonia, the present study has been planned to find out its role in ammonia management in the ureogenic freshwater air-breathing teleost, *Heteropneustes fossilis*.

Plan of Work:

The work has been planned as follows.

1. Optimum assay condition, physiological level and sub-cellular distribution of GDH (NADH and NAD^+ dependent) activity were studied in various tissues such as, liver, kidney, brain, muscle and gill of *H. fossilis*.

2. The alterations in GDH (NADH and NAD^+ dependent) activity was studied during 24 hrs cycle and annual cycle in the above mentioned tissues.

3. The effect of temperature acclimation on GDH (NADH and NAD^+ dependent) activity was studied in Summer (June) and Winter (December) in all the five tissues of *H. fossilis*.

4. Effect of water deprivation on GDH (NADH and NAD^+ dependent) activity in above mentioned tissues was studied by keeping the fish out side water for different time intervals.

5. The alterations in the concentration of total free amino acids (FAA) and the GDH (NADH and NAD^+ dependent) activity in

above mentioned tissues of *H. fossilis* were studied during starvation, refeeding and hyper-ammonia stress.

6. GDH was purified from the liver of *H. fossilis* during Summer and Winter. The fold of purification and percent of activity recovered were determined.

7. The kinetic studies, molecular characterization and regulation of the activity of purified GDH by various modulators such as ions, amino acids, nucleotides etc. were studied.

8. Immunological studies were conducted with summer and winter purified hepatic GDH to find out any possible structural change in the enzymes purified in the two different seasons.

MATERIALS AND METHODS

Animals

Freshwater air-breathing teleost Heteropneustes fossilis (30-40g size) were purchased from commercial sources. They were maintained in the laboratory at 20±2°C in plastic aquaria containing bacteria free filtered tap water with 12hrs: 12hrs light and dark period. Minced pork liver (1% body weight) and rice bran (0.2% body weight) were supplied as food and water changed on alternate days. The fishes were used after their acclimatization to the laboratory conditions for at least four weeks when the death rate become zero and food consumption normal. They were used in experiment one day after last food was given. No food was given during experimental period and the experiments were conducted under the same environmental conditions as acclimatized unless otherwise mentioned. Each set of data was collected from a group of at least five fishes.

Rabbits used for immunological studies were purchased from commercial sources. They were also maintained in the laboratory under the above conditions of light and temperature and fed with germinated gram, grass and vegetable. They were used after their acclimatization to the laboratory conditions.

EXPERIMENTAL SET UP

Physiological level, circadian and seasonal variation:

Five female fishes of similar size were killed by decapitation at 10-11 A.M. in the month of October (1987) for finding out the physiological level of GDH.

The GDH activity during 24hr cycle was studied in the month of August, 1989 (Summer) and January, 1990 (Winter). Five fishes each time were sacrificed at 3hrs interval starting from 6 A.M. to the 6 A.M. of next day. The seasonal variation in GDH activity was studied from June, 1988 till May, 1989. Five fishes were sacrificed each time on 1st day of every month at 10-11 A.M. Different tissues such as liver, kidney, brain, muscle and gill were immediately removed, washed in ice-cold potassium phosphate buffer pH-7.6 containing 0.25M sucrose and blotted dry. The activity of GDH (both NADH and NAD^+ dependent) and protein concentration were determined in each tissue. Where estimations could not be completed immediately those tissues were deep frozen at -20°C. All estimations were completed within 3-5 days of collection of tissue during which enzyme activity did not change.

Effect of temperature acclimation:

The experiments were conducted in middle of June 1988 (Summer) and December 1988 (Winter). Fishes were divided into three groups each having five fishes in plastic jars containing bacteria free filtered tap water with streptopenicillin (20mg/l) to stop microbial growth. Two groups were exposed to the two experimental temperatures of 10±2°C and 30±2°C in light and temperature controlled incubators. The third group was kept under laboratory conditions which served as control. The fishes were killed after 10 days and the tissues were collected as mentioned above.

Effect of water deprivation:

Each fish was kept in a separate plastic jar without water and covered with bilayers of cheese cloth under the laboratory conditions. The humidity

around the fish was about 80% as determined by a hygrometer. Control fishes were maintained in usual water medium under the same laboratory conditions. The experiment started at 6 A.M. and five fishes from each experimental set up and control were killed after different time intervals such as 1/2, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, and 36 hrs and the five tissues mentioned above were collected.

Effect of starvation,refeeding and ambient hyper-ammonia stress:

Fishes were divided into three experimental groups and the experiments were conducted upto 14 days. The fishes were maintained in plastic buckets containing 4 l of either bacteria free filtered tap water or NH_4Cl solution with streptopenicillin (20mg/l) to stop microbial growth. Group-I was the normal control with the fishes being fed and water changed on alternate days. Group-II fishes were kept without food with water changed on alternate days upto 7 days. After 7 days half of these fishes were continued to starve upto 14 days to serve as starved control and the other half were provided with food on alternate days like normal controls to find out the effect of starvation and refeeding. Group-III fishes were exposed to different concentrations of NH_4Cl (50, 75 and 100mM) for 14 days without food. The medium was changed on alternate days. Five fishes from each experimental set up were killed after 3, 7 and 14 days of experimentation and the above mentioned tissues were collected.

TISSUE PROCESSING

A 10% (w/v) homogenate of each fresh tissue or frozen tissue thawed on ice was prepared in potassium phosphate buffer pH-7.6 containing 0.32M

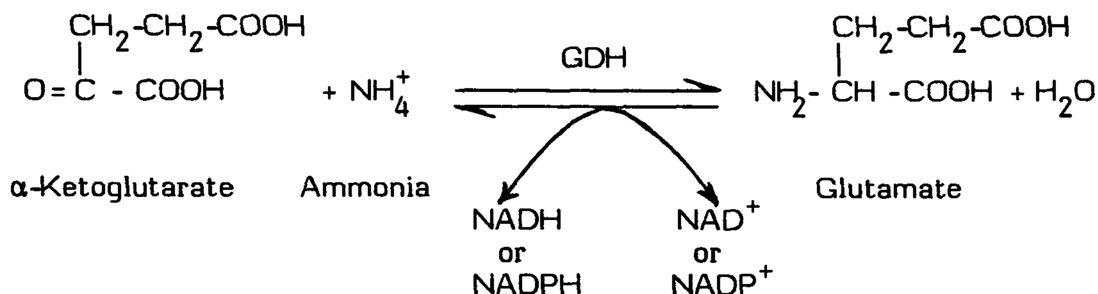
sucrose and 1mM EDTA using a Potter-Elvehjem type motor driven homogenizer fitted with a teflon pestle at 0±2°C. The homogenates were centrifuged at 600xg at 0±2°C for 30 min to remove the nuclei and cell debris. The supernatants were treated with Triton X-100 (0.5%) (v/v) for 60 min before use for GDH (NADH and NAD^+ dependent) assay and protein estimation. This treatment was found to release maximum GDH activity.

Different sub-cellular fractions such as - nuclear, mitochondrial and cytoplasmic were separated by differential centrifugation of the homogenates following the method of Johnson and Lardy (1967). The pellet of centrifugation at 600xg for 30min was resuspended in homogenizing medium and recentrifuged at 600xg for 30min. This pellet was called as nuclear fraction. The two above supernatants were pooled and centrifuged at 14,000xg for 60 min. The pellet was called mitochondrial fraction and supernatant as cytoplasmic fraction. The nuclear and mitochondrial fractions were suspended in the homogenizing medium. The sub-cellular fractions were treated with (0.5%) Triton X-100 and the enzyme activity assayed after 60 min. All these operations were done at 2±2°C, unless otherwise mentioned.

ESTIMATIONS

Enzyme assays:

Glutamate dehydrogenase (GDH): GDH activity both NADH dependent (reductive amination) and NAD^+ dependent (oxidative deamination) were assayed following the method of Olson and Anfinsen (1952) with necessary modifications in the concentrations of buffer and substrates.



The reaction mixture of 3.0ml for reductive amination reaction contained the following.

Potassium phosphate buffer (pH-7.6)	147.0 µ moles
Ammonium chloride	450.0µ moles
α -Ketoglutarate	14.0 µ moles
NADH/NADPH	0.6 µ moles
Enzyme extract	0.02 ml

The reaction mixture of 3.0ml for oxidative deamination reaction contained the following.

Potassium phosphate buffer (pH-8.5)	202.5µ moles
L-glutamate	50.0µ moles
$NAD^+/NADP^+$	6.0 µ moles
ADP	6.0 µ moles
Enzyme extract	0.05 ml

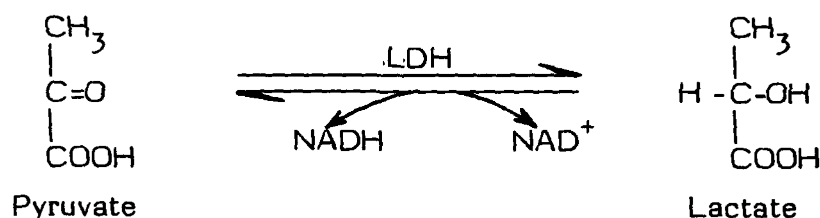
The assay mixture except coenzyme and one of the substrates was incubated in rectangular quartz cuvette (both experimental and reference having 1cm light path) at 30°C for 5 min in a uv-visible Spectrophotometer

(Beckman Model-26) fitted with a peltier type temperature control unit. After adjusting the optical density (O.D.) to zero, required amount of coenzyme and substrate were added to the experimental cuvette. The decrease or increase in O.D. at 340nm depending on the reductive amination or oxidative deamination respectively was recorded at 30 Sec intervals. The period of linear decrease or increase in O.D. was used for calculation of enzyme activity. The amount of coenzyme utilized per min was calculated taking 6.22×10^3 as molar extinction coefficient for NADH.

One unit of enzyme activity was defined as that amount which oxidized 1 µ mole of NADH (reductive amination) or reduced 1µ mole of NAD^+ (oxidative deamination) per min at 30°C.

Enzyme activity was expressed both as tissue activity- units per g wet wt. of tissue and specific activity units per mg protein.

Lactate dehydrogenase (LDH) [E.C. 1.1.1.27]:- LDH activity was assayed following the method of Markert and Masui (1969) with suitable modification in the concentration of the substrate.



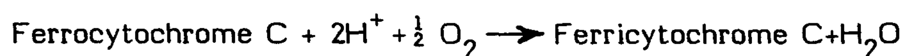
The reaction mixture of 3.0 ml contained the following

Potassium phosphate buffer (pH-7.0)	120.0 µ moles
Sodium pyruvate	1.0 µ mole
NADH	0.5 µ mole
Enzyme extract	0.005 ml

The enzyme activity was assayed and calculated like GDH (NADH dependent) reaction from decrease in O.D. at 340nm at 25°C.

One unit of enzyme was defined as that amount which oxidized 1 µ mole of NADH per min at 25°C.

Cytochrome oxidase (Cyt. Oxi) [E.C. 1.9.3.1.]:- Cytochrome oxidase activity was assayed as described by Wharton and Tzagoloff (1967).



$$\text{Ferrocytochrome C} + 2H^{+} + \tfrac{1}{2}O_2 \longrightarrow \text{Ferricytochrome C} + H_2O$$

The reaction mixture of 1.0 ml contained the following.

Potassium phosphate buffer (pH-7.0)	10.0 mM
Ferrocytochrome C	0.07 ml
Enzyme extract	0.02 ml

Ferrocytochrome C was prepared by dissolving 1% cytochrome C Type-III in 10mM potassium phosphate buffer pH-7.0. The solution was reduced with excess of potassium ascorbate. Excess ascorbate was removed by dialysis against same buffer for 18-24hrs with three changes of buffer.

The oxidation of reduced cytochrome C was measured as a decrease in O.D. at 550nm at 25°C in a uv-visible Spectrophotometer against the reagent blank oxidized with 1mM potassium ferricyanide.

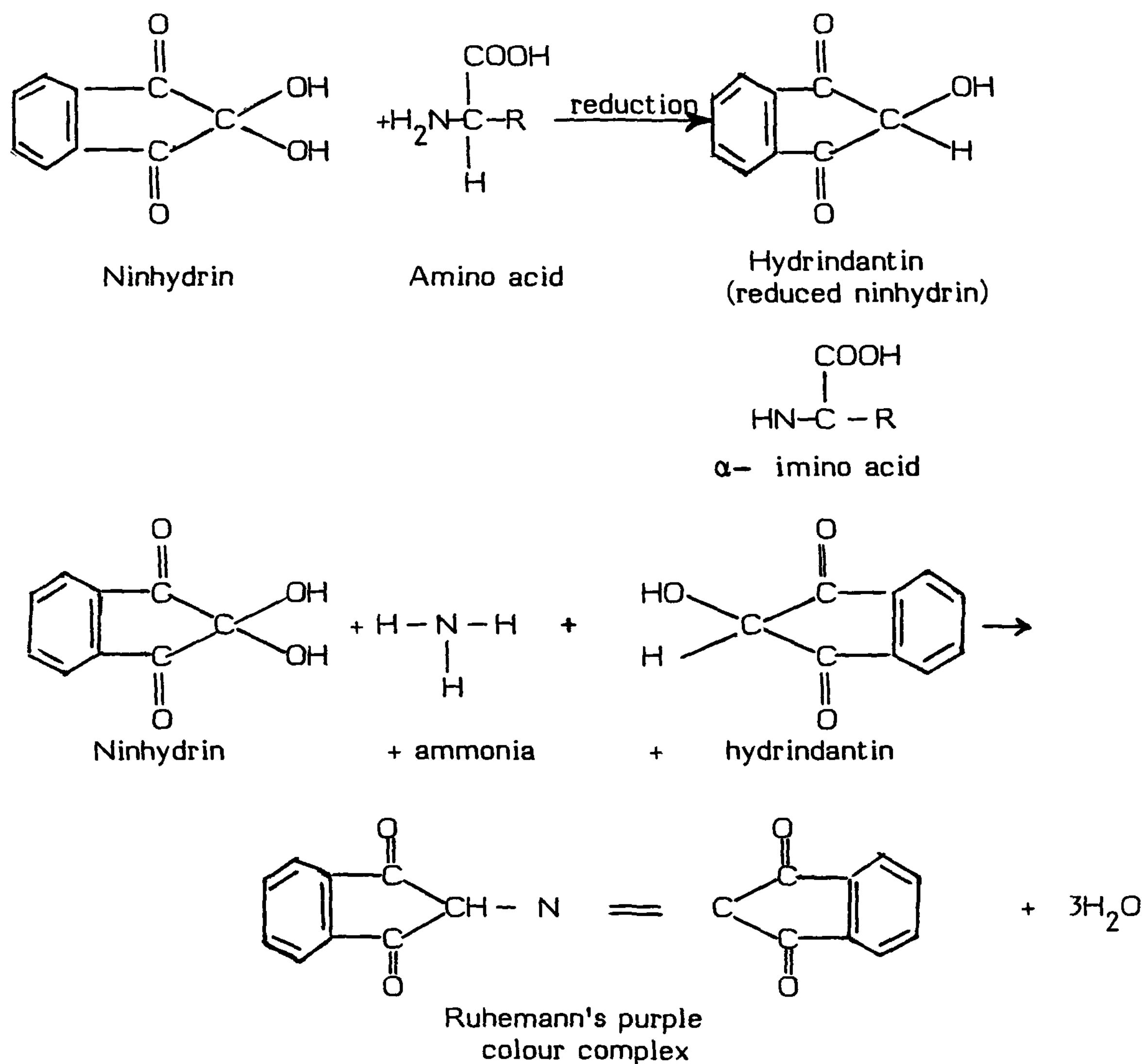
One unit of enzyme was defined as the amount which oxidized 1µ mole of reduced cytochrome C per min at 25°C. The molar extinction coefficient for cytochrome C $\Delta E_{(\text{reduced} \rightarrow \text{oxidized})}$ at 550nm as 19.6 $mM^{-1}Cm^{-1}$ was used to calculate cytochrome C oxidase activity.

Protein estimation:

Protein was estimated following the method of Lowry et al. (1951) using crystalline bovine serum albumin (BSA) as the standard.

Total free amino acid (FAA) estimation:

A portion of 10% homogenate was deproteinized with cold 5% PCA (v/v) in 1:1 ratio. The precipitated protein was separated by centrifugation at 5,000xg for 10min. The supernatant was used for the estimation of total FAA after neutralization with 0.1N NaOH. The estimation of total FAA was done following the method described by Spies (1957) using ninhydrin reagent.



To 0.05ml of neutralized and suitably diluted supernatant 1.0 ml of 2% ninhydrin reagent prepared in citrate buffer (100mM, pH-5.0) was added. The contents were mixed well, heated in a boiling water bath for 15 min and cooled at room temperature. 5.0ml of diluent (n-propanol and water mixed in the ratio of 1:1) was added and mixed thoroughly. The O.D. was measured at 570nm in a Spectrophotometer against reagent blank. The concentration of total FAA was determined using a linear standard graph prepared using glycine and expressed as μ mole per g wet wt. of tissue.

Purification of Hepatic GDH:

The purification of GDH from liver of H. fossilis was done in May, 1989 (Summer) and December, 1989 (Winter) due to the observed seasonal variation in the enzyme activity. The purification protocol was developed by modifications of various methods used earlier (Corman et al., 1967; King & Frieden, 1970; Hayashi & Ooshiro, 1977a; Julliard & dePaulet, 1978; Chee et al., 1978, 1979; McCarthy et al., 1980; Hayashi et al., 1982; Colon et al., 1986). The details of the protocol used have been described below. All operations were carried out at 0±2°C and in 50mM potassium phosphate buffer pH-7.6, unless otherwise mentioned.

Step-1 - Crude extract:- The fishes were killed irrespective of sex and weight in May and December, 1989 by decapitation and the liver was quickly removed, washed in buffer and kept deep frozen at - 20±2°C. A 10% (w/v) homogenate was prepared from 3-5g of liver tissue in buffer containing 0.32M sucrose and 1mM EDTA (Solution-A) using a Potter-Elvehjem type motor driven

homogenizer fitted with a teflon pestle. Centrifugation of the homogenate for 30min at 600xg yielded a low speed pellet which was resuspended in solution-A and recentrifuged as mentioned above. The first and second low-speed supernatants were pooled together and centrifuged at 14,000xg for 60min. The supernatant contained soluble GDH activity and the pellet contained the mitochondrial GDH which was the predominant form. The pellet was suspended in minimum volume of solution-A and mixed with an equal volume of TritonX-100 (1.0%). After 60min it was centrifuged for 30min at 14,000xg. The supernatant containing released mitochondrial GDH activity was called the crude extract and was used for further purification steps.

Step-2 - Ammonium sulphate fractionation:- Ammonium sulphate crystals were added without pH adjustment to the crude extract gradually with stirring to 20% saturation (114g/l). The mixture was centrifuged at 15,000xg for 30min. The pellet was discarded and the supernatant was adjusted to 50% saturation (189g/l) of ammonium sulphate with stirring for 2hrs. The mixture was then centrifuged at 15,000xg for 60min and the precipitate with the enzyme activity was collected. The precipitate was resuspended in phosphate buffer in a ratio of 10 ml/g tissue.

This solution was dialysed for 12hrs against the same buffer with at least three-four changes of the buffer. The dialysed solution was loaded to a previously equilibrated SephadexG-25 Column (24x2.5cm) for complete removal of the ions and eluted with the same buffer with a flow rate of 18-20ml/hr. 5.0ml fractions were collected in a Pharmacia Chromatography System (FRAC-100-Fraction Collector connected with a UV detecter & recorder). GDH activity was eluted in the void volume.

Step-3 - Ion Exchange Chromatography on DEAE - cellulose:- The enzyme activity obtained in void volume from SephadexG-25 column was directly applied on to DEAE-cellulose (anion exchanger) column (34x2.5cm) precycled and equilibrated with phosphate buffer. The unbound proteins were eluted out with buffer washing. The bound proteins were eluted with a linear NaCl gradient using a gradient mixer and with a flow rate of 12-15ml/hr. 5.0ml fractions were collected using a Pharmacia Chromatography System. The GDH activity was eluted from the column between 0.12 to 0.2M NaCl concentration. The fractions with high GDH activity were pooled and dialysed for 4-5hrs against phosphate buffer to remove NaCl.

Step-4 - Ion Exchange Chromatography on DEAE-Sephacel:- The dialysed sample obtained from Step-3 was loaded on to a DEAE-Sephacel (35x2.5cm) column equilibrated with phosphate buffer. No pre-cycling was required since DEAE-Sephacel was supplied pre-swollen. Unbound protein was washed out with starting buffer and the column was eluted with linear gradient of NaCl prepared in phosphate buffer with a flow rate of 14-16ml/hr. 5.0ml fractions were collected as mentioned above. The GDH activity was eluted between 0.1 and 0.19M NaCl. The fractions containing high GDH activity were pooled and dialysed for 4-5hrs against phosphate buffer to remove NaCl.

Step-5. Affinity Chromatography on Blue Sepharose:-The dialysed sample obtained from Step-4 was loaded on to a Blue Sepharose CL-6B (10x1.0cm) column previously washed and equilibrated with phosphate buffer at a flow rate of 8-10ml/hr. 5.0ml fractions were collected as mentioned above. The column was washed with the equilibrating buffer till it was free of the unbound proteins.

All the unwanted proteins came out with the equilibrating buffer except the dehydrogenases which were bound to Cibacron Blue F3GA dye in the column. The GDH was eluted by 1mM NADH in same buffer. The fractions containing high GDH activity were pooled and directly loaded on a Sephadex G-25 column previously equilibrated and eluted with phosphate buffer for removal of NADH. All the GDH activity was eluted in the void volume. The fractions with high activity were pooled together and the fold of purification and % recovery of activity calculated. This fraction was used for kinetic, regulatory, immunological and molecular studies. A portion of the pooled purified GDH was lyophilized and stored for further use.

Polyacrylamide gel electrophoresis (PAGE): Polyacrylamide gel electrophoresis of different tissue homogenates and different purification fractions were carried out according to Hames (1981) using 7.5% polyacrylamide gels (10x0.6cm). The ratio between acrylamide to N,N'-methylene bisacrylamide was 30:0.8. Anodic electrophoresis was carried out in an electrophoretic chamber at 0-4°C using 50mM potassium phosphate buffer (pH-8.0) at a constant current of 3-4mA per tube. Pre-run of the gel for 30min was done before loading (50-100 µl) of the sample containing 5-7 units of enzyme activity on each gel with 10% glycerol. The gels, after loading the sample, were run for 7-8hrs for different tissue homogenates and 14-16hrs for purification fractions. Duplicate gels were run for simultaneous protein and enzyme staining. At the end of the electrophoresis the gels were removed from the glass tubes using water jet. A set of gels were stained with specific stain for GDH (NAD^+ dependent) activity and the duplicate set for protein stain (Coomassie brillant blue) to detect the GDH and protein bands.

GDH specific staining:

Specific staining for GDH was carried out following the methods of Vander Helm (1962). The concentration of various components in the staining mixture was 25mM L-glutamate, 0.3mM NAD^+, 1mM ADP, 50mM potassium phosphate buffer (pH-8.5), 0.3mM NBT; and 0.08mM PMS. GDH activity on the gel was visualized as deep brown bands of reduced tetrazolium salt by incubating for 30min in the staining mixture at 37°C in dark. The gels ware rinsed in distilled water for several times after staining and photographed. The gels were preserved in distilled water.

Protein staining:- The proteins were fixed in the gel with 50% TCA for 30min. The staining was done as described by Hames (1981) with 0.1% Coomassie brillant blue prepared freshly in glacial acetic acid: methanol: water in the ratio of 1:6:8 by volume. The ratio of glacial acetic acid: methanol: water was modified to facilitate better destaining. The excess stain was removed by repeated washing with a mixture of glacial acetic acid: methanol: water prepared in the ratio of 3:2:35 by volume. Fixing, staining and destaining were carried out at room temperature. The gels were rinsed in distilled water, photographed and preserved in distilled water.

Absorption spectra of pure GDH:- The absorption spectra of the purified enzyme along with its various substrates for both NADH and NAD^+ dependent reactions were recorded between 200-400nm using a Jasco UV-visible (Model UVIDEC-610) recording Spectrophotometer.

Temperature optima and thermal stability:- The GDH activity was assayed at different temperatures such as, 25, 30, 37, 40, 45, 50, 55 and 60°C to

determine the temperature optima. A graph was plotted of enzyme activity against assay temperature.

The enzyme was pre-incubated at different temperature such as 30, 37, 40, 45, 50 and 55°C. GDH activity was assayed taking sample at different time intervals such as 0, 5, 10, 15, 20, 25, 30, 40 and 50min of incubation to find out the thermal stability of the GDH activity (both NADH and NAD^+ dependent). The alteration in the activity with relation to temperature and time was plotted on a graph.

In another set of experiment, thermal stability of GDH was studied at fixed temperature in presence of substrate and a modulator. The enzyme was incubated in three separate tubes — one with only phosphate buffer, another with one substrate and the third one with one substrate and ADP as a modulator. The incubation was done at 40°C for reductive amination and 45°C for oxidative deamination reaction. Aliquots for each were taken at different time intervals such as 5, 10, 15, 20, 25, 30, 40 and 50min and the enzyme activity assayed. The alteration in the activity was plotted against time on a graph to find out the role of the substrate and modulator on the thermal stability of pure enzyme.

pH optima:- GDH activity of the pure enzyme was assayed in potassium phosphate buffer with pH ranging from 6.5 to 11.0 [higher pH (9.0-11.0) was adjsuted with KOH]. The enzyme activity was plotted on a graph against the pH to find out the optimum pH for GDH activity.

Kinetic studies:- The Km and Vmax of the purified GDH for various substrates of the reverse reaction were determined by assaying the enzyme activity

at varying substrate concentrations. The concentration of α-ketoglutarate was varied between 0.1 and 6.0mM, ammonium chloride between 5 and 250mM, L-glutamate between 0.5 and 25mM, NADH between 0.01 and 0.35mM and NAD^+ between 0.15 and 3mM. Values of Km and Vmax were determined from Michaelis-Menten and Lineweaver-Burk plot of the data.

Products inhibition of GDH:- The assays were performed as mentioned above with several fixed concentrations of the inhibitor. Lineweaver-Burk plot of the data was prepared for determining the nature of inhibition. The Ki of the inhibitors were determined by Dixon plot and Ka was determined by Hill plot of the data.

The effect of nucleotides, amino acids, biogenic amines and various ions were studied. The enzyme assays were performed by pre-incubating the enzyme with different modulators at 30°C for 5min. Tris-HCl buffer was used instead of potassium phosphate buffer having same ionic strength and pH for various ions because some of the ions tested precipitated in phosphate buffer. The residual activity was calculated to find out the effect of individual effector studied.

Molecular weight determination:- *The molecular weight of the native enzyme* and its subunits were determined by using both exclusion chromatography (gel filtration) and PAGE both with and without SDS.

(i) Exclusion chromatography:- The molecular weight of the native enzyme by exclusion chromatography was determined following the method of Andrews (1970) using a column (40x2.5cm) of Sepharose 4B-200. The column was

equilibrated and eluted with 50mM potassium phosphate buffer pH-7.4 with a flow rate of 8-10ml/hr. 5.0ml fractions were collected using a Pharmacia Chromatography System. The column was calibrated by separate runs with the following marker proteins (2-4 mg/run in phosphate buffer) as standards: thyroglobulin (Mw. 6,69,000), ferritin (Mw. 4,40,000), catalase (Mw. 2,32,000) and aldolase (Mw. 1,58,000). Blue Dextran-2000 was used for void volume determination. Purified GDH 2-4mg in 2-4ml was also eluted through the column in the same manner. The apparent molecular weight of the purified GDH was interpolated from a plot of log molecular weight versus Kav for standard proteins and GDH. The value of Kav was calculated using the formula,

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

[Where Ve = elution volume,
Vo = void volume,
Vt = total volume of the packed bed]

The subunit molecular weight was determined by exclusion chromatography on Sepharose CL-6B column (40x2.5cm). The column was equilibrated and eluted with potassium phosphate buffer pH-7.4 containing 8M urea, 0.01M EDTA and 0.1M 2-mercaptoethanol at 25±2°C. The GDH and standard proteins were dissolved in above solution to make 1mg/ml to keep the subunits in separate form. 2-4ml of each sample was loaded on the column and the chromatography was done with a flow rate 6-8ml/hr. Blue Dextran-2000 was used for void volume determination. The column was calibrated by separate runs with the following marker proteins as standards: carbonic anhydrase (Mw. 29,000), ovalbumin (Mw. 43,000), catalase (subunit Mw. 57,500) and BSA (Mw. 67,000). Purified GDH subunit prepared as mentioned above were also eluted from the column in the same manner. The apparent subunit molecular

weight of purified GDH was determined from a graph plotted between log molecular weight and Kav for standard proteins and GDH subunits.

(ii) PAGE - The native enzyme molecular weight and subunit molecular weight were also determined by PAGE following the method of Laemmli (1970) discontinuous buffer system with some modifications in gel concentration in presence and absence of anionic detergent SDS. The composition of the gels used is given below.

Stock solution	Without SDS (Native enzyme)		With SDS (Subunit)	
	Stacking gel 3% (ml)	Resolving gel 6.5% (ml)	Stacking gel 5% (ml)	Resolving gel 10% (ml)
Acrylamide-bisacrylamide (30:0.8)	3.0	6.5	5.0	10.0
Stacking gel buffer[a]	3.75	-	3.75	-
Resolving gel buffer[b]	-	3.75	-	3.75
10% SDS	-	-	0.3	0.3
1.5% ammonium per sulphate	1.5	1.5	1.5	1.5
Water	21.75	18.25	19.45	14.45
TEMED	0.015	0.015	0.015	0.015

Final buffer concentration and pH:

(a) Stacking gel buffer - 125mM Tris-HCl, pH-6.8

(b) Resolving gel buffer-375mM Tris-HCl, pH-8.8

(c) Reservoir buffer- (i) Without SDS (native enzyme) - 25mM Tris and 192mM glycine, pH-8.3.

(ii) With SDS(subunit)-25mM Tris and 192mM glycine, pH-8.3 containing 1% SDS.

Preparation of standard protein solution and GDH sample for SDS-PAGE:- The enzyme sample was dialysed against 62.5mM Tris-HCl buffer pH-6.8 to remove potassium ions which would have otherwise precipitated with SDS. The standard proteins were dissolved in the same buffer at a concentration of 1mg/ml. The appropriate volume of standard and sample (GDH) proteins were then brought to a final concentration of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue and heated in a boiling water bath for 3min. About 15-20μg protein was loaded on each gel.

Anodic electrophoresis was carried out with a constant current of 3mA per tube for 6-7 hrs. The proteins were fixed, stained and destained as mentioned earlier.

The apparent molecular weight of native purified GDH and subunit was estimated from the calibration curve prepared by plotting log molecular weight versus Rm (relative mobility compared to bromophenol blue) for proteins of known molecular weight and GDH. The standard proteins used were: thyroglobulin (Mw. 6,69,000), urease (hexamer MW. 5,45,000, trimer Mw. 2,72,000), ferritin (Mw. 4,40,000), catalase (Mw. 2,32,000), aldolase (Mw. 1,58,000), BSA (dimer Mw. 1,32,000, monomer Mw. 66,000) and GDH (bovine liver from Sigma, Mw. 3,10,000-3,50,000) as reported (Olson & Anfinsen, 1952; Fisher et al., 1962; Frieden, 1965; Eisenberg & Tomkins, 1968; Eisenberg et al., 1976; McCarthy et al., 1980; Fisher, 1985).

The standard proteins used for subunit molecular weight were; BSA (Mw. 67,000), catalase (Mw. 57,000), ovalbumin (Mw. 43,000), aldolase (Mw. 38,994), carbonic anhydrase (Mw. 29,000), chymotrypsinogen A

(Mw. 25,000), ribonuclease A (Mw. 13,700) and GDH (bovine liver from Sigma, Mw. 53,000-57,000) as reported (Olson & Anfinsen, 1952; Fisher et al., 1962; Eisenberg & Tomkins, 1968; Weber & Osborn, 1969; Eisenberg & Reisler, 1970; Moon & Smith, 1973; Eisenberg et al.,1976; McCarthy et al.,1980; Fisher, 1985).

IMMUNOLOGICAL STUDIES

Preparation of antiserum:- The rabbits were immunized with antigen i.e. summer purified and winter purified GDH from liver of *H. fossilis* by multiple injections. Each rabbit was injected with 1ml of protein antigen (GDH) solution containing 0.25mg protein in100mM potassium phosphate buffer pH-7.4 containing 145mM NaCl (buffered saline) after it had been emulsified with an equal volume of FCA. The emulsification of FCA with protein solution was done by pumping in and out of the constituents in a 5.0ml glass hypodermic syringe fitted with a 20 gauge needle until a thick white emulsion was formed. The emulsion was injected (0.5ml at each site) sub-cutaneously between the shoulder blades and above the sacrum (between the hips) of the rabbit.

A second (first booster) injection of protein antigen was administered on 30th day with the emulsion prepared with incomplete Freund's adjuvant instead of complete Freund's adjuvant.

A third (second booster) injection of protein antigen was administered on 45th day with aluminium hydroxide gel (alum-precipitate). 0.5ml of the protein solution containing 2mg/ml of protein was mixed with twice its volume (1ml) of aluminium hydroxide gel. Phosphate buffered saline was added (0.5ml) to make it 2.0ml solution. The mixture was slowly stirred and kept for 3-4hrs

at room temperature. As a result, the protein was adsorbed on to the resulting aluminium hydroxide precipitate. The mixture was injected (0.5ml at each site) intramuscularly into the legs of the rabbit.

The rabbits were bled after 7 days of third (second booster) injection. The ear was gently rubbed and a longitudinal nick was made in the marginal ear vein. Venous blood dripping from the nick was collected in a centrifuge tube. The blood clot was stirred gently to prevent it from adhering to the wall of the tube and then left overnight at 0-4°C. Precaution was taken to prevent haemolysis. Next day the straw coloured serum was pipetted out and centrifuged for 15min at 1,500xg. The supernatant (antiserum) was stored at -20°C until used as antibody source.

Immunodiffusion:

Immunodiffusion was carried out following the method of Ouchterlony (1959) in agarose gel. 1% agarose gel (including 0.02% sodium azide) containing phosphate buffered saline as mentioned above was heated over a boiling water bath. It was cooled to about 40-50°C and slowly poured on a microscopic slide to have a gel thickness of about 0.1cm. It was left for 30min till the agarose was cooled and set. Smooth wells were punched in the gel (approximately 0.5cm in diameter). Antiserum was placed in the central well and different antigens were placed in the adjacent wells (about 20µl each). The plate was covered and left in a humid environment for 48hrs for diffusion. White precipitin lines appeared due to antigen-antibody reaction between the central (antiserum) and adjacent (antigen) wells. The plate was washed for overnight at 0-4°C in phosphate buffered saline followed by distilled water washing for 2hrs.

The gel plate was dried for 10-12 hrs at 37°C and then stained for protein in Coomassie brillant blue (prepared as mentioned for PAGE), destained in 7% acetic acid and photographed. The gel plates were dried and preserved at room temperature.

The GDH (NADH and NAD^+ dependent) activity were assayed with varying amounts of antiserum in the reaction mixture as described by di Prisco and Casola (1975). Aliquots (10-100µl) of the two antisera (from summer and winter purified GDH) were incubated for 15min at 37°C with summer and winter purified GDH separately. The residual activity was assayed and the effect was plotted on a graph as percent activity versus amount of antiserum.

Chemicals:

All the chemicals used were of analytical grade. Enzymes, coenzymes, substrates, nucleotides, molecular weight markers for electrophoresis DEAE-cellulose, Sepharose 4B-200, SDS, 2-mercaptoethanol, NBT, carbamyl phosphate, biogenic amines, acetylcholine, FCA and dialysis tubings were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.. Incomplete Freund's adjuvant was obtained from Difco Laboratory Ltd., U.S.A.. Aluminium hydroxide gel was obtained from Denmark. Sephadex G-25, DEAE-Sephacel, Blue Sepharose CL-6B, Sepharose CL-6B, molecular weight markers for column chromatography were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Other chemicals used were obtained from indigenous sources. Deionized double glass distilled ammonia free water was used in all preparations.

Statistical analysis and presentation of data:

The data was calculated from at least five observations for each point and presented as mean ± standard deviation (S.D.). The level of significance

between two sets of data were calculated by student's"t" test (Croxton et al., 1982) and p value above 0.05 were taken as non-significant (N.S.). Besides the presentation of data in tabulated form, graphs and histograms were prepared to highlight the results.

RESULTS

The data are presented as tables to indicate the standard deviations and the levels of significance and figures to indicate the patterns of change.

Effect of buffer on stability of GDH activity: (Fig.1)

GDH (NADH and NAD^+ dependent) activity in the liver homogenates prepared in potassium phosphate and Tris-HCl buffer was initially observed to be equal. In the homogenate prepared in Tris-HCl buffer the enzyme activity decreased within 2-3 hrs and continued to decrease with time when kept at 0±2°C. However, GDH activity was fairly stable at least upto 36 hrs at 0±2°C in the homogenate prepared in potassium phosphate buffer (Fig.1).

ADP and nicotinamide coenzyme requirement: (Table 1; Fig.2)

Oxidative deamination (NAD^+ dependent) activity of GDH could not be detected in different tissues such as liver, kidney, brain, muscle and gill whereas considerable amount of reductive amination (NADH dependent) activity could be assayed in all the tissues in absence of ADP in the assay mixture (Table 1). The oxidative deamination activity of GDH could be assayed only in presence of ADP. The reductive amination activity was increased by about 8-10 fold in presence of ADP. The reductive amination activity in absence of ADP, however, was higher than the oxidative deamination activity in presence of ADP in all the tissues. 2mM ADP in the assay mixture

was found to be optimum to get maximum enzyme activity of both the reactions of GDH (Fig.2).

NADH and NADPH were used equally well as coenzyme for the reductive amination reaction of GDH in all the tissues studied (Table 1). However, the oxidative deamination reaction of GDH showed specificity for NAD^+ and failed to show any enzyme activity in presence of $NADP^+$ as the coenzyme (Table 1).

Physiological level of GDH in various tissues: (Table 2; Fig.3)

Significant amount of GDH activity both reductive amination (in absence of ADP) and oxidative deamination (in presence of ADP) could be detected in the five tissues of H. fossilis studied (Table 2). The liver had maximum GDH activity and kidney showed half the activity present in liver followed by gill, muscle and brain. Both total and specific activity showed similar pattern of distribution. The reductive amination activity was 2.5-5 times greater than the oxidative deamination activity in all the tissues.

Specific staining for GDH (NAD^+ dependent) activity on polyacrylamide gel after electrophoresis of crude homogenates from liver, kidney, brain, muscle and gill tissues of H. fossilis showed a single enzyme band in each case (Fig.3). However, the migration of GDH on the gels was not same for all the tissues and Rm values were different (Fig.3).

Sub-cellular distribution of GDH: (Tables 3-6)

Treatment with different concentrations of Triton X-100 showed maximum (68-88%) release of GDH activity at 0.5% concentration in the

homogenates of all the tissues (Table 3). Treatment with Triton X-100 (0.5%) for different time intervals showed maximum release of activity after 60 min in all the tissues (Table 4).

GDH (NADH and NAD^+ dependent) activity was primarily mitochondrial (60-75%) in various tissues of H. fossilis (Tables 5&6). The distribution of the. marker enzyme activities compared well with the sub-cellular fractions. Cytochrome oxidase activity was (75-85%) in mitochondrial and lactate dehydrogenase (LDH) activity was (77-82%) in cytoplasmic fraction.

Circadian (24hr) cycle: (Tables 7-10; Figs.4-7)

There was no significant change in GDH (NADH and NAD^+ dependent) total and specific activity during 24 hr cycle in various tissues of H. fossilis studied during August, 1989 (Summer) (Tables 7&8; Figs.4&5) and January, 1990 (Winter) (Tables 9&10; Figs.6&7).

Annual cycle: (Tables 11&12; Figs.8&9)

GDH (NADH and NAD^+ dependent) activity in various tissues was determined at monthly intervals for 12 months from June, 1988 to May 1989. Reductive amination (NADH dependent) activity was found maximum in Summer (May-June) and minimum in Winter (October-November). In contrast, oxidative deamination (NAD^+ dependent) activity was maximum in Winter (November-March) and minimum in Summer (June-July) in the five tissues of H. fossilis studied. The ratio of NADH/NAD^+ dependent GDH activity showed the highest level in Summer (June) and lowest level in Winter (November) (Tables 11&12; Figs.8&9).

Effect of temperature: (Tables 13&14)

GDH (NADH dependent) total and specific activity due to the exposure of the fishes maintained at 20°C ambient temperature to 30°C for 10 days in June 1989 (Summer) (Table 13) showed no significant alteration except for small but significant induction in brain. NAD^+ dependent activity of GDH was induced only in liver. However, exposure to 10°C for 10 days resulted in general decrease in NADH dependent activity and increase in NAD^+ dependent activity in most of the tissues. In a similar experiment conducted during December, 1989 (Winter) Table 14) the results were much different. The NADH dependent activity was induced and NAD^+ dependent activity was inhibited in general in all the tissues on exposure to both 30°C and 10°C for 10 days. The effects were more pronounced at 30°C than 10°C.

Effect of water deprivation: (Tables 15-20; Figs.10-13)

There was a significant induction of GDH (both reductive amination and oxidative deamination) activity in various tissues within 3 hrs of emersion of H. fossilis (Tables 15-18; Figs.10-13). The rate of induction was faster upto 15-18 hrs of emersion after which the increase was rather slow. Liver showed a biphasic pattern within 15 hrs of emersion. Induction of NAD^+ dependent GDH activity in muscle declined after reaching the peak at 15 hrs of water deprivation (Tables 17&18; Figs.10-13). The maximum percentage of induction of both NADH and NAD^+ dependent activity of GDH was highest in brain followed by liver, gill, kidney and muscle. The ratio of NADH/NAD^+ dependent GDH activity did not show any significant variation in all the tissues during the 36 hrs of water deprivation (Tables 19&20; Figs. 10&11).

Effect of starvation, refeeding and hyper-ammonia stress: (Tables 21-29; Figs.14-22)

There was a significant increase in total free amino acids (FAA) in various tissues of H. fossilis studied during starvation (Tables 21&22; Figs.14&15). Total FAA level was increased with increasing time of starvation. The recovery in free amino acid pool was observed in various tissues of H. fossilis after refeeding of the fishes from 7 to 14 days (Table 21; Figs. 14&15). Very significant increase in total FAA was also observed in the five tissues studied during exposure of H. fossilis to hyper-ammonia ambient medium. The increase was directly proportional to the concentration of NH_4Cl in the medium and the time of treatment (Table 22; Fig.16).

Both NADH and NAD^+ dependent GDH activity showed significant induction in various tissues of H. fossilis studied during starvation except in brain (Table 23; Figs.17&19). The specific activity showed greater induction than the total activity in five tissues studied and it increased with increasing time of starvation (Tables 23&24; Figs.17-20). The rate of induction was found higher for oxidative deamination activity than the reductive amination activity except in muscle where the trend was reverse. Maximum induction of GDH (NAD^+ dependent) activity was observed in liver followed by kidney, gill, muscle and brain. On the other hand muscle showed highest induction of NADH dependent GDH activity followed by gill, liver, kidney and brain during starvation. The recovery of GDH (NADH and NAD^+ dependent) activity was observed in various tissues of H. fossilis during refeeding of the fishes from 7 to 14 days. However, the ratio of NADH/NAD^+ dependent activity did not show any significant variation during starvation.

H. fossilis exposed to hyper-ammonia ambient medium showed significant induction of total and specific activity of both NADH and NAD^+ dependent GDH activity in liver and kidney tissues (Tables 25&26; Figs. 17,18,21&22) of NADH dependent activity was observed in brain at 50 and 75mM NH_4Cl on 3rd day and of NAD^+ dependent activity on 7th and 14th day at 50mM NH_4Cl concentration (Table 27; Figs. 17,18,21&22). There was no significant alteration in the GDH activity in muscle and gill tissues under hyper-ammonia ambient medium (Tables 28&29; Figs.17,18,21&22). At 75mM NH_4Cl concentration the maximum induction was observed after 7 days of treatment. Total and specific activities showed similar pattern of induction during hyper-ammonia stress. NAD^+ dependent GDH activity showed greater induction than NADH dependent GDH activity in liver and kidney. The maximum rate of induction of NAD^+ dependent activity was observed in kidney followed by liver and brain whereas NADH dependent activity showed maximum induction in liver followed by kidney and brain during hyper-ammonia stress. The ratio of NADH/NAD^+ dependent activity did not show any significant change in various tissues of H. fossilis during hyper-ammonia stress.

Purification of GDH from liver of H. fossilis: (Table 30; Figs.23&24a,b)

GDH was purified from the liver of H. fossilis during Summer (May) and Winter (December). The purification protocol has been summarized in Table 30. The enzyme was eluted as a single peak from all the three columns used during purification (Fig.23). The degree of purification achieved were 500 fold and 420 fold with the yield of 48% and 57% of the enzyme activity during the purification done in Summer (May) and Winter (December) respectively.

The protein staining on polyacrylamide gel showed a single protein band of the purified GDH (Figs.24a,b). Specific staining for GDH (NAD^+ dependent) activity also showed a single enzyme band throughout the purification steps both during Summer (Fig.24a) and Winter (Fig.24b). It was observed that the purified GDH in potassium phosphate buffer (pH-7.6) was stable without any loss of activity for at least one month during which all studies were completed.

Kinetics: (Table 31; Figs.25-28)

Kinetic studies were performed with summer purified GDH from liver of H. fossilis as there was no apparent difference observed between the summer and winter purified enzyme. The apparent Michaelis constant (Km) for the substrates NH_4^+, α-ketoglutarate and coenzyme NADH for reductive amination reaction (in absence of ADP) were 25.0, 0.38 and 0.07mM respectively. The Vmax was 1.3 units/ml. The apparent Km for reductive amination reaction (in presence of ADP) were 23.8, 0.35 and 0.07mM for NH_4^+,α-ketoglutarate and NADH respectively and Vmax was 11.7 units/ml. The apparent Km for the substrate and coenzyme of the oxidative deamination reaction (in presence of ADP) was 4.76 and 0.28mM for L-glutamate and NAD^+ respectively. The Vmax was 0.64 units/ml. All the substrates except NAD^+ at higher concentrations inhibited GDH activity (Figs.25-28).

Absorption spectra: (Figs.29&30)

The absorption spectra of purified GDH from liver of H. fossilis showed another peak at 228nm besides the protein peak at 280nm. Presence of various reactants shifted the peak towards 260nm. The complete reaction

mixture showed a broad peak between 260-280nm. The peak at 340 nm was specific for the presence of NADH which increased in oxidative deamination reaction and decreased in reductive amination reaction (Figs.29&30).

Temperature optima and thermal stability: (Table 31; Figs.31&32)

The maximum NADH and NAD^+ dependent GDH activity were obtained at 30°C and 45°C respectively. The enzyme was rapidly inactivated above 50°C. The half life of the enzyme at 45°C was 8 min for NADH dependent and 25 min for NAD^+ dependent activity. The presence of substrate (α-ketoglutarate or L-glutamate) and ADP in the incubation medium helped to some extent the purified GDH from thermal denaturation NAD^+ dependent activity of GDH had better tolerance for temperature increase than NADH dependent activity (Figs.31&32).

pH optima: (Table 31; Fig.33)

The optimum pH for NADH and NAD^+ dependent GDH activity was between 7.6 to 8.0 and 8.5 to 9.5 respectively. NADH dependent activity was always higher than NAD^+ dependent activity at all the pH studied between 6.5-11.0 (Table 31; Fig.33).

Amino acid (Substrate) specificity: (Table 32)

The purified GDH showed strong specificity for L-glutamate as substrate in the oxidative deamination reaction. The enzyme activity was either absent or very low when L-glutamate was replaced by twenty other amino acids individually in the reaction mixture (Table 32).

Coenzyme specificity: (Table 33)

The purified GDH in the reductive amination direction used NADH or NADPH equally well as coenzyme for its activity. However, in the oxidative deamination reaction the enzyme showed specificity for NAD^+ for its activity and failed to use $NADP^+$ as the coenzyme (Table 33).

Effect of various nucleotides: (Tables 33&34; Figs.34-39)

Oxidative deamination activity could not be detected whereas reductive amination activity could be assayed at an appreciable level in absence of ADP in the assay mixture. In presence of ADP the oxidative deamination reaction could be assayed and the reductive amination activity was increased by 9 fold (Table 33).

ADP and GTP showed allosteric activation and inhibition effect on NADH and NAD^+ dependent activity of purified GDH respectively (Table 34; Figs.34&35). NADH dependent activity was slightly induced at lower concentrations of ATP and AMP whereas, NAD^+ dependent activity was significantly inhibited. IMP was found to be an inhibitor for both the activities of GDH (Table 34, Fig.34). The pattern of inhibition by IMP and ATP was non-competitive (Fig.36). The apparent Ka for ADP was 0.06mM for NADH dependent GDH activity (Table 34; Fig.37). The apparent Ki for ATP was 0.11mM for NAD^+ dependent GDH activity (Table 34; Fig.38). The apparent Ki for GTP was 0.53×10^{-2} and 0.42×10^{-2}mM for NADH (in absence of ADP) and NAD^+ (in presence of ADP) dependent activity respectively and 1.13×10^{-2}mM for NADH (in presence of ADP) dependent activity (Table 34; Fig .39).

Effect of various ions: (Table 35)

All the metal ions studied inhibited both NADH and NAD^+ dependent activity of purified GDH (Table 35). The inhibition was complete by mercury, zinc, silver and ferric ions above 25mM concentration. Both NADH and NAD^+ dependent activity of GDH were inhibited by all the negative ions studied except PO_4^{2-} which activated both the activities (Table 35).

Effect of acetylcholine, biogenic amines and some amino acid derivatives: (Table 36)

Acetylcholine, biogenic amines (norepinephrine, epinephrine, DOPA and serotonin) and some amino acid derivatives (γ-aminobutyric acid, urea and carbamyl phosphate) inhibited both NADH and NAD^+ dependent activity of purified hepatic GDH (Table 36).

Effect of varying substrates: (Figs.40-42)

A family of lines intersecting on the abscissa was obtained, when the concentrations of NH_4^+ and α-ketoglutarate were varied and NADH were kept at a constant saturating level (Fig.40). When the concentrations of NADH and α-ketoglutarate were varied and NH_4^+ were kept at a constant saturating level, the double reciprocal plots were parallel (Fig.4). The point of intersection on the ordinate was obtained with L-glutamate and NAD^+ against each other (Fig.42).

Product inhibition: (Table 37; Figs.43-47)

All the products at higher concentrations inhibited their respective reaction. The inhibition by the products L-glutamate and NAD^+ with relation

to α-ketoglutarate as the substrate for NADH dependent activity were competitive and non-competitive respectively (Table 37; Fig.43). On the other hand, the products α-ketoglutarate, NH_4^+ and NADH with relation to L-glutamate as the substrate for NAD^+ dependent activity were non-competitive, competitive and un-competitive respectively (Table 37; Fig.44). However, with relation to NAD^+, α-ketoglutarate and NH_4^+ showed non-competitive and NADH competitive type of inhibition (Table 37; Fig.45). The apparent Ki for NAD^+ and L-glutamate with relation to α-ketoglutarate as the substrate for NADH dependent activity were 0.31 and 4.7mM respectively whereas apparent Ki for L-glutamate was 5.6mM with relation to NH_4^+ as the substrate (Table 37; Fig.46). The apparent Ki for α-ketoglutarate, NH_4^+ and NADH with relation to L-glutamate as the substrate for NAD^+ dependent reaction were 0.24, 10.0 and 0.02mM respectively (Tables 37, Fig.47).

Effect of amino acids and keto acids: (Tables 38&39; Figs.48-53)

All the amino acids and keto acids studied inhibited both NADH and NAD^+ dependent activity of GDH at higher concentrations except L-leucine and DL-isoleucine (Table 38). The pattern of inhibition for L-cysteine, L-aspartic acid, L-histidine and L-lysine for NADH dependent activity was studied. The inhibition was competitive for L-cysteine and non-competitive for L-aspartic acid, L-histidine and L-lysine. The inhibition pattern of L-alanine, L-cysteine, L-histidine and L-lysine for NAD^+ dependent activity were competitive, un-competitive, and non-competitive respectively (Table 39; Figs.48-50). The apparent Ki for L-cysteine, L-aspartic acid, L-histidine and L-lysine for NADH dependent activity were 0.67; 0.56, 0.12 and 0.30mM respectively and apparent Ki for L-alanine, L-cysteine, L-histidine and L-lysine

for NAD^+ dependent activity were 3.5, 0.11, 1.33 and 0.16mM respectively (Table 39; Figs.51-53).

Effect of antibody: (Fig.54)

The antibody raised against summer and winter purified GDH showed inhibition of both NADH and NAD^+ dependent activity of both summer and winter purified GDH. However, the rate of inhibition was complete for NAD^+ dependent activity and the NADH dependent activity was inhibited by about 80% at that concentration of antibody (Fig.54).

Determination of molecular weight: (Table 31; Figs.55-58)

The apparent molecular weight of the native enzyme as determined by exclusion chromatography and PAGE was 3,38,844 and 3,38,064 respectively (Table 31; Figs.55a, 56&57). There was only one type of subunit with molecular weight of about 56,000 as determined in presence of dissociating agents such as 8M urea in exclusion chromatography and SDS in PAGE respectively (Table 31; Figs.55b, 56&58). There was no dissociation or association observed when the enzyme was loaded on the column or on PAGE at various dilutions. The results indicated that hepatic GDH of *H. fossilis* was composed of six identical subunits (hexamer).

Immunodiffusion: (Fig.59)

Single precipitin line was obtained with summer and winter purified GDH (antigen) when cross reacted with antibody raised against summer and winter purified GDH (Fig.59).

TABLE 1. Effect of ADP and nicotinamide coenzymes on GDH total activity (units/g wet wt.) in various tissues of *H. fossilis*. (Mean±S.D.)

	LIVER	KIDNEY	BRAIN	MUSCLE	GILL
NADH-ADP	7.33 ± 0.72	4.05 ± 0.54	2.16 ± 0.45	2.25 ± 0.36	2.61 ± 0.27
NADH+ADP	76.42 ± 9.20	38.55 ± 8.50	22.46 ± 7.90	20.43 ± 7.50	22.68 ± 6.85
NADPH-ADP	7.71 ± 0.76	4.15 ± 0.55	2.22 ± 0.26	1.94 ± 0.26	0.77 ± 0.14
NADPH+ADP	68.45 ±10.45	32.61 ± 9.35	20.50 ± 8.45	17.75 ± 9.31	18.50 ± 7.45
NAD^+-ADP	BLD	BLD	BLD	BLD	BLD
NAD^++ADP	2.88 ± 0.27	1.17 ± 0.27	0.69 ± 0.09	0.54 ± 0.18	0.63 ± 0.09
NADP-ADP	BLD	BLD	BLD	BLD	BLD
NADP+ADP	BLD	BLD	BLD	BLD	BLD

BLD - Below limit of detection.

Reaction mixture of 3ml each contained:

(for NADH/NADPH dependent reaction)

Potassium phosphate buffer pH-7.6 - 147 μ moles
Ammonium chloride - 450 μ moles
α - ketoglutarate - 14 μ moles
NADH / NADPH - 0.6 μ moles
+ADP - 6 μ moles

(for $NAD^+/NADP^+$ dependent reaction)

Potassium phosphate buffer pH-8.5 - 202.5 μ moles
L-glutamate 50 μ moles
$NAD^+/NADP^+$ 6 μ moles
+ADP 6 μ moles

TABLE 2. Total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio (NADH/NAD^+) in various tissues of *H. fossilis*. (Mean±S.D.)

Tissue	NADH - dependent GDH		NAD^+- dependent GDH		NADH/NAD^+- dependent GDH ratio	
	T.A.	S.A.	T.A.	S.A.	T.A.	S.A.
Liver	7.33 ± 0.72	9.90 ± 1.80	2.88 ± 0.27	3.60 ± 0.36	2.53 ± 0.43	2.66 ± 0.38
Kidney	4.05 ± 0.54	4.50 ± 0.63	1.17 ± 0.27	1.30 ± 0.45	3.45 ± 0.44	3.90 ± 1.14
Brain	2.16 ± 0.45	3.60 ± 0.81	0.69 ± 0.09	1.40 ± 0.45	3.04 ± 0.32	2.90 ± 0.65
Muscle	2.25 ± 0.36	3.20 ± 0.45	0.54 ± 0.18	0.81 ± 0.18	4.34 ± 1.27	3.60 ± 0.55
Gill	2.61 ± 0.27	4.70 ± 0.45	0.63 ± 0.09	1.10 ± 0.36	4.29 ± 0.75	4.90 ± 1.43

T.A. - Total activity

S.A. - Specific activity

TABLE 3. Release of GDH(NADH and NAD^+ dependent) activity (units/g wet wt.) in various tissue homogenates of *H. fossilis* treated with different concentrations of Triton X-100 for 60 min. (Mean±S.D.)

TritonX-100 (%)	LIVER		KIDNEY		BRAIN		MUSCLE		GILL	
	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+
0	4.34±0.46	1.74±0.38	2.41±0.38	0.96±0.16	1.45±0.16	0.41±0.09	1.40±0.26	0.38±0.08	1.54±0.34	0.38±0.07
0.1	5.05±0.46 (16)	1.93±0.40 (11)	2.69±0.46 (12)	1.21±0.26 (26)	1.69±0.38 (17)	0.59±0.16 (44)	1.54±0.26 (10)	0.44±0.09 (16)	1.74±0.26 (13)	0.54±0.10 (42)
0.2	5.74±0.50 (32)	2.25±0.38 (29)	2.89±0.46 (20)	1.28±0.26 (33)	1.83±0.40 (26)	0.62±0.20 (51)	1.69±0.30 (21)	0.54±0.10 (42)	1.93±0.26 (25)	0.58±0.10 (53)
0.3	6.95±0.55 (60)	2.41±0.40 (39)	3.38±0.46 (40)	1.45±0.30 (51)	2.12±0.38 (46)	0.64±0.20 (56)	1.93±0.34 (38)	0.58±0.10 (53)	2.38±0.30 (55)	0.64±0.16 (68)
0.4	7.77±0.60 (79)	2.89±0.40 (66)	3.93±0.50 (63)	1.56±0.38 (63)	2.38±0.38 (64)	0.68±0.20 (66)	2.25±0.38 (61)	0.64±0.16 (68)	2.65±0.38 (72)	0.68±0.16 (79)
0.5	7.94±0.65 (83)	3.22±0.46 (85)	4.20±0.50 (74)	1.65±0.40 (72)	2.48±0.40 (71)	0.69±0.26 (68)	2.41±0.40 (72)	0.65±0.16 (71)	2.83±0.46 (84)	0.71±0.26 (87)
1.0	7.92±0.60 (83)	3.20±0.40 (84)	4.20±0.46 (74)	1.66±0.46 (73)	2.48±0.38 (71)	0.70±0.26 (71)	2.41±0.46 (72)	0.63±0.14 (66)	2.81±0.46 (83)	0.69±0.20 (82)

Figure in parentheses indicate percentage increased in enzyme activity.

TABLE 4. Release of GDH (NADH and NAD^+ dependent) activity (units/g wet wt.) in various tissue homogenates of *H. fossilis* treated with Triton X-100 (0.5%) for different times. (Mean±S.D.)

Time after Triton X-100 treatment (min)	LIVER		KIDNEY		BRAIN		MUSCLE		GILL	
	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+	NADH	NAD^+
0	4.34±0.46	1.74±0.38	2.41±0.38	0.96±0.16	1.45±0.16	0.41±0.09	1.40±0.26	0.38±0.08	1.54±0.34	0.38±0.07
10	4.82±0.46 (11)	1.83±0.36 (5)	2.41±0.40 (0)	1.06±0.26 (10)	1.54±0.34 (6)	0.48±0.09 (17)	1.45±0.26 (4)	0.41±0.09 (8)	1.64±0.32 (7)	0.48±0.10 (26)
20	5.79±0.55 (33)	1.93±0.38 (11)	2.65±0.40 (10)	1.16±0.26 (21)	1.74±0.36 (20)	0.54±0.11 (32)	1.54±0.34 (10)	0.48±0.10 (26)	1.74±0.34 (13)	0.54±0.11 (42)
30	6.75±0.55 (56)	2.32±0.38 (33)	2.89±0.46 (20)	1.25±0.25 (30)	1.83±0.38 (26)	0.58±0.12 (42)	1.74±0.36 (24)	0.58±0.11 (53)	1.93±0.38 (25)	0.58±0.12 (53)
40	7.72±0.65 (78)	2.70±0.40 (55)	3.14±0.42 (30)	1.35±0.32 (41)	1.93±0.38 (33)	0.64±0.16 (56)	1.93±0.36 (38)	0.62±0.12 (63)	2.12±0.36 (38)	0.64±0.15 (68)
50	7.85±0.55 (81)	2.89±0.40 (66)	3.86±0.46 (60)	1.54±0.34 (60)	2.12±0.36 (46)	0.68±0.16 (66)	2.12±0.38 (51)	0.64±0.15 (68)	2.41±0.40 (57)	0.68±0.16 (79)
60	7.95±0.65 (83)	3.25±0.46 (87)	4.16±0.45 (73)	1.61±0.38 (68)	2.49±0.40 (72)	0.72±0.26 (76)	2.41±0.46 (72)	0.67±0.16 (76)	2.89±0.50 (88)	0.69±0.16 (82)
70	7.93±0.55 (83)	3.25±0.40 (87)	4.15±0.46 (72)	1.62±0.38 (69)	2.49±0.40 (72)	0.70±0.24 (71)	2.43±0.40 (74)	0.66±0.20 (74)	2.89±0.46 (88)	0.69±0.16 (82)

Figure in parentheses indicate percentage increase in enzyme activity.

TABLE 5. Sub-cellular distribution of total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) in various tissues of *H. fossilis*. (Mean±S.D.)

Fraction	Enzyme	LIVER	KIDNEY	BRAIN	MUSCLE	GILL
Homogenate	NADH	7.95±0.55	4.16±0.45	2.41±0.40	2.41±0.46	2.89±0.50
	NAD^+	3.25±0.46	1.51±0.30	0.72±0.34	0.63±0.26	0.69±0.26
	Cyt.Oxi	2.01±0.26	1.82±0.18	1.62±0.36	1.28±0.30	1.45±0.34
	LDH	72.02±7.45	46.55±5.43	39.58±4.25	35.79±3.89	37.45±4.05
Sub-cellular fractions						
Nuclear (N)	NADH	0.96±0.26(9)	0.48±0.21(9)	0.38±0.16(11)	0.39±0.21(11)	0.58±0.36(15)
	NAD^+	0.38±0.16(9)	0.08±0.02(5)	0.05±0.01(6)	0.06±0.01(8)	0.08±0.01(10)
	Cyt.Oxi.	0.19±0.04(8)	0.15±0.01(7)	0.10±0.01(6)	0.07±0.01(5)	0.09±0.01(6)
	LDH	8.20±2.25(9)	4.05±1.75(8)	3.86±0.90(9)	3.25±0.90(8)	3.86±1.38(9)
Mitochondrial (M)	NADH	7.68±0.55(73)	3.84±0.40(70)	2.31±0.36(67)	2.25±0.34(65)	2.45±0.50(61)
	NAD^+	2.89±0.46(72)	1.16±0.34(67)	0.58±0.26(75)	0.52±0.21(72)	0.54±0.26(68)
	Cyt.Oxi.	1.82±0.15(77)	1.62±0.46(78)	1.35±0.16(80)	1.16±0.26(85)	1.32±0.26(82)
	LDH	12.34±2.55(14)	6.75±1.15(12)	5.31±1.75(12)	3.68±1.67(10)	4.82±1.24(11)
Cytoplasmic (C)	NADH	1.93±0.40(18)	1.16±0.36(21)	0.77±0.24(22)	0.84±0.26(24)	0.96±0.40(24)
	NAD^+	0.77±0.16(19)	0.48±0.14(28)	0.15±0.02(19)	0.14±0.01(20)	0.17±0.01(22)
	Cyt.Oxi.	0.35±0.03(15)	0.32±0.12(15)	0.24±0.02(14)	0.13±0.01(10)	0.20±0.01(12)
	LDH	66.33±5.51(77)	44.25±4.65(80)	35.25±4.55(79)	32.46±3.92(82)	34.66±4.35(80)
Total recovery (N+M+C)	NADH	10.57±2.15(133)	5.48±0.51(132)	3.46±0.36(144)	3.48±0.61(144)	3.99±0.55(138)
	NAD^+	4.04±0.94(124)	1.72±0.36(114)	0.78±0.26(108)	0.72±0.34(114)	0.79±0.36(114)
	Cyt.Oxi.	2.36±0.09(117)	2.09±0.38(115)	1.69±0.25(104)	1.36±0.30(106)	1.61±0.24(111)
	LDH	86.87±9.52(121)	55.05±6.25(118)	44.42±5.15(112)	39.39±4.28(110)	43.34±5.22(116)

Cyt.Oxi. - Cytochrome Oxidase and LDH - Lactate dehydrogenase (units/g wet wt.)
Figure in parentheses indicate percentage recovered activity compared to homogenate.

TABLE 6. Sub-cellular distribution of specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis. (Mean±S.D.)

Fraction	Enzyme	LIVER	KIDNEY	BRAIN	MUSCLE	GILL
Homogenate	NADH	5.6±0.9	3.6±0.9	4.7±1.5	4.3±0.9	4.8±0.8
	NAD^+	2.3±0.6	1.3±0.6	1.4±0.2	1.1±0.1	1.2±0.5
	Cyt.Oxi.	1.4±0.3	1.6±0.5	3.2±0.8	2.3±0.2	2.4±0.5
	LDH	51.0±15.0	40.0±6.0	79.0±9.0	64.0±4.6	63.0±6.0
Sub-cellular fractions						
Nuclear (N)	NADH	3.7±0.7(17)	3.1±0.8(14)	4.0±0.8(9)	2.8±0.6(16)	4.7±0.9(22)
	NAD^+	1.5±0.4(17)	0.5±0.1(8)	0.5±0.1(10)	0.4±0.1(11)	0.6±0.1(15)
	Cyt.Oxi.	0.8±0.2(16)	0.8±0.1(9)	1.1±0.9(10)	0.5±0.1(7)	0.7±0.1(8)
	LDH	39.0±4.0(26)	26.0±4.5(25)	40.0±6.0(20)	20.0±3.2(14)	32.0±5.1(19)
Mitochondrial (M)	NADH	16.2±1.2(75)	17.0±4.2(79)	15.0±4.1(69)	12.2±1.5(70)	13.8±3.3(64)
	NAD^+	6.1±1.1(73)	5.1±0.9(84)	3.8±0.3(80)	2.8±0.4(78)	3.0±0.5(73)
	Cyt.Oxi.	3.8±0.7(76)	7.3±1.6(86)	9.0±2.6(83)	6.3±0.8(88)	7.4±1.8(85)
	LDH	26.0±5.0(20)	30.0±4.9(28)	35.0±5.6(18)	20.0±3.2(14)	27.0±4.0(17)
Cytoplasmic (C)	NADH	2.0±0.5(9)	1.3±0.5(7)	2.6±0.2(12)	2.5±0.5(14)	2.9±0.5(14)
	NAD^+	0.8±0.1(10)	0.5±0.1(8)	0.5±0.1(10)	0.4±0.1(11)	0.5±0.1(12)
	Cyt.Oxi.	0.4±0.09(8)	0.4±0.1(5)	0.7±0.2(7)	0.4±0.1(5)	0.6±0.1(7)
	LDH	70.0±12.0(54)	50.0±7.0(47)	120.0±12.0(62)	101.0±11.0(72)	104.0±12.0(64)
Total recovery (N+M+C)	NADH	6.3±0.9(113)	4.4±0.5(122)	6.5±1.0(138)	5.5±0.6(128)	6.6±0.9(138)
	NAD^+	2.4±0.4(104)	1.4±0.2(108)	1.5±0.1(107)	1.2±0.1(109)	1.3±0.2(108)
	Cyt.Oxi.	1.4±0.2(100)	1.7±0.3(106)	3.2±0.4(100)	2.4±0.4(104)	2.7±0.5(113)
	LDH	52.0±6.6(102)	44.0±4.0(110)	83.0±9.6(105)	64.0±7.0(100)	72.0±7.6(114)

Cyt.Oxi. - Cytochrome Oxidase and LDH - Lactate dehydrogenase (units/mg protein)x10^2
Figure in parentheses indicate percentage recovered activity compared to homogenate.

TABLE 7. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during 24 hr cycle in August, 1989 (Summer). (Mean±S.D.)

Tissue	Enzyme	DAY					NIGHT			
		6 hr Control	9 hr	12 hr	15 hr	18 hr	21 hr	24 hr	3 hr	6 hr (next day)
Liver	NADH	7.83±0.72	8.04±0.80 N.S.[a]	7.95±0.70 N.S.	8.06±0.75 N.S.	7.79±0.58 N.S.	7.88±0.73 N.S.	7.74±0.58 N.S.	7.87±0.65 N.S.	7.88±0.75 N.S.
	NAD^+	2.55±0.26	2.70±0.30 N.S.[a]	2.62±0.30 N.S.	2.58±0.24 N.S.	2.64±0.35 N.S.	2.57±0.26 N.S.	2.50±0.22 N.S.	2.55±0.26 N.S.	2.52±0.25 N.S.
	$NADH/NAD^+$	3.07	2.98	3.03	3.12	2.95	3.07	3.10	3.09	3.13
Kidney	NADH	4.05±0.45	4.12±0.50 N.S.[a]	4.02±0.45 N.S.	3.95±0.46 N.S.	3.83±0.54 N.S.	4.10±0.45 N.S.	3.90±0.45 N.S.	3.83±0.38 N.S.	4.01±0.50 N.S.
	NAD^+	1.35±0.27	1.38±0.30 N.S.[a]	1.25±0.26 N.S.	1.29±0.26 N.S.	1.20±0.22 N.S.	1.38±0.30 N.S.	1.20±0.28 N.S.	1.25±0.26 N.S.	1.35±0.30 N.S.
	$NADH/NAD^+$	3.00	2.99	3.22	3.06	3.19	2.97	3.25	3.06	2.97
Brain	NADH	2.46±0.35	2.50±0.40 N.S.[a]	2.57±0.46 N.S.	2.48±0.40 N.S.	2.52±0.38 N.S.	2.45±0.35 N.S.	2.41±0.38 N.S.	2.50±0.40 N.S.	2.57±0.46 N.S.
	NAD^+	0.73±0.22	0.75±0.26 N.S.[a]	0.80±0.30 N.S.	0.76±0.26 N.S.	0.83±0.30 N.S.	0.74±0.26 N.S.	0.70±0.22 N.S.	0.78±0.22 N.S.	0.82±0.26 N.S.
	$NADH/NAD^+$	3.37	3.33	3.21	3.26	3.04	3.31	3.44	3.21	3.13
Muscle	NADH	2.25±0.36	2.30±0.28 N.S.[a]	2.28±0.26 N.S.	2.35±0.28 N.S.	2.25±0.28 N.S.	2.20±0.28 N.S.	2.27±0.48 N.S.	2.32±0.30 N.S.	2.28±0.28 N.S.
	NAD^+	0.65±0.18	0.70±0.22 N.S.[a]	0.66±0.22 N.S.	0.72±0.26 N.S.	0.68±0.22 N.S.	0.62±0.26 N.S.	0.66±0.30 N.S.	0.75±0.26 N.S.	0.70±0.26 N.S.
	$NADH/NAD^+$	3.46	3.29	3.45	3.26	3.31	3.55	3.44	3.09	3.26
Gill	NADH	2.55±0.40	2.65±0.46 N.S.[a]	2.60±0.48 N.S.	2.57±0.40 N.S.	2.62±0.46 N.S.	2.57±0.38 N.S.	2.65±0.40 N.S.	2.45±0.35 N.S.	2.50±0.38 N.S.
	NAD^+	0.83±0.26	0.88±0.30 N.S.[a]	0.84±0.26 N.S.	0.78±0.22 N.S.	0.80±0.22 N.S.	0.74±0.20 N.S.	0.79±0.26 N.S.	0.72±0.22 N.S.	0.74±0.26 N.S.
	$NADH/NAD^+$	3.07	3.01	3.10	3.29	3.28	3.47	3.35	3.40	3.38

a – p value compared to control

TABLE 8. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of *H. fossilis* during 24 hr cycle in August, 1989 (Summer). (Mean±S.D.)

Tissue	Enzyme	DAY					NIGHT			
		6 hr Control	9 hr	12 hr	15 hr	18 hr	21 hr	24 hr	3 hr	6 hr (next day)
Liver	NADH	9.95±0.75	10.38±1.06 N.S.[a]	10.25±0.99 N.S.	9.99±0.95 N.S.	10.30±1.00 N.S.	10.15±1.09 N.S.	9.96±0.99 N.S.	9.85±0.95 N.S.	10.10±1.05 N.S.
	NAD^+	3.35±0.46	3.42±0.50 N.S.[a]	3.40±0.46 N.S.	3.38±0.40 N.S.	3.46±0.44 N.S.	3.38±0.40 N.S.	3.45±0.46 N.S.	3.30±0.38 N.S.	3.50±0.50 N.S.
	$NADH/NAD^+$	2.97	3.04	3.01	2.96	2.98	3.00	2.89	2.98	2.89
Kidney	NADH	4.55±0.60	4.38±0.40 N.S.[a]	4.62±0.46 N.S.	4.42±0.38 N.S.	4.65±0.50 N.S.	4.45±0.40 N.S.	4.64±0.50 N.S.	4.40±0.46 N.S.	4.52±0.46 N.S.
	NAD^+	1.50±0.38	1.37±0.26 N.S.[a]	1.48±0.35 N.S.	1.35±0.26 N.S.	1.52±0.38 N.S.	1.42±0.26 N.S.	1.50±0.40 N.S.	1.40±0.30 N.S.	1.45±0.38 N.S.
	$NADH/NAD^+$	3.03	3.20	3.12	3.27	3.06	3.13	3.09	3.14	3.12
Brain	NADH	3.95±0.46	3.83±0.40 N.S.[a]	4.05±0.46 N.S.	3.85±0.38 N.S.	4.00±0.40 N.S.	3.88±0.38 N.S.	4.04±0.46 N.S.	3.94±0.40 N.S.	4.06±0.50 N.S.
	NAD^+	1.25±0.26	1.20±0.20 N.S.[a]	1.35±0.30 N.S.	1.22±0.22 N.S.	1.35±0.30 N.S.	1.20±0.26 N.S.	1.32±0.26 N.S.	1.25±0.26 N.S.	1.30±0.30 N.S.
	$NADH/NAD^+$	3.16	3.19	3.00	3.16	2.96	3.23	3.06	3.15	3.12
Muscle	NADH	3.75±0.45	3.90±0.55 N.S.[a]	3.70±0.46 N.S.	3.83±0.45 N.S.	3.65±0.40 N.S.	3.78±0.46 N.S.	3.64±0.38 N.S.	3.80±0.40 N.S.	3.60±0.38 N.S.
	NAD^+	1.15±0.22	1.28±0.26 N.S.[a]	1.10±0.20 N.S.	1.20±0.26 N.S.	1.09±0.22 N.S.	1.16±0.26 N.S.	1.05±0.20 N.S.	1.20±0.26 N.S.	1.14±0.22 N.S.
	$NADH/NAD^+$	3.26	3.05	3.36	3.19	3.35	3.26	3.47	3.17	3.16
Gill	NADH	4.70±0.50	4.82±0.46 N.S.[a]	4.65±0.40 N.S.	4.86±0.50 N.S.	4.50±0.46 N.S.	4.75±0.40 N.S.	4.60±0.38 N.S.	4.45±0.40 N.S.	4.65±0.46 N.S.
	NAD^+	1.40±0.38	1.50±0.40 N.S.[a]	1.45±0.38 N.S.	1.48±0.40 N.S.	1.35±0.38 N.S.	1.45±0.40 N.S.	1.40±0.26 N.S.	1.38±0.30 N.S.	1.45±0.38 N.S.
	$NADH/NAD^+$	3.36	3.21	3.21	3.28	3.33	3.28	3.29	3.22	3.21

a – p value compared to control

TABLE 9. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during 24 hr cycle in January, 1990 (Winter). (Mean±S.D.)

Tissue	Enzyme	DAY					NIGHT			
		6 hr Control	9 hr	12 hr	15 hr	18 hr	21 hr	24 hr	3 hr	6 hr (next day)
Liver	NADH	10.61±1.25	10.85±0.89 N.S.[a]	10.52±0.99 N.S.	11.05±1.30 N.S.	10.97±1.08 N.S.	10.45±1.01 N.S.	11.09±1.28 N.S.	10.25±1.04 N.S.	10.78±1.19 N.S.
	NAD^+	5.79±0.82	5.65±0.65 N.S.[a]	5.85±0.55 N.S.	6.04±0.63 N.S.	6.28±0.75 N.S.	5.65±0.60 N.S.	5.92±0.72 N.S.	5.49±0.66 N.S.	5.55±0.65 N.S.
	$NADH/NAD^+$	1.83	1.92	1.80	1.83	1.75	1.85	1.87	1.87	1.94
Kidney	NADH	5.79±0.65	5.66±0.76 N.S.[a]	6.12±0.85 N.S.	5.95±0.74 N.S.	6.25±0.90 N.S.	6.08±0.87 N.S.	6.34±0.95 N.S.	5.50±0.68 N.S.	5.65±0.72 N.S.
	NAD^+	2.41±0.46	2.38±0.45 N.S.[a]	2.65±0.55 N.S.	2.52±0.50 N.S.	2.74±0.62 N.S.	2.68±0.60 N.S.	2.89±0.65 N.S.	2.38±0.46 N.S.	2.41±0.40 N.S.
	$NADH/NAD^+$	2.40	2.38	2.31	2.36	2.28	2.27	2.19	2.31	2.34
Brain	NADH	2.41±0.38	2.65±0.45 N.S.[a]	2.38±0.40 N.S.	2.45±0.46 N.S.	2.41±0.38 N.S.	2.54±0.40 N.S.	2.49±0.50 N.S.	2.72±0.45 N.S.	2.70±0.46 N.S.
	NAD^+	0.77±0.16	0.82±0.22 N.S.[a]	0.68±0.15 N.S.	0.78±0.25 N.S.	0.72±0.20 N.S.	0.77±0.15 N.S.	0.80±0.26 N.S.	0.85±0.30 N.S.	0.80±0.22 N.S.
	$NADH/NAD^+$	3.13	3.23	3.50	3.14	3.35	3.30	3.11	3.20	3.38
Muscle	NADH	2.35±0.26	2.41±0.38 N.S.[a]	2.79±0.46 N.S.	2.55±0.40 N.S.	2.38±0.26 N.S.	2.63±0.38 N.S.	2.57±0.46 N.S.	2.75±0.55 N.S.	2.45±0.30 N.S.
	NAD^+	0.73±0.12	0.77±0.16 N.S.[a]	0.90±0.22 N.S.	0.85±0.18 N.S.	0.77±0.16 N.S.	0.87±0.18 N.S.	0.82±0.18 N.S.	0.91±0.25 N.S.	0.78±0.26 N.S.
	$NADH/NAD^+$	3.22	3.13	3.10	3.00	3.09	3.02	3.13	3.02	3.14
Gill	NADH	2.89±0.38	2.77±0.34 N.S.[a]	2.83±0.46 N.S.	2.65±0.26 N.S.	2.58±0.22 N.S.	2.71±0.34 N.S.	2.89±0.40 N.S.	2.70±0.36 N.S.	2.65±0.26 N.S.
	NAD^+	0.92±0.22	0.88±0.18 N.S.[a]	0.90±0.26 N.S.	0.83±0.15 N.S.	0.77±0.14 N.S.	0.87±0.16 N.S.	0.95±0.26 N.S.	0.86±0.16 N.S.	0.83±0.12 N.S.
	$NADH/NAD^+$	3.14	3.15	3.14	3.19	3.35	3.11	3.04	3.14	3.19

a - p value compared to control

TABLE 10. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during 24 hr cycle in January, 1990 (Winter). (Mean±S.D.)

Tissue	Enzyme	DAY					NIGHT			
		6 hr Control	9 hr	12 hr	15 hr	18 hr	21 hr	24 hr	3 hr	6 hr (next day)
Liver	NADH	9.70±0.95	10.05±1.05 N.S.[a]	9.58±0.89 N.S.	10.45±1.15 N.S.	9.88±0.99 N.S.	10.25±1.04 N.S.	10.40±1.10 N.S.	9.94±1.00 N.S.	9.85±0.99 N.S.
	NAD^+	5.20±0.75	5.31±0.80 N.S.[a]	5.15±0.65 N.S.	5.65±0.90 N.S.	5.45±0.85 N.S.	5.62±0.89 N.S.	5.58±0.75 N.S.	5.40±0.68 N.S.	5.46±0.75 N.S.
	$NADH/NAD^+$	1.87	1.89	1.86	1.85	1.81	1.82	1.86	1.84	1.80
Kidney	NADH	5.58±0.84	5.65±0.90 N.S.[a]	5.72±0.78 N.S.	5.45±0.65 N.S.	6.02±0.90 N.S.	5.75±0.89 N.S.	5.38±0.68 N.S.	5.85±0.85 N.S.	5.92±0.89 N.S.
	NAD^+	2.82±0.35	2.85±0.40 N.S.[a]	2.89±0.45 N.S.	2.65±0.38 N.S.	2.93±0.45 N.S.	2.75±0.44 N.S.	2.55±0.25 N.S.	2.77±0.40 N.S.	2.85±0.45 N.S.
	$NADH/NAD^+$	1.98	1.98	1.98	2.06	2.05	2.09	2.11	2.11	2.08
Brain	NADH	4.54±0.48	4.65±0.60 N.S.[a]	4.48±0.55 N.S.	4.75±0.65 N.S.	4.83±0.65 N.S.	4.72±0.70 N.S.	4.68±0.77 N.S.	4.78±0.50 N.S.	4.60±0.58 N.S.
	NAD^+	1.41±0.25	1.45±0.22 N.S.[a]	1.38±0.18 N.S.	1.48±0.18 N.S.	1.54±0.22 N.S.	1.48±0.26 N.S.	1.44±0.25 N.S.	1.63±0.30 N.S.	1.40±0.32 N.S.
	$NADH/NAD^+$	3.22	3.21	3.25	3.21	3.14	3.19	3.25	2.93	3.29
Muscle	NADH	3.65±0.38	3.78±0.46 N.S.[a]	3.54±0.40 N.S.	3.85±0.46 N.S.	3.70±0.40 N.S.	3.68±0.45 N.S.	3.65±0.45 N.S.	3.55±0.40 N.S.	3.75±0.46 N.S.
	NAD^+	1.20±0.30	1.25±0.26 N.S.[a]	1.18±0.22 N.S.	1.30±0.26 N.S.	1.23±0.24 N.S.	1.24±0.20 N.S.	1.16±0.16 N.S.	1.18±0.18 N.S.	1.22±0.18 N.S.
	$NADH/NAD^+$	3.04	3.02	3.00	2.96	3.01	2.97	3.15	3.01	3.07
Gill	NADH	4.82±0.60	4.93±0.65 N.S.[a]	4.78±0.45 N.S.	4.65±0.65 N.S.	4.80±0.75 N.S.	4.68±0.55 N.S.	4.50±0.50 N.S.	4.75±0.55 N.S.	4.65±0.50 N.S.
	NAD^+	1.52±0.46	1.65±0.50 N.S.[a]	1.50±0.40 N.S.	1.48±0.38 N.S.	1.54±0.40 N.S.	1.45±0.38 N.S.	1.46±0.38 N.S.	1.52±0.46 N.S.	1.56±0.46 N.S.
	$NADH/NAD^+$	3.17	2.99	3.19	3.14	3.12	3.23	3.08	3.13	2.98

a – p value compared to control

TABLE 11. Alteration in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during annual cycle (1988-1989) studied at monthly interval. (Mean±S.D.)

Tissue	Enzyme	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Liver	NADH	9.55 ±0.87	10.80 ±0.99	10.71 ±1.25	11.09 ±0.76	13.41 ±0.96	14.47 ±0.76	11.09 ±1.07	8.87 ±0.55	9.65 ±0.34	7.14 ±0.41	5.82 ±0.76	7.81 ±0.63
	NAD^+	4.59 ±0.37	3.93 ±0.42	4.40 ±0.37	4.32 ±0.40	3.78 ±0.22	2.93 ±0.16	2.74 ±0.16	2.92 ±0.15	3.71 ±0.29	4.63 ±0.31	5.71 ±0.64	5.25 ±0.37
	$NADH/NAD^+$	2.08	2.75	2.43	2.57	3.55	4.94	4.05	3.04	2.60	1.54	1.02	1.49
Kidney	NADH	5.02 ±0.55	5.31 ±0.76	5.31 ±0.76	5.50 ±0.55	6.56 ±0.79	5.98 ±0.55	5.02 ±0.55	4.24 ±0.40	4.44 ±0.63	3.67 ±0.55	2.31 ±0.40	4.15 ±0.55
	NAD^+	2.05 ±0.22	2.24 ±0.32	2.20 ±0.22	2.28 ±0.46	1.89 ±0.28	1.50 ±0.16	1.43 ±0.22	1.69 ±0.16	1.62 ±0.22	1.46 ±0.32	1.89 ±0.44	2.51 ±0.30
	$NADH/NAD^+$	2.45	2.37	2.41	2.41	3.47	3.99	3.51	2.51	2.74	2.51	1.22	1.65
Brain	NADH	2.22 ±0.40	2.70 ±0.55	2.70 ±0.55	3.18 ±0.55	3.09 ±0.27	3.28 ±0.41	2.31 ±0.40	2.79 ±0.41	2.22 ±0.26	2.03 ±0.40	1.54 ±0.53	2.22 ±0.55
	NAD^+	0.85 ±0.22	0.93 ±0.16	1.01 ±0.16	0.97 ±0.14	0.81 ±0.16	0.81 ±0.26	0.81 ±0.16	1.05 ±0.10	0.85 ±0.11	0.73 ±0.34	1.01 ±0.16	1.01 ±0.16
	$NADH/NAD^+$	2.61	2.90	2.67	3.28	3.81	4.05	2.85	2.66	2.61	2.78	1.53	2.20
Muscle	NADH	2.22 ±0.55	2.03 ±0.40	2.31 ±0.40	2.51 ±0.40	2.60 ±0.26	2.12 ±0.26	1.74 ±0.26	1.93 ±0.34	1.45 ±0.34	1.54 ±0.41	1.93 ±0.40	1.83 ±0.40
	NAD^+	0.69 ±0.10	0.73 ±0.16	0.89 ±0.22	0.85 ±0.11	0.81 ±0.16	0.62 ±0.16	0.62 ±0.26	0.62 ±0.16	0.73 ±0.16	0.62 ±0.16	0.66 ±0.17	0.73 ±0.16
	$NADH/NAD^+$	3.22	2.78	2.60	2.95	3.21	3.42	2.81	3.11	1.99	2.48	2.92	2.51
Gill	NADH	2.31 ±0.40	2.60 ±0.55	2.51 ±0.40	2.60 ±0.55	2.79 ±0.41	2.12 ±0.26	1.93 ±0.33	2.22 ±0.26	1.54 ±0.40	1.45 ±0.34	1.54 ±0.40	1.93 ±0.34
	NAD^+	0.73 ±0.16	0.93 ±0.16	0.93 ±0.16	0.93 ±0.16	0.89 ±0.11	0.73 ±0.26	0.73 ±0.26	0.85 ±0.20	0.80 ±0.16	0.73 ±0.16	0.81 ±0.16	0.81 ±0.16
	$NADH/NAD^+$	3.16	2.80	2.70	2.80	3.13	2.90	2.64	2.61	1.93	1.99	1.90	2.38

TABLE 12. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during annual cycle (1988-1989) studies at monthly interval. (Mean±S.D.)

Tissue	Enzyme	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Liver	NADH	7.60 ±0.82	9.72 ±0.90	9.72 ±1.18	9.62 ±0.82	11.10 ±1.05	8.90 ±0.82	7.32 ±0.73	5.89 ±0.50	8.61 ±0.92	6.78 ±0.73	6.05 ±0.73	6.15 ±0.70
	NAD^+	3.61 ±0.40	3.61 ±0.53	4.01 ±0.34	3.81 ±0.40	3.09 ±0.34	1.80 ±0.09	1.80 ±0.09	1.90 ±0.20	3.31 ±0.40	4.39 ±0.45	5.90 ±0.55	3.32 ±0.34
	$NADH/NAD^+$	2.11	2.69	2.42	2.52	3.59	4.94	4.07	3.10	2.60	1.54	1.03	1.85
Kidney	NADH	4.50 ±0.40	5.40 ±0.55	5.89 ±0.85	6.01 ±0.50	6.73 ±0.75	4.88 ±0.50	4.20 ±0.40	3.62 ±0.40	5.01 ±0.55	4.92 ±0.50	3.82 ±0.38	3.80 ±0.38
	NAD^+	1.80 ±0.34	2.29 ±0.34	2.50 ±0.30	2.50 ±0.34	1.91 ±0.20	1.20 ±0.16	1.20 ±0.16	1.41 ±0.20	1.80 ±0.20	2.02 ±0.36	2.21 ±0.22	2.30 ±0.20
	$NADH/NAD^+$	2.50	2.36	2.36	2.40	3.52	4.07	3.50	2.57	2.78	2.44	1.73	1.65
Brain	NADH	4.80 ±0.75	5.38 ±0.73	5.89 ±0.85	5.81 ±0.50	6.60 ±0.75	6.01 ±0.73	4.29 ±0.40	5.30 ±0.45	4.70 ±0.45	6.29 ±0.73	3.48 ±0.40	4.31 ±0.45
	NAD^+	1.60 ±0.34	1.89 ±0.38	2.20 ±0.20	2.20 ±0.38	2.00 ±0.40	1.49 ±0.34	1.49 ±0.20	1.89 ±0.20	1.81 ±0.26	2.30 ±0.34	2.31 ±0.26	2.01 ±0.20
	$NADH/NAD^+$	3.00	2.85	2.68	2.64	3.30	4.03	2.88	2.80	2.60	2.74	1.51	2.14
Muscle	NADH	3.40 ±0.85	3.01 ±0.55	3.21 ±0.55	3.21 ±0.45	2.91 ±0.55	4.00 ±0.40	3.31 ±0.30	3.39 ±0.34	2.50 ±0.26	4.60 ±0.45	3.80 ±0.45	3.22 ±0.34
	NAD^+	1.10 ±0.30	1.10 ±0.38	1.30 ±0.26	1.10 ±0.20	1.00 ±0.20	1.20 ±0.16	1.10 ±0.20	1.20 ±0.20	1.20 ±0.16	2.20 ±0.34	1.90 ±0.26	1.31 ±0.16
	$NADH/NAD^+$	3.09	2.74	2.47	2.92	2.91	3.33	3.01	2.83	2.08	2.09	2.00	2.46
Gill	NADH	3.90 ±0.55	3.90 ±0.65	4.29 ±0.65	3.09 ±0.40	3.10 ±0.38	4.32 ±0.40	3.81 ±0.38	4.20 ±0.45	2.59 ±0.26	4.60 ±0.50	4.02 ±0.50	3.38 ±0.38
	NAD^+	1.20 ±0.30	1.40 ±0.34	1.59 ±0.34	1.09 ±0.20	1.00 ±0.09	1.31 ±0.26	1.31 ±0.20	1.40 ±0.26	1.91 ±0.30	2.40 ±0.38	2.12 ±0.34	1.39 ±0.26
	$NADH/NAD^+$	3.25	2.79	2.70	2.83	3.10	3.30	2.91	3.00	1.36	1.92	1.90	2.43

TABLE 13. Effect of exposure to various temperatures for 10 days in June, 1989 (ambient temp. 20±2°C) on GDH (NADH and NAD^+ dependent) activity and their ratio ($NADH/NAD^+$) in various tissues of *H. fossilis* (Mean±S.D.)

Tissue	Enzyme	10±2°C		20±2°C control		30±2°C	
		T.A.	S.A.	T.A.	S.A.	T.A.	S.A.
Liver	NADH	8.26±0.65 (-27)[a] <0.001[b]	5.95±0.55 (-35) <0.001	11.25±1.12	9.14±1.01	12.45±1.25 (11)[a] N.S.[b]	9.93±0.99 (9) N.S.
	NAD^+	4.89±0.50 (45)[a] <0.001[b]	3.52±0.38 (28) <0.01	3.38±0.40	2.75±0.34	5.84±0.55 (73)[a] <0.001[b]	4.66±0.46 (70) <0.001
	$NADH/NAD^+$	1.69	1.69	3.33	3.32	2.13	2.13
Kidney	NADH	4.45±0.46 (-19)[a] <0.02[b]	3.96±0.40 (-21) <0.005	5.50±0.55	5.03±0.46	5.75±0.55 (5)[a] N.S.[b]	5.21±0.55 (4) N.S.
	NAD^+	2.55±0.40 (32)[a] <0.05[b]	2.27±0.26 (28) <0.02	1.93±0.34	1.77±0.26	2.21±0.34 (15)[a] N.S.[b]	2.00±0.26 (13) N.S.
	$NADH/NAD^+$	1.75	1.74	2.85	2.84	2.60	2.61
Brain	NADH	2.22±0.46 (-19)[a] N.S.[b]	4.32±0.46 (-16) <0.05	2.74±0.38	5.15±0.50	3.38±0.38 (23)[a] <0.05[b]	6.76±0.65 (31) <0.005
	NAD^+	0.83±0.20 (14)[a] N.S.[b]	1.61±0.26 (18) N.S.	0.73±0.16	1.37±0.34	0.93±0.16 (27)[a] N.S.[b]	1.86±0.26 (36) <0.05
	$NADH/NAD^+$	2.67	2.68	3.75	3.76	3.63	3.63
Muscle	NADH	1.93±0.38 (-14)[a] N.S.[b]	3.28±0.34 (-16) <0.02	2.25±0.34	3.90±0.34	2.37±0.26 (5)[a] N.S.[b]	4.07±0.38 (4) N.S.
	NAD^+	0.81±0.16 (3)[a] N.S.[b]	1.38±0.30 (1) N.S.	0.79±0.20	1.37±0.26	0.93±0.16 (18)[a] N.S.[b]	1.60±0.16 (17) N.S.
	$NADH/NAD^+$	2.38	2.38	2.85	2.85	2.55	2.54
Gill	NADH	2.01±0.38 (-18)[a] N.S.[b]	3.69±0.38 (-13) <0.05	2.45±0.38	4.26±0.40	2.68±0.34 (9)[a] N.S.[b]	4.41±0.46 (4) N.S.
	NAD^+	0.83±0.16 (14)[a] N.S.[b]	1.52±0.26 (20) N.S.	0.73±0.10	1.27±0.26	0.93±0.20 (27)[a] N.S.[b]	1.53±0.34 (21) N.S.
	$NADH/NAD^+$	2.42	2.43	3.36	3.35	2.88	2.88

T.A. - Total activity (units/g wet wt.), S.A. - Specific activity (units/mg protein) $\times 10^2$

a - % change compared to control; b - p value compared to control.

TABLE 14. Effect of exposure to various temperatures for 10 days in December, 1989 (ambient temp. 20±2°C) on GDH (NADH and NAD^+ dependent) activity and their ratio ($NADH/NAD^+$) in various tissues of *H. fossilis*. (Mean±S.D.)

Tissue	Enzyme	10±2°C		20±2°C control		30±2°C	
		T.A.	S.A.	T.A.	S.A.	T.A.	S.A.
Liver	NADH	10.95±0.90 (38)[a] <0.001[b]	8.66±0.85 (41) <0.001	7.93±0.65	6.13±0.55	14.25±1.25 (80)[a] <0.001[b]	10.90±0.99 (78) <0.001
	NAD^+	4.06±0.40 (-21)[a] <0.005[b]	3.21±0.38 (-19) <0.02	5.15±0.50	3.98±0.40	3.38±0.38 (-34)[a] <0.001[b]	2.59±0.38 (-35) <0.001
	$NADH/NAD^+$	2.70	2.70	1.54	1.54	4.22	4.21
Kidney	NADH	5.38±0.55 (23)[a] <0.02[b]	5.09±0.55 (27) <0.01	4.38±0.46	4.02±0.40	6.45±0.65 (47)[a] <0.001[b]	5.64±0.55 (40) <0.001
	NAD^+	2.75±0.38 (8)[a] N.S.[b]	2.60±0.34 (12) N.S.	2.54±0.38	2.33±0.34	2.22±0.30 (-13)[a] N.S.[b]	1.94±0.26 (-17) N.S.
	$NADH/NAD^+$	1.96	1.96	1.72	1.73	2.91	2.91
Brain	NADH	2.89±0.46 (20)[a] N.S.[b]	5.54±0.55 (25) <0.01	2.41±0.34	4.44±0.46	3.38±0.34 (40)[a] <0.005[b]	6.71±0.65 (51) <0.001
	NAD^+	0.93±0.16 (-20)[a] N.S.[b]	1.78±0.26 (-17) N.S.	1.16±0.20	2.14±0.26	0.81±0.26 (-30)[a] <0.05[b]	1.61±0.30 (-25) <0.02
	$NADH/NAD^+$	3.11	3.11	2.08	2.07	4.17	4.17
Muscle	NADH	2.41±0.30 (9)[a] N.S.[b]	3.94±0.38 (20) <0.05	2.22±0.30	3.29±0.38	2.79±0.38 (26)[a] <0.05[b]	4.19±0.46 (27) <0.01
	NAD^+	0.73±0.16 (-10)[a] N.S.[b]	1.19±0.26 (-1) N.S.	0.81±0.16	1.20±0.20	0.65±0.16 (-20)[a] N.S.[b]	0.98±0.10 (-18) N.S.
	$NADH/NAD^+$	3.30	3.31	2.74	2.74	4.29	4.28
Gill	NADH	2.93±0.38 (22)[a] N.S.[b]	4.94±0.46 (17) <0.05	2.41±0.38	4.24±0.40	3.22±0.34 (34)[a] <0.01[b]	5.21±0.55 (23) <0.02
	NAD^+	0.93±0.26 (-8)[a] N.S.[b]	1.57±0.30 (-12) N.S.	1.01±0.26	1.78±0.26	0.93±0.26 (-8)[a] N.S.[b]	1.51±0.26 (-15) N.S.
	$NADH/NAD^+$	3.15	3.15	2.39	2.38	3.46	3.45

T.A. - Total activity (units/g wet wt.), S.A. - Specific activity (units/mg protein)$\times 10^2$
a - % change compared to control; b - p value compared to control.

TABLE 15. Alterations in the total activity (units/g wet wt.) of GDH (NADH dependent) in various tissues of *H. fossilis* during water deprivation. (Mean±S.D.)

Tissue	Time (in hours) of water deprivation															
	0 Control	$\frac{1}{2}$	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	7.33 ±0.72	8.97 ±0.63[a] (22) < 0.01[b]	9.27 ±0.81 (27) <0.005	9.54 ±0.54 (30) <0.001	9.72 ±0.71 (33) <0.001	9.99 ±0.80 (36) <0.001	11.07 ±1.26 (51) < 0.001	12.42 ±1.71 (69) <0.001	14.80 ±0.80 (102) <0.001	14.94 ±0.45 (104) < 0.001	15.03 ±0.54 (105) <0.001	15.48 ±0.45 (111) <0.001	15.54 ±0.54 (112) < 0.001	15.75 ±0.45 (115) <0.001	15.93 ±0.90 (117) <0.001	16.92 ±0.72 (131) < 0.001
Kidney	4.05 ±0.54	4.14 ±0.72[a] (2) N.S.[b]	4.41 ±0.63 (9) N.S.	4.77 ±0.81 (18) N.S.	5.04 ±0.36 (25) < 0.01	5.49 ±0.72 (36) <0.01	6.21 ±0.81 (53) <0.005	6.57 ±0.54 (62) < 0.001	6.66 ±0.36 (64) <0.001	6.84 ±0.54 (69) <0.001	6.29 ±0.54 (55) < 0.001	6.03 ±0.54 (49) <0.001	6.30 ±0.72 (56) < 0.001	6.66 ±0.54 (64) <0.001	6.30 ±0.72 (56) < 0.001	6.30 ±0.59 (56) < 0.001
Brain	2.16 ±0.45	2.61 ±0.27[a] (21) N.S.[b]	2.79 ±0.63 (29) N.S.	3.15 ±0.27 (46) < 0.005	3.33 ±0.36 (54) <0.005	3.45 ±0.36 (60) <0.005	3.78 ±0.27 (75) < 0.001	4.41 ±0.54 (104) <0.001	4.59 ±0.45 (113) <0.001	4.77 ±0.63 (121) <0.001	4.68 ±0.63 (117) < 0.001	4.63 ±0.72 (114) <0.001	5.13 ±0.72 (138) <0.001	5.04 ±0.54 (133) <0.001	5.13 ±0.81 (138) <0.001	5.13 ±0.63 (138) <0.001
Muscle	2.25 ±0.36	2.52 ±0.18[a] (12) N.S.[b]	2.70 ±0.27 (20) N.S.	2.70 ±0.54 (20) N.S.	2.79 ±0.18 (24) <0.02	2.97 ±0.45 (32) <0.025	3.33 ±0.45 (48) <0.005	3.51 ±0.45 (56) < 0.005	3.33 ±0.45 (48) <0.005	3.42 ±0.45 (52) <0.005	3.24 ±0.36 (44) < 0.005	3.33 ±0.45 (48) <0.005	3.42 ±0.36 (52) <0.005	3.33 ±0.45 (48) < 0.005	3.42 ±0.45 (52) <0.005	3.51 ±0.45 (56) < 0.005
Gill	2.61 ±0.27	2.61 ±0.27[a] (0) N.S.[b]	2.70 ±0.27 (3) N.S.	2.79 ±0.18 (7) N.S.	2.88 ±0.36 (10) N.S.	3.33 ±0.27 (28) < 0.005	3.42 ±0.36 (31) <0.005	3.58 ±0.45 (37) < 0.005	3.42 ±0.54 (31) <0.02	3.51 ±0.54 (35) <0.02	3.33 ±0.45 (28) <0.02	3.33 ±0.45 (28) < 0.02	3.51 ±0.45 (35) <0.005	3.78 ±0.36 (45) < 0.001	4.23 ±0.18 (62) < 0.001	4.77 ±0.27 (83) < 0.001

a - % change compared to control
b - p value compared to control

TABLE 16. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH dependent) in various tissues of <u>H. fossilis</u> during water deprivation. (Mean±S.D.)

Tissue	Time (in hours) of water deprivation															
	0 Control	½	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	9.90 ±1.80	11.70 ±0.90 (18)[a] N.S.[b]	12.60 ±0.90 (27) <0.02	12.60 ±0.90 (27) <0.02	13.00 ±1.80 (31) <0.05	13.50 ±1.80 (36) <0.02	15.30 ±1.80 (55) <0.005	16.20 ±1.80 (64) <0.001	19.80 ±1.80 (100) <0.001	19.80 ±0.90 (100) <0.001	19.80 ±0.90 (100) <0.001	20.70 ±0.90 (109) <0.001	20.70 ±0.90 (109) <0.001	20.70 ±0.90 (109) <0.001	21.10 ±0.80 (113) <0.001	21.60 ±0.90 (118) <0.001
Kidney	4.50 ±0.63	4.70 ±0.90 (4)[a] N.S.[b]	5.20 ±0.90 (16) N.S.	5.40 ±0.63 (20) N.S.	5.60 ±0.36 (24) <0.01	6.30 ±0.90 (40) <0.01	7.20 ±0.90 (60) <0.001	7.70 ±0.90 (71) <0.001	7.49 ±0.90 (66) <0.001	7.70 ±0.63 (71) <0.001	7.40 ±0.63 (64) <0.001	6.70 ±0.45 (49) <0.001	7.00 ±0.90 (56) <0.001	7.49 ±0.63 (66) <0.001	7.20 ±0.27 (60) <0.001	7.20 ±0.90 (60) <0.001
Brain	3.60 ±0.81	4.50 ±0.45 (25)[a] N.S.[b]	5.40 ±0.90 (50) <0.02	5.80 ±0.45 (61) <0.001	6.10 ±0.90 (69) <0.005	6.10 ±0.90 (69) <0.005	6.70 ±0.45 (86) <0.001	7.20 ±0.90 (100) <0.001	8.00 ±0.90 (122) <0.001	8.30 ±0.90 (131) <0.001	8.80 ±0.36 (144) <0.001	8.80 ±0.90 (144) <0.001	9.00 ±1.80 (150) <0.001	8.80 ±0.36 (144) <0.001	8.50 ±0.90 (136) <0.001	8.50 ±0.90 (136) <0.001
Muscle	3.20 ±0.45	3.80 ±0.36 (19)[a] <0.05[b]	4.10 ±0.45 (28) <0.02	4.10 ±0.45 (28) <0.02	4.30 ±0.36 (34) <0.005	4.70 ±0.75 (47) <0.005	5.20 ±0.90 (63) <0.005	5.60 ±0.90 (75) <0.001	5.40 ±0.90 (69) <0.005	5.40 ±0.90 (69) <0.005	5.20 ±0.90 (63) <0.005	5.20 ±0.90 (63) <0.005	5.40 ±0.90 (69) <0.005	5.20 ±0.90 (63) <0.005	5.40 ±0.90 (69) <0.005	5.80 ±0.90 (81) <0.001
Gill	4.70 ±0.45	4.70 ±0.45 (0)[a] N.S.[b]	5.00 ±0.45 (6) N.S.	5.20 ±0.36 (11) N.S.	5.40 ±0.63 (15) N.S.	6.30 ±0.90 (34) <0.01	6.50 ±0.90 (38) <0.005	6.80 ±0.90 (45) <0.005	6.50 ±0.90 (38) <0.005	6.50 ±0.90 (38) <0.005	6.10 ±0.90 (30) <0.02	6.30 ±0.90 (34) <0.01	6.10 ±0.90 (30) <0.02	6.70 ±0.90 (43) <0.005	7.20 ±0.90 (53) <0.001	8.80 ±0.90 (87) <0.001

a - % change compared to control

b - p value compared to control

TABLE 17. Alterations in the total activity (units/g wet wt.) of GDH (NAD^+ dependent) in various tissues of H. fossilis during water deprivation. (Mean ± S.D.)

Tissue	Time (in hours) of water deprivation															
	0 Control	$\frac{1}{2}$	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	2.88 ±0.27	3.15 ±0.27 (9)[a] N.S.[b]	3.15 ±0.54 (9) N.S.	3.33 ±0.27 (16) < 0.05	3.42 ±0.27 (19) <0.02	3.87 ±0.56 (34) < 0.01	4.23 ±0.72 (47) <0.005	4.50 ±0.18 (56) <0.001	4.86 ±0.45 (69) <0.001	5.49 ±0.45 (91) <0.001	5.85 ±0.54 (103) <0.001	6.03 ±0.36 (109) <0.001	6.05 ±0.45 (110) <0.001	6.29 ±0.27 (118) <0.001	6.39 ±0.45 (122) <0.001	6.48 ±0.36 (125) < 0.001
Kidney	1.17 ±0.27	1.26 ±0.18 (8)[a] N.S.[b]	1.31 ±0.18 (12) N.S.	1.35 ±0.18 (15) N.S.	1.44 ±0.18 (23) N.S.	1.71 ±0.18 (46) <0.01	2.07 ±0.36 (77) < 0.005	2.43 ±0.18 (108) <0.001	2.07 ±0.18 (77) <0.001	2.08 ±0.18 (78) <0.001	2.30 ±0.36 (97) <0.001	2.25 ±0.36 (92) <0.001	2.34 ±0.36 (100) <0.001	2.38 ±0.27 (103) <0.001	2.16 ±0.36 (85) < 0.005	2.16 ±0.36 (85) < 0.005
Brain	0.69 ±0.09	0.81 ±0.09 (17)[a] N.S.[b]	0.96 ±0.18 (39) <0.02	0.99 ±0.09 (44) <0.001	1.17 ±0.18 (70) <0.001	1.35 ±0.09 (96) <0.001	1.35 ±0.18 (96) < 0.001	1.39 ±0.18 (101) < 0.001	1.21 ±0.18 (75) <0.001	1.44 ±0.18 (109) <0.001	1.39 ±0.18 (101) < 0.001	1.39 ±0.27 (101) <0.001	1.62 ±0.27 (135) <0.001	1.76 ±0.27 (155) < 0.001	1.53 ±0.16 (122) < 0.001	1.57 ±0.27 (128) < 0.001
Muscle	0.54 ±0.18	0.58 ±0.18 (7)[a] N.S.[b]	0.58 ±0.18 (7) N.S.	0.62 ±0.18 (15) N.S.	0.69 ±0.18 (28) N.S.	0.77 ±0.18 (43) N.S.	0.85 ±0.09 (57) < 0.01	0.89 ±0.09 (65) < 0.005	0.93 ±0.18 (72) <0.01	0.89 ±0.09 (65) < 0.005	0.89 ±0.09 (65) < 0.005	0.85 ±0.09 (57) <0.01	0.85 ±0.09 (57) <0.01	0.77 ±0.18 (43) N.S.	0.77 ±0.18 (43) N.S.	0.72 ±0.18 (33) N.S.
Gill	0.63 ±0.09	0.66 ±0.09 (5)[a] N.S.[b]	0.72 ±0.18 (14) N.S.	0.77 ±0.09 (22) < 0.05	0.81 ±0.18 (29) N.S.	0.81 ±0.18 (29) N.S.	0.89 ±0.18 (41) < 0.02	0.96 ±0.18 (52) < 0.01	1.08 ±0.09 (71) <0.001	0.94 ±0.18 (49) < 0.01	0.90 ±0.18 (43) <0.02	0.95 ±0.09 (51) <0.001	1.08 ±0.18 (71) <0.001	1.17 ±0.18 (86) < 0.001	1.17 ±0.18 (86) < 0.001	1.20 ±0.18 (91) < 0.001

a - % change compared to control
b - p value compared to control

TABLE 18. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NAD^+ dependent) in various tissue of <u>H. fossilis</u> during water deprivation. (Mean±S.D.)

Tissue	Time (in hours) of water deprivation															
	0 Control	½	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	3.60 ±0.36	4.50 ±0.36 (25)[a] <0.005[b]	4.50 ±0.36 (25) <0.005	4.70 ±0.36 (31) <0.005	4.70 ±0.36 (31) <0.005	5.40 ±0.90 (50) <0.005	5.60 ±0.90 (56) <0.005	6.30 ±0.36 (75) <0.001	6.30 ±0.63 (75) <0.001	7.20 ±0.90 (100) <0.001	7.80 ±0.90 (117) <0.001	7.90 ±0.36 (119) <0.001	8.10 ±0.68 (125) <0.001	8.60 ±0.45 (139) <0.001	8.10 ±0.27 (125) <0.001	8.50 ±0.25 (136) <0.001
Kidney	1.30 ±0.45	1.60 ±0.36 (23)[a] N.S.[b]	1.60 ±0.36 (23) N.S.	1.80 ±0.36 (39) N.S.	1.80 ±0.36 (39) N.S.	1.90 ±0.18 (46) <0.025	2.20 ±0.45 (69) <0.02	2.70 ±0.45 (108) <0.005	2.40 ±0.36 (85) <0.005	2.40 ±0.36 (85) <0.005	2.30 ±0.45 (77) <0.01	2.50 ±0.36 (92) <0.005	2.50 ±0.36 (92) <0.005	2.60 ±0.27 (100) <0.001	2.30 ±0.45 (77) <0.01	2.30 ±0.45 (77) <0.01
Brain	1.40 ±0.45	1.50 ±0.27 (7)[a] N.S.[b]	1.70 ±0.27 (21) N.S.	1.80 ±0.18 (29) N.S.	1.90 ±0.36 (36) N.S.	2.50 ±0.36 (79) <0.005	2.30 ±0.45 (64) <0.02	2.50 ±0.36 (79) <0.005	2.30 ±0.45 (64) <0.02	2.70 ±0.27 (93) <0.001	2.50 ±0.36 (79) <0.005	2.30 ±0.45 (64) <0.02	2.70 ±0.45 (93) <0.005	3.20 ±0.45 (129) <0.001	2.70 ±0.45 (93) <0.005	2.70 ±0.45 (93) <0.005
Muscle	0.81 ±0.18	0.90 ±0.18 (11)[a] N.S.[b]	0.90 ±0.18 (11) N.S.	0.99 ±0.18 (22) N.S.	1.10 ±0.36 (36) N.S.	1.20 ±0.36 (48) N.S.	1.30 ±0.36 (61) <0.025	1.40 ±0.45 (73) <0.025	1.44 ±0.45 (78) <0.02	1.40 ±0.45 (73) <0.025	1.44 ±0.45 (78) <0.02	1.30 ±0.36 (61) <0.025	1.30 ±0.36 (61) <0.025	1.10 ±0.36 (36) N.S.	1.10 ±0.36 (36) N.S.	1.30 ±0.36 (61) <0.025
Gill	1.10 ±0.36	1.30 ±0.45 (18)[a] N.S.	1.44 ±0.45 (31) N.S.	1.60 ±0.36 (46) N.S.	1.44 ±0.45 (31) N.S.	1.60 ±0.36 (46) N.S.	1.80 ±0.36 (64) <0.02	1.90 ±0.36 (73) <0.01	2.20 ±0.45 (100) <0.005	1.90 ±0.45 (73) <0.02	1.90 ±0.45 (73) <0.02	1.90 ±0.45 (73) <0.02	2.10 ±0.36 (91) <0.005	2.30 ±0.45 (109) <0.005	2.20 ±0.36 (100) <0.005	2.20 ±0.36 (100) <0.005

a - % change compared to control
b - p value compared to control

TABLE 19. Alterations in the ratio of the GDH (NADH/NAD^+ dependent) total activity in various tissues of _H. fossilis_ during water deprivation. (Mean±S.D.)

Tissue	Time (in hours) of water deprivation															
	O Control	1/2	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	2.53 ±0.43	2.85 ±0.15	2.96 ±0.27	2.88 ±0.22	2.86 ±0.57	2.71 ±0.45	2.81 ±0.41	2.78 ±0.26	3.03 ±0.63	2.74 ±0.29	2.57 ±0.24	2.56 ±0.18	2.57 ±0.28	2.48 ±0.14	2.52 ±0.29	2.59 ±0.29
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Kidney	3.45 ±0.44	3.32 ±0.85	3.44 ±0.73	3.52 ±0.53	3.53 ±0.57	3.77 ±0.47	2.81 ±0.52	2.82 ±0.53	3.79 ±0.45	3.45 ±0.59	3.06 ±0.54	2.84 ±0.58	3.03 ±0.43	2.74 ±0.76	3.09 ±0.60	2.91 ±0.39
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Brain	3.04 ±0.32	3.27 ±0.50	2.76 ±0.56	3.16 ±0.29	3.02 ±0.39	2.70 ±0.22	2.89 ±0.27	3.13 ±0.47	3.83 ±0.85	3.25 ±0.29	3.38 ±0.46	3.47 ±0.55	3.12 ±0.27	2.90 ±0.59	3.90 ±0.94	3.26 ±0.33
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Muscle	4.34 ±1.27	4.55 ±0.73	4.89 ±0.74	4.83 ±0.55	4.12 ±0.73	3.63 ±0.48	3.92 ±0.62	4.16 ±0.59	3.71 ±0.43	3.95 ±0.46	3.47 ±0.55	3.93 ±0.58	4.24 ±0.59	4.07 ±0.65	4.63 ±0.55	5.16 ±1.16
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Gill	4.29 ±0.75	4.29 ±0.75	3.69 ±0.96	3.87 ±0.66	4.15 ±0.74	4.24 ±0.79	4.33 ±0.73	4.41 ±0.72	3.42 ±0.59	4.12 ±0.90	3.60 ±0.73	3.76 ±0.49	3.08 ±1.25	3.15 ±0.95	3.24 ±0.93	4.00 ±0.64
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

a - p value compared to control

TABLE 20. Alterations in the rato of the GDH ($NADH/NAD^+$ dependent) specific activity in various tissues of *H. fossilis* during water deprivation. (Mean±S.D.)

Tissue	Time (in hours) of water deprivation														
	0 Control	½	1	2	3	6	9	12	15	18	21	24	27	30	33	36
Liver	2.66 ±0.38	2.81 ±0.33	2.95 ±0.28	2.74 ±0.19	2.76 ±0.27	2.77 ±0.27	2.76 ±0.39	2.74 ±0.37	2.95 ±0.29	2.88 ±0.20	2.62 ±0.29	2.60 ±0.23	2.58 ±0.31	2.50 ±0.31	2.55 ±0.26	2.68 ±0.12
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Kidney	3.90 ±1.14	3.30 ±0.52	3.40 ±0.56	3.00 ±0.75	3.48 ±0.32	3.46 ±0.45	3.28 ±0.48	3.10 ±0.55	2.74 ±0.95	3.30 ±0.50	3.26 ±0.48	3.07 ±0.76	2.98 ±0.88	2.77 ±0.95	3.20 ±0.47	3.20 ±0.51
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Brain	2.90 ±0.65	3.37 ±0.57	2.93 ±0.38	3.22 ±0.50	3.07 ±0.48	2.73 ±0.33	3.20 ±0.68	3.03 ±0.38	3.45 ±0.64	3.17 ±0.34	3.33 ±0.58	3.73 ±0.89	3.48 ±0.58	2.78 ±0.51	3.43 ±0.62	3.23 ±0.55
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Muscle	3.60 ±0.55	4.39 ±0.65	4.56 ±0.94	4.01 ±0.78	3.75 ±0.53	4.35 ±0.78	3.49 ±0.57	4.45 ±0.84	3.90 ±0.52	3.64 ±0.48	3.50 ±0.28	4.09 ±0.58	4.30 ±0.78	4.60 ±0.94	4.20 ±0.69	4.22 ±0.82
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Gill	4.90 ±1.43	4.40 ±0.78	3.90 ±0.72	3.50 ±0.76	3.90 ±0.74	3.40 ±0.88	3.60 ±0.82	3.33 ±0.99	3.53 ±0.94	3.70 ±0.85	3.40 ±0.84	3.50 ±0.85	3.00 ±1.43	3.00 ±1.45	3.20 ±1.28	4.27 ±0.57
		N.S.[a]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

a - p value compared to control

TABLE 21. Alterations in the total free amino acids (µ moles/g wet wt.) in various tissues of _H. fossilis_ during starvation and refeeding. (Mean±S.D.)

Tissue	Normal control	Starvation			Refeeding from 7 to 14 days
		3 days	7 days	14 days	
Liver	112.00±10.63	135.51±9.55	144.49±9.49	153.08±11.03	139.90±10.15
		(21)[a]	(29)	(37)	(25)
		< 0.01[b]	< 0.001	< 0.001	< 0.005
Kidney	98.69±7.58	114.19±7.21	126.69±8.45	146.18±7.79	120.55±9.05
		(16)[a]	(28)	(48)	(22)
		< 0.02[b]	< 0.001	< 0.001	< 0.005
Brain	67.72±4.81	77.60±5.74	82.45±6.25	91.24±9.57	83.64±8.60
		(15)[a]	(22)	(35)	(24)
		< 0.02[b]	< 0.005	< 0.005	< 0.02
Muscle	52.93±5.56	65.45±5.99	73.50±5.95	79.22±9.23	75.15±7.55
		(24)[a]	(39)	(50)	(42)
		< 0.01[b]	< 0.001	< 0.001	< 0.001
Gill	51.10±4.79	55.77±5.59	69.65±4.98	69.84±8.65	68.70±7.40
		(9)[a]	(36)	(37)	(34)
		N.S.[b]	< 0.001	< 0.005	< 0.005

a - % change compared to normal control

b - p value compared to normal control

TABLE 22. Alterations in the total free amino acids (µmoles/g wet wt.) in various tissues of _H. fossilis_ during starvation and hyper-ammonia stress. (Mean±S.D.)

	Tissue	Normal	3 days	7 days	14 days
Normal/ Starved Control	Liver	112.00±10.63	135.51±9.55 (21)[a]	144.49±9.49 (29)	153.08±11.03 (37)
	Kidney	98.69±7.58	114.19±7.21 (16)[a]	126.69±8.45 (28)	146.18±7.79 (48)
	Brain	67.72±4.81	77.60±5.74 (15)[a]	82.45±6.25 (22)	91.24±9.57 (35)
	Muscle	52.93±5.56	65.48±5.99 (24)[a]	73.50±5.95 (39)	79.22±9.23 (50)
	Gill	51.10±4.79	55.77±5.59 (9)[a]	69.65±4.98 (36)	69.84±8.65 (37)
50mM (NH_4Cl) medium	Liver		181.20±8.65 (34)[b] <0.001[c]	231.06±12.49 (60) <0.001	276.34±15.64 (81) < 0.001
	Kidney		148.85±7.93 (30)[b] <0.001[c]	195.75±9.35 (55) <0.001	232.50±14.85 (59) < 0.001
	Brain		90.34±7.30 (16)[b] <0.02[c]	119.80±7.65 (45) <0.001	150.75±10.38 (65) < 0.001
	Muscle		87.55±6.84 (34)[b] <0.001[c]	116.45±9.35 (58) <0.001	139.82±10.15 (76) < 0.001
	Gill		69.75±5.75 (25)[b] <0.005[c]	92.40±8.55 (33) <0.001	99.89±9.98 (43) < 0.001
75mM (NH_4Cl) medium	Liver		219.15±10.86 (62)[b] <0.001[c]	254.75±15.35 (76) <0.001	290.45±18.25 (90) < 0.001
	Kidney		168.70±9.92 (48)[b] <0.001[c]	216.45±10.12 (71) <0.001	249.10±17.05 (70) < 0.001
	Brain		114.62±9.35 (48)[b] <0.001[c]	132.50±9.24 (61) <0.001	170.64±13.36 (87) < 0.001
	Muscle		98.75±9.80 (51)[b] <0.001[c]	128.72±10.65 (75) <0.001	147.70±11.40 (86) < 0.001
	Gill		75.46±8.99 (35)[b] <0.005[c]	104.35±8.20 (50) <0.001	115.85±10.78 (66) < 0.001
100mM (NH_4Cl) medium	Liver		221.33±13.35 (63)[b] <0.001[c]	Did not survive	
	Kidney		170.50±10.75 (49)[b] <0.001[c]		
	Brain		116.45±9.48 (50)[b] <0.001[c]		
	Muscle		99.80±10.02 (52)[b] <0.001[c]		
	Gill		78.38±8.79 (41)[b] <0.005[c]		

a - % change compared to normal control
b - % change compared to starved control
c - p value compared to starved control

TABLE 23. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of *H. fossilis* during starvation and refeeding. (Mean ± S.D.)

Tissue	Normal control			Starvation									Refeeding from 7 to 14 days		
				3 days			7 days			14 days					
	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$
Liver	7.42 ±0.55	5.11 ±0.55	1.45	9.45 ±0.55 (27)[a] < 0.001[b]	9.74 ±0.68 (91) <0.001	0.97	8.87 ±0.81 (20) <0.01	11.19 ± 1.25 (119) <0.001	0.79	9.45 ±0.55 (27) <0.001	10.22 ± 0.79 (100) <0.001	0.92	9.45 ±0.55 (27)[a] <0.001[b]	7.33 ±0.41 (43) <0.001	1.29
Kidney	3.67 ±0.55	2.41 ±0.34	1.52	4.24 ±0.63 (16)[a] N.S.[b]	4.34 ±0.90 (80) < 0.005	0.98	3.96 ±0.40 (8) N.S.	4.92 ±0.72 (104) <0.001	0.80	4.63 ±0.55 (26) <0.025	3.86 ±0.34 (60) <0.001	1.20	4.34 ±0.34 (18) <0.05	3.09 ±0.55 (28) <0.05	1.41
Brain	2.03 ±0.40	2.03 ±0.40	1.00	2.22 ±0.26 (9)[a] N.S.[b]	2.60 ±0.43 (28) N.S.	0.85	2.22 ±0.26 (9) N.S.	2.41 ±0.34 (19) N.S.	0.92	2.41 ±0.34 (19) N.S.	2.51 ±0.41 (24) N.S.	0.96	2.31 ±0.40 (14) N.S.	1.83 ±0.40 (-10) N.S.	1.26
Muscle	1.16 ±0.27	1.16 ±0.27	1.00	1.93 ±0.34 (66)[a] < 0.005[b]	1.93 ±0.34 (66) <0.005	1.00	1.74 ±0.26 (50) <0.01	1.83 ±0.40 (58) <0.02	0.95	1.74 ±0.26 (50) < 0.01	1.45 ±0.34 (25) N.S.	1.20	1.25 ±0.27 (8) N.S.	0.96 ±0.34 (-17) N.S.	1.30
Gill	1.35 ±0.41	1.34 ±0.27	1.01	2.22 ±0.26 (64)[a] < 0.005[b]	2.31 ±0.40 (72) <0.005	0.96	2.03 ±0.40 (50) < 0.05	1.93 ±0.34 (44) <0.02	1.05	2.12 ±0.43 (57) <0.02	2.23 ±0.26 (66) <0.001	0.95	1.54 ±0.41 (14) N.S.	1.25 ±0.27 (-7) N.S.	1.23

a - % change compared to normal control

b - p value compared to normal control

TABLE 24. Alteration in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio (NADH/NAD^+) in various tissues of <u>H. fossilis</u> during starvation and refeeding. (Mean±S.D.)

Tissue	Normal control			Starvation									Refeeding from 7 to 14 days		
				3 days			7 days			14 days					
	NADH	NAD^+	NADH/NAD^+	NADH	NAD^+	NADH/NAD^+	NADH	NAD^+	NADH/NAD^+	NADH	NAD^+	NADH/NAD^+	NADH	NAD^+	NADH/NAD^+
Liver	4.80 ±0.50	3.30 ±0.40	1.45	6.40 ±0.50 (33)[a] <0.001[b]	6.60 ±0.65 (100) <0.001	0.97	6.70 ±0.90 (40) <0.005	8.30 ±0.80 (152) <0.001	0.81	7.40 ±0.70 (54) <0.001	8.00 ±0.90 (142) <0.001	0.93	6.30 ±0.80 (31)[a] <0.01[b]	4.90 ±0.46 (49) <0.001	1.29
Kidney	2.80 ±0.40	1.84 ±0.36	1.52	3.20 ±0.40 (14)[a] N.S.[b]	3.30 ±0.50 (79) <0.001	0.97	3.40 ±0.40 (21) <0.05	4.20 ±0.60 (128) <0.001	0.81	4.10 ±0.50 (46) <0.005	3.40 ±0.30 (85) <0.001	1.21	3.40 ±0.30 (21) <0.05	2.40 ±0.36 (30) <0.05	1.42
Brain	3.40 ±0.46	3.40 ±0.46	1.00	4.00 ±0.50 (18)[a] N.S.[b]	4.70 ±0.96 (38) < 0.05	0.85	4.60 ±0.50 (35) <0.005	4.90 ±0.90 (44) < 0.02	0.94	5.50 ±0.60 (62) <0.001	5.70 ±0.90 (68) < 0.001	0.96	4.00 ±0.40 (18) N.S.	3.20 ±0.36 (-6) N.S.	1.25
Muscle	2.10 ±0.30	2.10 ±0.30	1.00	3.40 ±0.50 (62)[a] <0.001[b]	3.40 ±0.50 (62) <0.001	1.00	3.20 ±0.40 (52) <0.005	3.40 ±0.46 (62) <0.001	0.94	4.30 ±0.50 (105) <0.001	3.60 ±0.60 (71) <0.001	1.19	2.30 ±0.40 (10) N.S.	1.70 ±0.26 (-19) N.S.	1.35
Gill	2.80 ±0.36	2.80 ±0.40	1.00	4.60 ±0.50 (64)[a] < 0.001[b]	4.80 ±0.75 (71) <0.001	0.96	4.90 ±0.80 (75) <0.001	4.60 ±0.50 (64) < 0.001	1.07	5.10 ±0.90 (82) <0.001	5.40 ±0.90 (93) <0.001	0.94	3.10 ±0.46 (11) N.S.	2.50 ±0.36 (-11) N.S.	1.24

a = % change compared to normal control

b = p value compared to normal control

TABLE 25. Alterations in the total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in the liver of _H. foissilis_ during starvation and hyper-ammonia stress. (Mean±S.D.)

		Normal			3 days			7 days			14 days		
		NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+
Normal/ Starved Control	T.A.	7.42 ±0.55	5.11 ±0.55	1.45	9.45±0.55 (27)[a] <0.001[b]	9.74±0.68 (91) <0.001	0.97	8.87±0.81 (20) <0.01	11.19±1.25 (119) <0.001	0.79	9.45±0.55 (27) <0.001	10.22±0.79 (100) <0.001	0.92
	S.A.	4.80 ±0.50	3.30 ±0.40	1.45	6.40±0.50 (33)[a] <0.001[b]	6.60±0.65 (100) <0.001	0.97	6.70±0.90 (40) <0.005	8.30±0.80 (152) <0.001	0.81	7.40±0.70 (54) <0.001	8.00±0.90 (142) <0.001	0.93
50mM (NH_4Cl medium	T.A.				10.99±0.28 (16)[c] <0.001[d]	14.76±0.80 (52) <0.001	0.74	10.42±0.81 (18) <0.02	14.76±0.55 (32) <0.001	0.71	13.50±0.70 (43) <0.001	16.11±1.30 (58) <0.001	0.84
	S.A.				7.40±1.30 (16)[c] N.S.[d]	9.90±1.30 (50) <0.001	0.75	7.80±0.50 (16) <0.05	11.00±0.50 (33) <0.001	0.71	10.50±0.90 (42) <0.001	12.5±1.10 (56) <0.001	0.84
75mM (NH_4Cl) medium	T.A.				14.95±1.00 (58)[c] <0.001[d]	15.05±0.79 (55) <0.001	0.99	12.83±1.26 (45) <0.001	23.54±0.92 (110) <0.001	0.55	15.05±0.63 (59) <0.001	19.19±1.82 (88) <0.001	0.78
	S.A.				10.10±1.20 (58)[c] <0.001[d]	10.2±0.70 (55) <0.001	0.99	9.30±0.90 (39) <0.005	17.00±0.90 (105) <0.001	0.55	11.50±0.50 (55) <0.001	14.70±1.30 (84) <0.001	0.78
100mM (NH_4Cl) medium	T.A.				13.50±1.13 (43)[c] <0.001[d]	14.95±0.90 (53) <0.001	0.90	Did not survive					
	S.A.				8.90±0.80 (39)[c] <0.001[d]	9.80±0.60 (49) <0.001	0.91						

T.A. - Total activity, S.A. - Specific activity

a - % change compared to normal control, c - % change compared to starved control

b - p value compared to normal control , d - p value compared to starved control

TABLE 26. Alterations in the total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in the kidney of _H. foissilis_ during starvation and hyper-ammonia stress. (Mean±S.D.)

		Normal			3 days			7 days			14 days		
		NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+	NADH	NAD^+	NADH/ NAD^+
Normal/ starved control	T.A.	3.67 ±0.55	2.41 ±0.34	1.52	4.24±0.63 (16)[a] N.S.[b]	4.34±0.90 (80) < 0.005	0.98	3.96±0.40 (8) N.S.	4.92±0.72 (104) <0.001	0.80	4.63±0.55 (26) <0.025	3.86±0.34 (60) <0.001	1.20
	S.A.	2.80 ±0.40	1.84 ±0.36	1.52	3.20±0.40 (14)[a] N.S.[b]	3.30±0.50 (79) < 0.001	0.97	3.40±0.40 (21) < 0.05	4.20±0.60 (128) <0.001	0.81	4.10±0.50 (46) <0.005	3.40±0.30 (85) < 0.001	1.21
50mM (NH_4Cl) medium	T.A.				5.21±0.79 (23)[c] N.S.[d]	5.11±1.00 (18) N.S.	1.02	5.11±0.26 (29) <0.001	5.59±0.88 (14) N.S.	0.91	5.59±0.55 (21) < 0.025	5.31±0.59 (38) < 0.005	1.05
	S.A.				4.10±0.60 (28)[c] < 0.025[d]	4.00±0.80 (21) N.S.	1.03	4.50±0.30 (32) <0.005	4.80±0.80 (14) N.S.	0.94	4.90±0.50 (20) < 0.05	4.70±0.40 (38) <0.001	1.04
75mM (NH_4Cl) medium	T.A.				5.40±1.33 (27)[c] N.S.[d]	5.11±0.43 (18) N.S.	1.06	6.17±0.63 (56) < 0.001	10.71±0.53 (118) <0.001	0.58	6.85±1.15 (48) <0.005	6.17±0.79 (60) <0.001	1.11
	S.A.				4.40±1.00 (38)[c] < 0.05[d]	4.10±0.50 (24) <0.05	1.07	5.30±0.60 (56) <0.001	9.20±0.40 (119) <0.001	0.58	6.10±0.90 (49) <0.005	5.60±0.70 (65) <0.001	1.09
100mM (NH_4Cl) medium	T.A.				6.27±0.76 (48)[c] < 0.005[d]	6.08±0.94 (40) <0.02	1.03	Did not survive					
	S.A.				4.70±0.20 (47)[c] <0.001[d]	4.60±0.60 (39) <0.01	1.02						

T.A. - Total activity, S.A. - Specific activity

a - % change compared to normal control, c - % change compared to starved control

b - p value compared to normal control , d - p value compared to starved control

TABLE 27. Alterations in the total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in the brain of *H. foissilis* during starvation and hyper-ammonia stress. (Mean±S.D.)

		Normal			3 days			7 days			14 days		
		NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$
Normal/ starved control	T.A.	2.03 ±0.40	2.03 ±0.40	1.00	2.22±0.26 (9)[a] N.S.[b]	2.60±0.43 (28) N.S.	0.85	2.22±0.26 (9) N.S.	2.41±0.34 (19) N.S.	0.92	2.41±0.34 (19) N.S.	2.51±0.41 (24) N.S.	0.96
	S.A.	3.40 ±0.46	3.40 ±0.46	1.00	4.00±0.50 (18)[a] N.S.[b]	4.70±0.96 (38) < 0.05	0.85	4.60±0.50 (35) <0.005	4.90±0.90 (44) < 0.02	0.94	5.50±0.60 (62) < 0.001	5.70±0.90 (68) <0.001	0.96
50mM (NH_4Cl) medium	T.A.				3.18±0.27 (43)[c] < 0.001[d]	2.89±0.34 (11) N.S.	1.10	2.89±0.34 (30) <0.01	3.17±0.55 (32) < 0.05	0.91	2.60±0.26 (8) N.S.	2.99±0.41 (19) N.S.	0.87
	S.A.				6.10±0.50 (53)[c] <0.001[d]	5.60±0.70 (19) N.S.	1.09	5.90±0.90 (28) <0.025	6.50±1.00 (33) <0.05	0.91	6.80±1.10 (24) <0.05	7.80±1.20 (37) <0.02	0.87
75mM (NH_4Cl) medium	T.A.				3.18±0.43 (43)[c] < 0.005[d]	3.09±0.55 (19) N.S.	1.03	2.41±0.34 (9) N.S.	2.89±0.69 (20) N.S.	0.83	2.41±0.34 (0) N.S.	2.41±0.33 (-4) N.S.	1.00
	S.A.				6.00±0.70 (50)[c] <0.001[d]	5.90±1.10 (26) N.S.	1.02	5.00±0.80 (9) N.S.	6.10±1.10 (24) N.S.	0.82	6.10±1.00 (11) N.S.	6.00±0.70 (5) N.S.	1.02
100mM (NH_4Cl) medium	T.A.				2.60±0.26 (17)[c] <0.05[d]	3.18±0.43 (22) N.S.	0.82	Did not survive					
	S.A.				4.50±0.60 (13)[c] N.S.[d]	5.50±1.00 (17) N.S.	0.82						

T.A. - Total activity, S.A. - Specific activity

a - % change compared to normal control, c - % change compared to starved control

b - p value compared to normal control, d - p value compared to starved control

TABLE 28. Alterations in the total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in the muscle of *H. foissilis* during starvation and hyper-ammonia stress. (Mean±S.D.)

		Normal			3 days			7 days			14 days		
		NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$
Normal/ starved control	T.A.	1.16 ±0.27	1.16 ±0.27	1.00	1.93±0.34 (66)[a] <0.005[b]	1.93±0.34 (66) <0.005	1.00	1.74±0.26 (50) <0.01	1.83±0.40 (58) <0.02	0.95	1.74±0.26 (50) <0.01	1.45±0.34 (25) N.S.	1.20
	S.A.	2.10 ±0.30	2.10 ±0.30	1.00	3.40±0.50 (62)[a] < 0.001[b]	3.40±0.50 (62) < 0.001	1.00	3.20±0.40 (52) <0.005	3.40±0.46 (62) < 0.001	0.94	4.30±0.50 (105) < 0.001	3.60±0.60 (71) < 0.001	1.23
50mM (NH_4Cl) medium	T.A.				2.21±0.26 (15)[c] N.S.[d]	2.02±0.40 (5) N.S.	1.09	2.12±0.26 (22) < 0.05	2.03±0.40 (11) N.S.	1.04	1.64±0.26 (-6) N.S.	1.83±0.21 (26) N.S.	0.90
	S.A.				3.30±0.60 (-3)[c] N.S.[d]	3.10±0.90 (-9) N.S.	1.07	3.90±0.80 (22) N.S.	3.81±0.80 (12) N.S.	1.05	3.70±0.50 (-14) N.S.	4.21±0.60 (17) N.S.	0.90
75mM (NH_4Cl) medium	T.A.				2.22±0.26 (15)[c] N.S.[d]	2.12±0.26 (10) N.S.	1.05	1.93±0.34 (11) N.S.	1.93±0.34 (5) N.S.	1.00	1.74±0.26 (0) N.S.	1.45±0.34 (0) N.S.	1.20
	S.A.				4.00±0.50 (18)[c] N.S.[d]	3.80±0.60 (12) N.S.	1.05	3.50±0.70 (9) N.S.	3.60±0.80 (6) N.S.	1.00	3.60±0.50 (-16) N.S.	3.56±0.70 (-1) N.S.	1.20
100mM (NH_4Cl) medium	T.A.				1.83±0.40 (-5)[c] N.S.[d]	1.93±0.34 (0) N.S.	0.95	Did not survive					
	S.A.				3.10±0.50 (-9)[c] N.S.[d]	3.30±0.70 (-3) N.S.	0.94						

T.A. - Total activity, S.A. - Specific activity

a - % change compared to normal control, c - % change compared to starved control

b - p value compared to normal control, d - p value compared to starved control

TABLE 29. Alterations in the total activity (units/g wet wt.) and specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in the gill of *H. foissilis* during starvation and hyper-ammonia stress. (Mean±S.D.)

		Normal			3 days			7 days			14 days		
		NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$	NADH	NAD^+	$NADH/NAD^+$
Normal/ starved control	T.A.	1.35 ±0.41	1.34 ±0.27	1.01	2.22±0.26 (64)[a] <0.005[b]	2.31±0.40 (72) <0.005	0.96	2.03±0.40 (50) <0.05	1.93±0.34 (44) <0.02	1.05	2.21±0.43 (57) <0.02	2.23±0.26 (66) <0.001	0.95
	S.A.	2.80 ±0.36	2.80 ±0.40	1.00	4.60±0.50 (64)[a] <0.001[b]	4.80±0.75 (71) <0.001	0.96	4.90±0.80 (75) <0.001	4.60±0.50 (64) <0.001	1.07	5.10±0.90 (82) <0.001	5.40±0.90 (93) <0.001	0.94
50mM (NH_4Cl) medium	T.A.				2.31±0.40 (4)[c] N.S.[d]	2.60±0.43 (13) N.S.	0.89	2.41±0.33 (19) N.S.	2.12±0.26 (10) N.S.	1.14	2.32±0.26 (5) N.S.	2.60±0.26 (17) N.S.	0.86
	S.A.				5.30±0.80 (15)[c] N.S.[d]	5.90±1.00 (23) N.S.	0.90	5.60±0.80 (14) N.S.	4.90±0.60 (7) N.S.	1.14	5.20±1.20 (2) N.S.	6.05±0.60 (12) N.S.	0.87
75mM (NH_4Cl) medium	T.A.				2.51±0.40 (13)[c] N.S.[d]	2.31±0.40 (0) N.S.	1.09	2.12±0.26 (4) N.S.	2.22±0.26 (15) N.S.	0.95	2.32±0.26 (5) N.S.	2.21±0.26 (-1) N.S.	1.00
	S.A.				5.80±1.20 (26)[c] N.S.[d]	5.30±0.80 (10) N.S.	1.09	5.30±0.50 (8) N.S.	5.60±1.00 (22) N.S.	0.95	5.56±0.60 (9) N.S.	5.62±1.00 (4) N.S.	1.00
100mM (NH_4Cl) medium	T.A.				2.41±0.34 (9)[c] N.S.[d]	2.31±0.40 (0) N.S.	1.04	Did not survive					
	S.A.				5.40±0.80 (17)[c] N.S.[d]	5.20±1.00 (8) N.S.	1.04						

T.A. - Total activity, S.A. - Specific activity

a - % change compared to normal control, c - % change compared to starved control

b - p value compared to normal control, d - p value compared to starved control

TABLE 30. Purification protocol for GDH from liver of *H. fossilis*.

	Purification steps	Volume (ml)	Enzyme activity (units / ml)	Protein (mg/ml)	Specific activity (units/mg P.)	Fold of Purification	Total activity (units)	% Recovery
May (Summer)	Crude extract	45	1.21	9.53	0.13	1	[illegible]4.45	100
	$(NH_4)_2SO_4$ precipitate (20-50%)	35	1.25	7.95	0.16	1	43.75	80
	DEAE -Cellulose	35	0.96	0.07	13.71	106	33.60	62
	DEAE-Sephacel	30	0.93	0.06	15.50	119	32.55	60
	Blue Sepharose-CL - 6B	20	1.30	0.02	65.00	500	26.00	48
December (Winter)	Crude extract	30	0.98	12.85	0.08	1	29.40	100
	$(NH_4)_2SO_4$ precipitate (20-50%)	20	1.11	7.45	0.15	2	22.20	76
	DEAE-Cellulose	20	1.21	0.10	12.10	151	24.20	82
	DEAE-Sephacel	15	1.30	0.09	14.94	187	19.50	66
	Blue Sepharose-CL - 6B	10	1.68	0.05	33.60	420	16.80	57

TABLE 31. Properties of purified GDH from liver of <u>H. fossilis</u>.

		NADH dependent GDH		NAD^+ dependent GDH	
Kinetics	Substrate	K_m(mM)	V_{max} (units/ml)	K_m(mM)	V_{max} (units/ml)
	NH_4^+ (-ADP)	25.00	1.30		
	NH_4^+ (+ADP)	23.80	11.70		
	α-ketoglutarate (-ADP)	0.38	1.30		
	α-ketoglutarate (+ADP)	0.35	11.70		
	NADH (-ADP)	0.07	1.30		
	NADH (+ADP)	0.07	11.70		
	L-glutamate (+ADP)			4.76	0.64
	NAD^+ (+ADP)			0.28	0.64
pH optima		7.6 - 8.0		8.5 - 9.5	
Temperature optima		30°C		45°C	
Half life at 45°C		8 min		25 min	
Molecular weight of native enzyme (Hexamer)			3,38, 064; 3,38, 844		
Molecular weight of subunit (monomer)			56,000		

TABLE 32. Substrate affinity of the purified GDH (NAD^+ dependent) activity from liver of *H. fossilis*.

Substrate (16.67 mM)	Activity (%)
L-Glutamate	100
L-Alanine	3
DL-Valine	2
L-Leucine	14
DL-Isoleucine	6
L-Proline	3
DL-Phenylalanine	2
L-Tryptophan	4
DL-Methionine	5
L-Glycine	0
L-Serine	1
L-Threonine	2
L-Tyrosine	4
L-Aspargine	1
L-Glutamine	0
L-Cysteine	0
L-Aspartic acid	0
L-Arginine	3
L-Histidine	2
L-Lysine	4
DL-Ornithine	3

TABLE 33. Effect of ADP and nicotinamide coenzymes on purified GDH activity (units/ml) from liver of *H. fossilis*. (Mean ± S.D.)

Enzyme with/ without ADP	Concentrations of coenzymes (mM)									
	0.05	0.10	0.15	0.20	0.25	0.50	1.00	1.50	2.00	2.50
NADH-ADP	0.54 ±0.04	0.82 ±0.06	1.21 ±0.08	1.30 ±0.09	1.30 ±0.16	-	-	-	-	-
NADH + ADP	4.77 ±0.55	7.38 ±0.99	10.45 ±1.06	11.70 ±1.25	11.70 ±1.45	-	-	-	-	-
NADPH-ADP	0.34 ±0.03	0.44 ±0.04	0.48 ±0.04	0.58 ±0.05	0.68 ±0.05	-	-	-	-	-
NADPH+ADP	2.65 ±0.38	3.17 ±0.46	3.36 ±0.46	4.00 ±0.55	4.76 ±0.55	-	-	-	-	-
NAD^+-ADP	-	-	-	-	-	BLD	BLD	BLD	BLD	BLD
NAD^++ADP	-	-	-	-	-	0.38 ±0.03	0.52 ±0.04	0.61 ±0.04	0.64 ±0.05	0.64 ±0.04
$NADP^+$-ADP	-	-	-	-	-	BLD	BLD	BLD	BLD	BLD
$NADP^+$+ADP	-	-	-	-	-	BLD	BLD	BLD	BLD	BLD

Constant concentration of ADP-2mM, BLD-Below limit of defection.

TABLE 34. Modulation of GDH activity purified from liver of *H. fossilis* by nucleotides.

Nucleotide	Enzyme	Modulation	Pattern	Nature of inhibition	Ki (app.) (mM)	K_a (app.) (mM)
ADP	NADH	activation	Sigmoidal			0.06
	NAD^+	activation	Sigmoidal			
ATP	NADH	activation	Hyperbolic			
	NAD^+	inhibition	Sigmoidal	NC	0.11	
GTP	NADH-ADP	inhibition	Sigmoidal		0.53×10^{-2}	
	NADH+ADP	inhibition	Sigmoidal		1.13×10^{-2}	
	NAD^+ +ADP	inhibition	Sigmoidal		0.42×10^{-2}	
AMP	NADH	activation	Hyperbolic			
	NAD^+	inhibition	?			
IMP	NADH	inhibition	Hyperbolic	NC		
	NAD^+	inhibition	?	NC		

NC - Non-competitive, K_i - Inhibition constant, K_a - Activation constant.

TABLE 35. Effect of various ions on GDH activity (% change) purified from liver of *H. fossilis*. (+ induction, - inhibition)

Ions	NADH dependent GDH				NAD^+ dependent GDH			
	Concentrations (mM)				Concentrations (mM)			
	10	15	25	50	10	15	25	50
$CdCl_2$	-18	-30	-45	-64	-15	-26	-69	-75
$CaCl_2$	-22	-39	-62	-67	-12	-24	-49	-64
$HgCl_2$	-62	-82	-100	-100	-55	-88	-100	-100
$MnCl_2$	-37	-59	-78	-100	-48	-66	-64	-78
$MgCl_2$	-19	-33	-51	-67	-37	-63	-75	-82
$FeCl_3$	-65	-89	-100	-100	-77	-88	-100	-100
$CuSO_4$	0	0	-11	-9	0	0	-8	-15
$MgSO_4$	-35	-53	-69	-83	-27	-42	-58	-75
$MnSO_4$	-30	-41	-58	-72	-18	-34	-51	-68
$ZnSO_4$	-68	-80	-94	-100	-65	-73	-87	-100
K_2SO_4	-48	-57	-52	-48	-53	-67	-66	-75
K_2HPO_4	+45	+62	+74	+85	+23	+49	+63	+74
$CaCO_3$	0	0	-9	-11	0	-14	-18	-15
Na_2CO_3	0	-14	-22	-27	0	-11	-23	-21
$AgNO_3$	-82	-94	-100	-100	-79	-90	-100	-100
NaF	0	-12	-17	-21	-10	-18	-24	-38
NaI	0	-8	-14	-25	-15	-24	-35	-48
$C_6H_5Li_3O_7$	-29	-48	-48	-46	-30	-44	-56	-65

TABLE 36. Effect of acetylcholine, biogenic amines and some amino acid derivatives on GDH activity (% change) purified from liver of *H. fossilis* (-inhibition)

	NADH dependent GDH Concentrations (mM)				NAD^+ dependent GDH Concentrations (mM)			
	1	5	10	50	1	5	10	50
Acetylcholine	-22	-15	-18	-22	-28	-45	-36	-50
Norepinephrine	-22	-35	-32	-36	-30	-35	-40	-35
Epinephrine	-15	-15	-28	-22	-25	-30	-28	-34
3,4-Dihydroxy Phenylalanine	-15	-46	-64	-68	- 8	-15	-19	-21
Serotonine	-28	-36	-64	-64	-30	-64	-75	-81
Aminobutyric acid	-33	-45	-55	-50	-15	-26	-33	-44
Urea	-21	-35	-62	-75	-15	-32	-66	-82
Carbamylphosphate	-24	-55	-64	-77	-26	-47	-69	-82

TABLE 37. Nature of inhibition and inhibition constant of various products on GDH activity purified from liver of *H. fossilis.*

Enzyme	Inhibitor	Varied substrate	Fixed substrate(s)	Nature of inhibition	K_i(app) (mM)
GDH NADH dependent	L-glutamate	α-Ketoglutarate	NH_4^+ & NADH	C	4.70
	L-glutamate	NH_4^+	α-Ketoglutarate & NADH	-	5.60
	NAD^+	α-Ketoglutarate	NH_4^+ & NADH	NC	0.31
GDH NAD^+ dependent	α-Ketoglutarate	L-glutamate	NAD^+	NC	0.24
	NH_4^+	L-glutamate	NAD^+	C	10.00
	NADH	L-glutamate	NAD^+	UC	0.02
	α-Ketoglutarate	NAD^+	L-glutamate	NC	
	NH_4^+	NAD^+	L-glutamate	NC	
	NADH	NAD^+	L-glutamate	C	

C - Competitive, NC - Non-competitive, UC - Un-competitive, K_i - Inhibition constant.

TABLE 38. Effect of amino acids and keto acids on GDH activity (% change) purified from liver of *H. fossilis*. (+ induction, - inhibition)

Amino/keto acids	NADH dependent GDH					NAD^+ dependent GDH				
Concs(mM)	0.5	1	3	5	9	0.5	1	3	5	9
L-Alanine	0	-16	-27	-25	-31	0	-35	-38	-42	-55
DL-Valine	0	0	-20	-37	-34	0	0	0	-6	-10
L-Leucine	0	+5	+12	+23	+35	0	0	+5	0	+8
DL-Isoleucine	0	0	+5	+7	+10	0	0	0	+6	+15
L-Proline	0	0	-24	-35	-41	0	0	0	-19	-22
DL-Phenyl alanine	0	-11	0	-15	-13	-7	-20	-24	-33	-47
L-Tryptophan	-25	-36	-36	-43	-43	-14	-25	-34	-45	-52
DL-Methionine	0	0	0	-22	-24	0	0	0	0	0
L-Glycine	0	0	0	0	-14	0	0	0	0	0
L-Serine	0	0	0	-25	-34	0	-8	0	-25	-15
L-Threonine	0	-20	-31	-45	-48	0	0	0	0	-19
L-Tyrosine	-55	-53	-48	-56	-55	-30	-42	-50	-58	-55
L-Asparagine	0	-13	-20	-38	-50	0	0	-10	-27	-38
L-Glutamine	0	-5	-18	-36	-41	0	-12	-25	-16	-21
L-Cysteine	-52	-68	-79	-82	-92	-45	-58	-77	-72	-80
L-Aspartic acid	-26	-55	-67	-78	-83	0	-11	-25	-31	-40
L-Glytamic acid	0	0	0	-15	-26	0	0	0	0	0
L-Arginine	0	0	-35	-40	-45	0	0	0	-11	-19
L-Histidine	-30	-45	-50	-58	-66	-26	-48	-59	-65	-68
L-Lysine	-22	-37	-48	-52	-68	-40	-55	-64	-77	-85
DL-Ornithine	0	0	-8	-15	-37	0	0	0	-17	-30
Pyruvate	0	0	-5	-18	-35	0	0	0	-12	-30
Oxaloacetate	0	0	0	-15	-28	0	0	0	-9	-19

TABLE 39. Nature of inhibition and inhibition constant of some amino acids on GDH activity purified from liver of *H. fossilis*.

Amino acid	NADH dependent GDH		NAD^+ dependent GDH	
	Nature of inhibition	Ki (app.) (mM)	Nature of inhibition	Ki (app.) (mM)
L-Alanine	ND	ND	C	3.50
L-Cysteine	C	0.67	UC	0.11
L-Aspartic acid	NC	0.56	ND	ND
L-Histidine	NC	0.12	NC	1.33
L-Lysine	NC	0.30	NC	0.16

C - Competitive

NC - Non-competitive

UC - Uncompetitive

ND - Not determined.

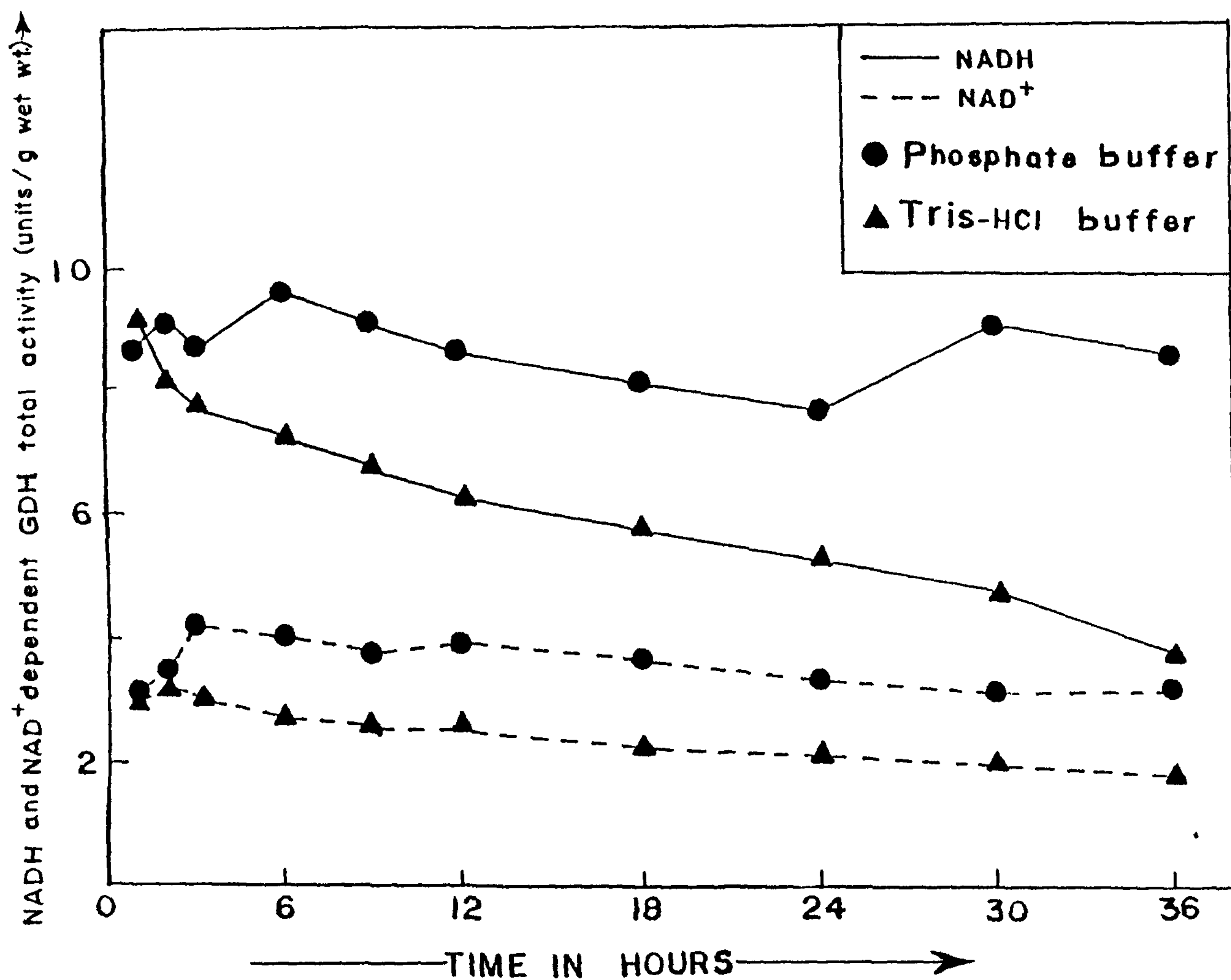


Fig.1. Effect of different buffers phosphate and Tris-HCl on GDH (NADH and NAD^+ dependent) activity in the liver homogenate of <u>H. fossilis</u>.

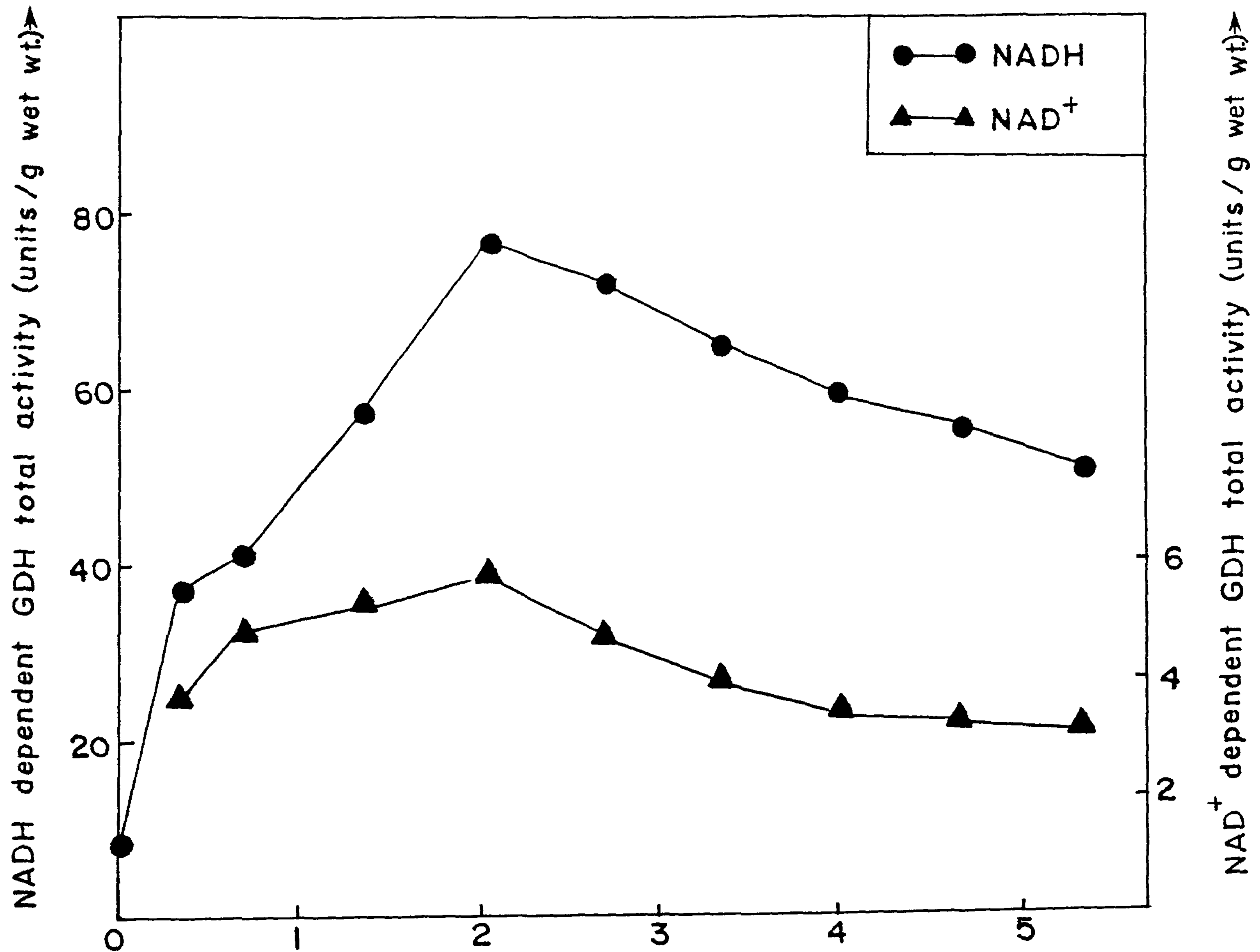
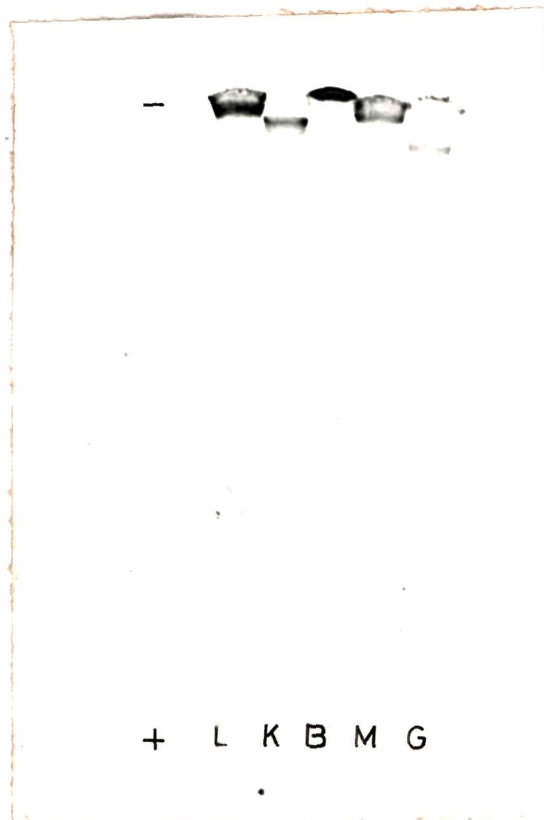


Fig.2. Effect of different concentrations of ADP on GDH (NADH and NAD^+ dependent) activity in the liver homogenate of _H. fossilis_.



Rm value (Mean ± S.D.)

Liver - 0.024 ± 0.003
Kidney - 0.028 ± 0.003
Brain - 0.011 ± 0.002
Muscle - 0.024 ± 0.004
Gill - 0.050 ± 0.005

Fig.3. Specific staining for GDH (NAD$^+$ dependent) activity on polyacrylamide gel (PAGE) of crude homogenates from liver, kidney, brain, muscle and gill tissues of H. fossilis.

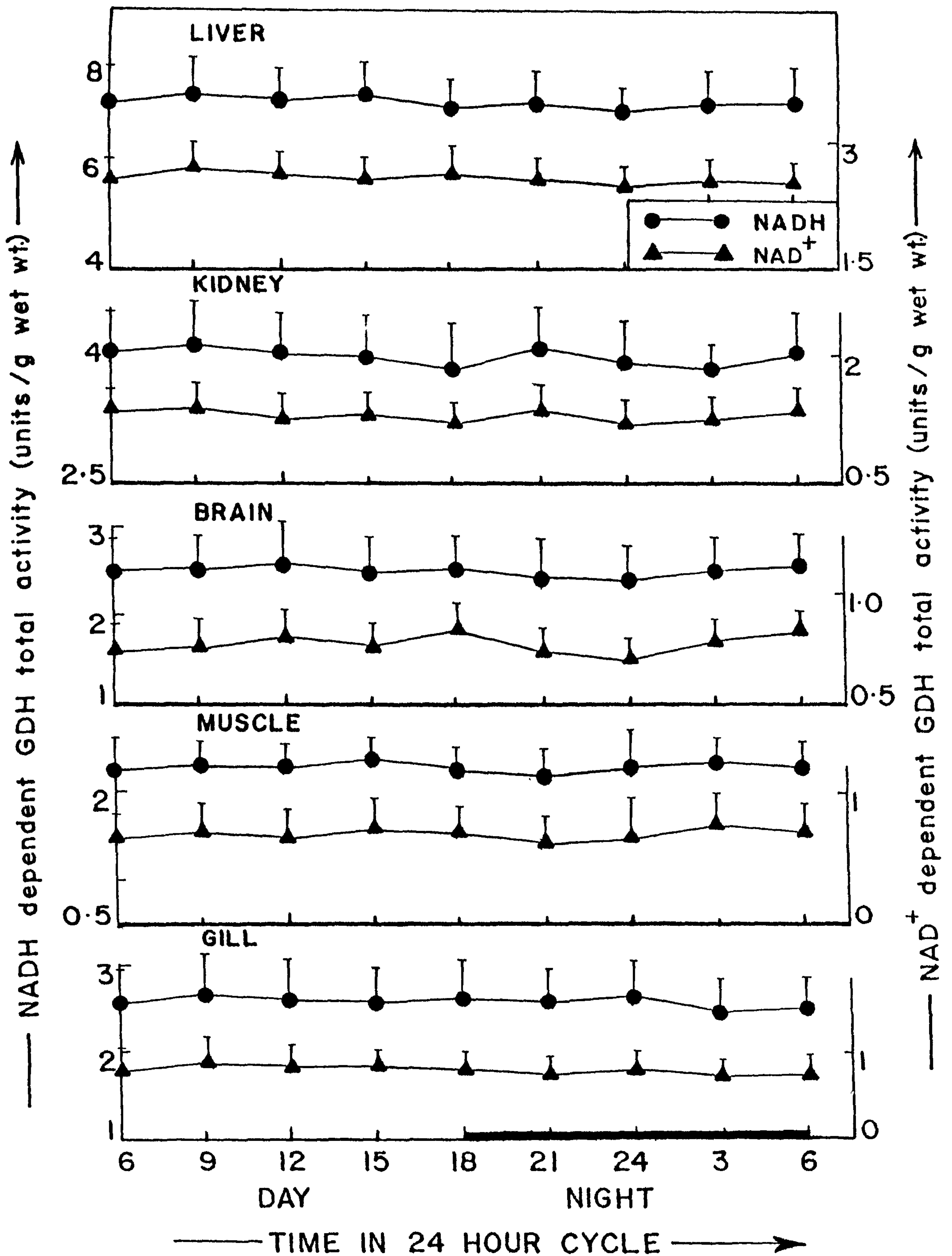


Fig. 4. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during 24 hr cycle in August, 1989 (Summer).

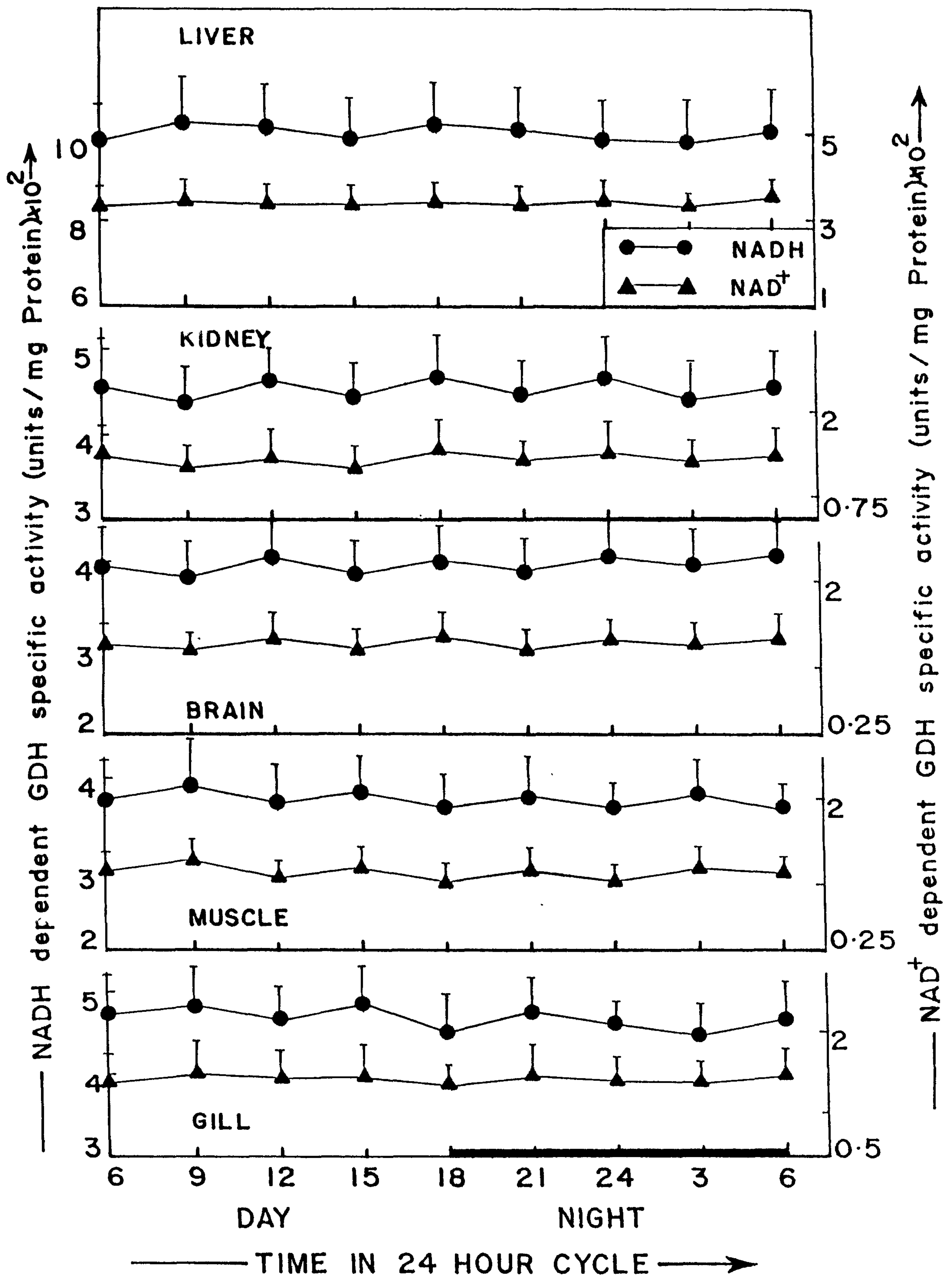


Fig.5. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during 24 hr cycle in August, 1989 (Summer).

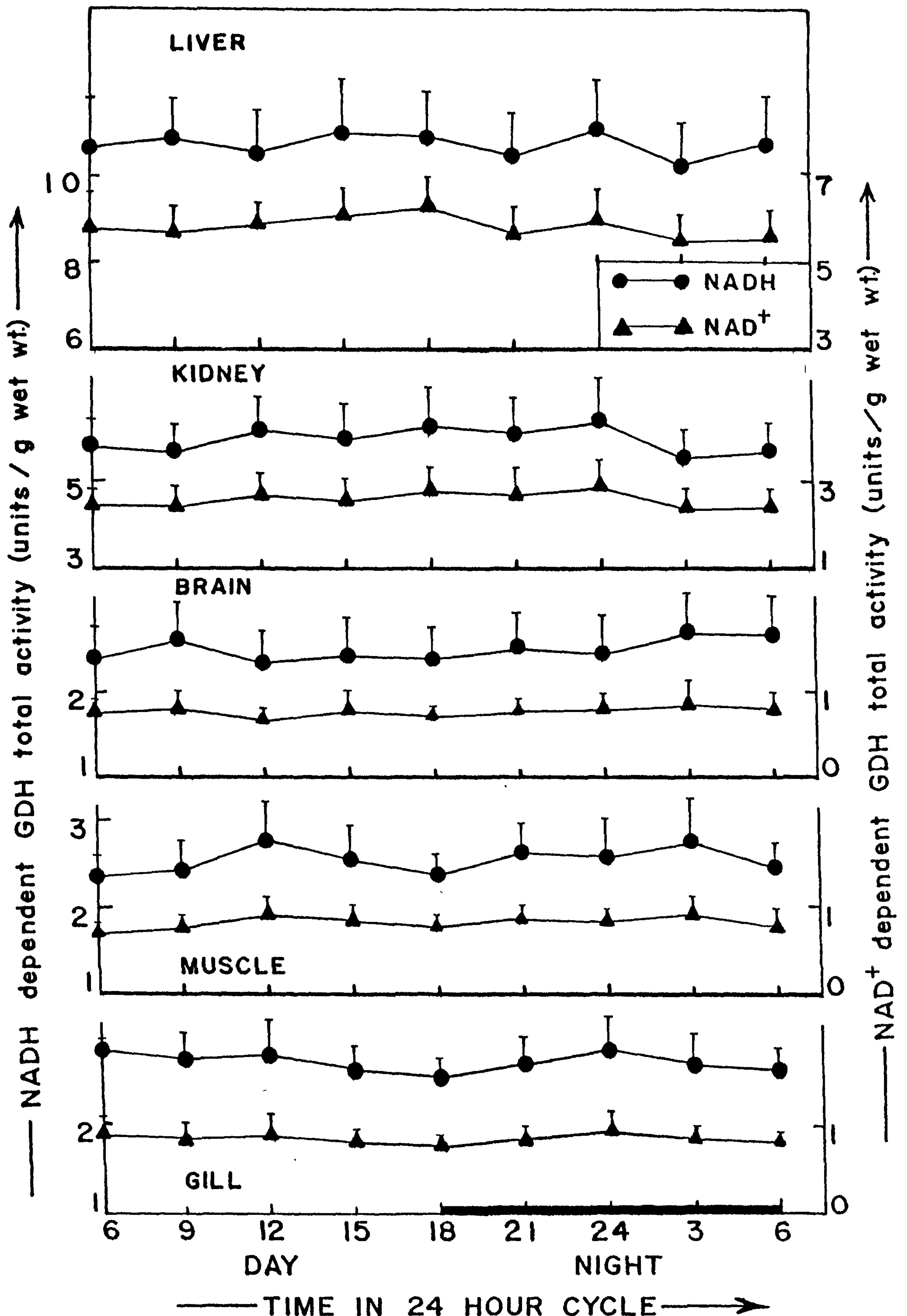


Fig.6. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during 24 hr cycle in January, 1990 (Winter).

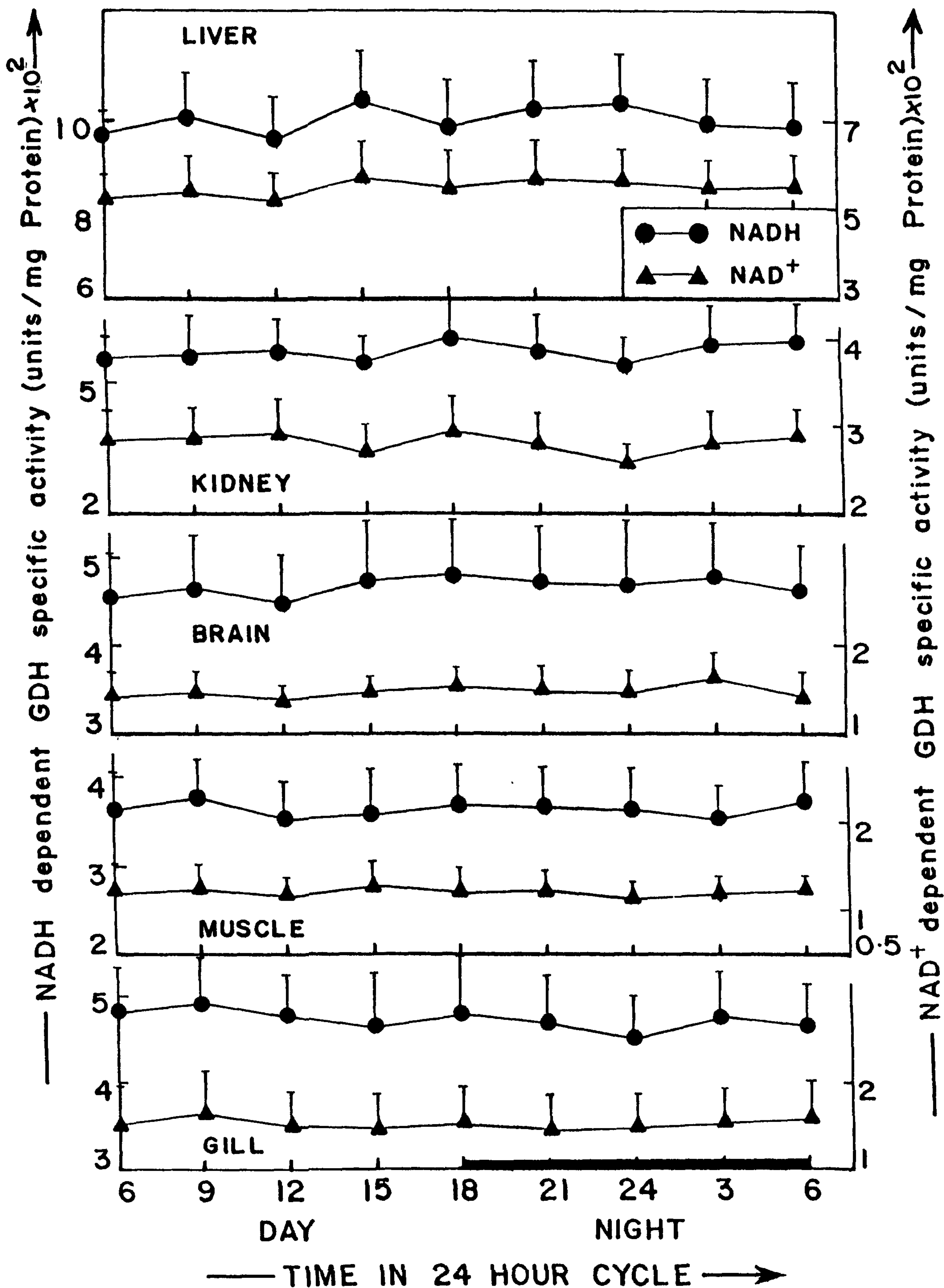


Fig.7. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during 24 hr cycle in January, 1990 (Winter).

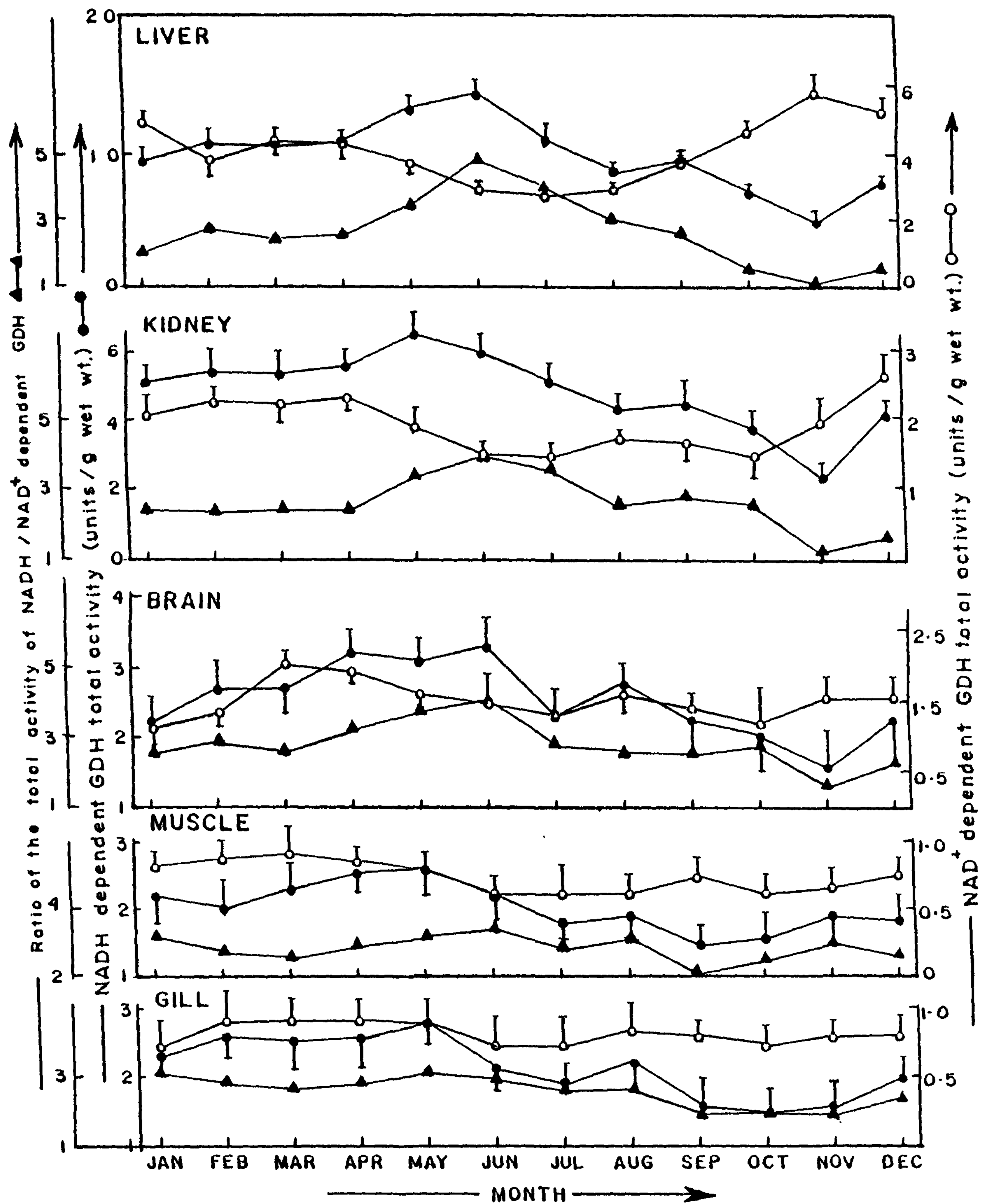


Fig.8. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during annual cycle (1988-1989) studied at monthly interval.

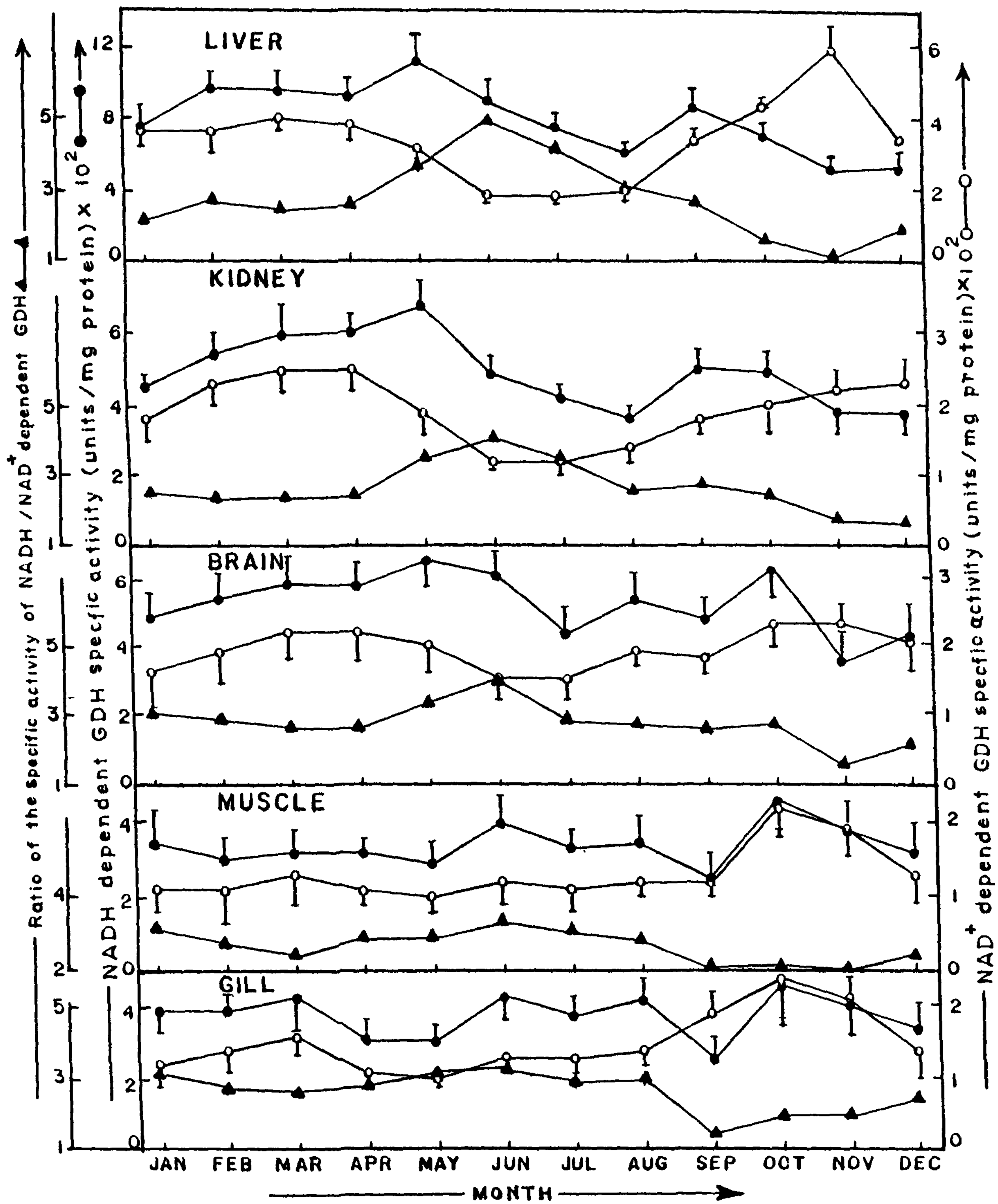


Fig.9. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) and their ratio (NADH/NAD^+) in various tissues of *H. fossilis* during annual cycle (1988-1989) studied at monthly interval.

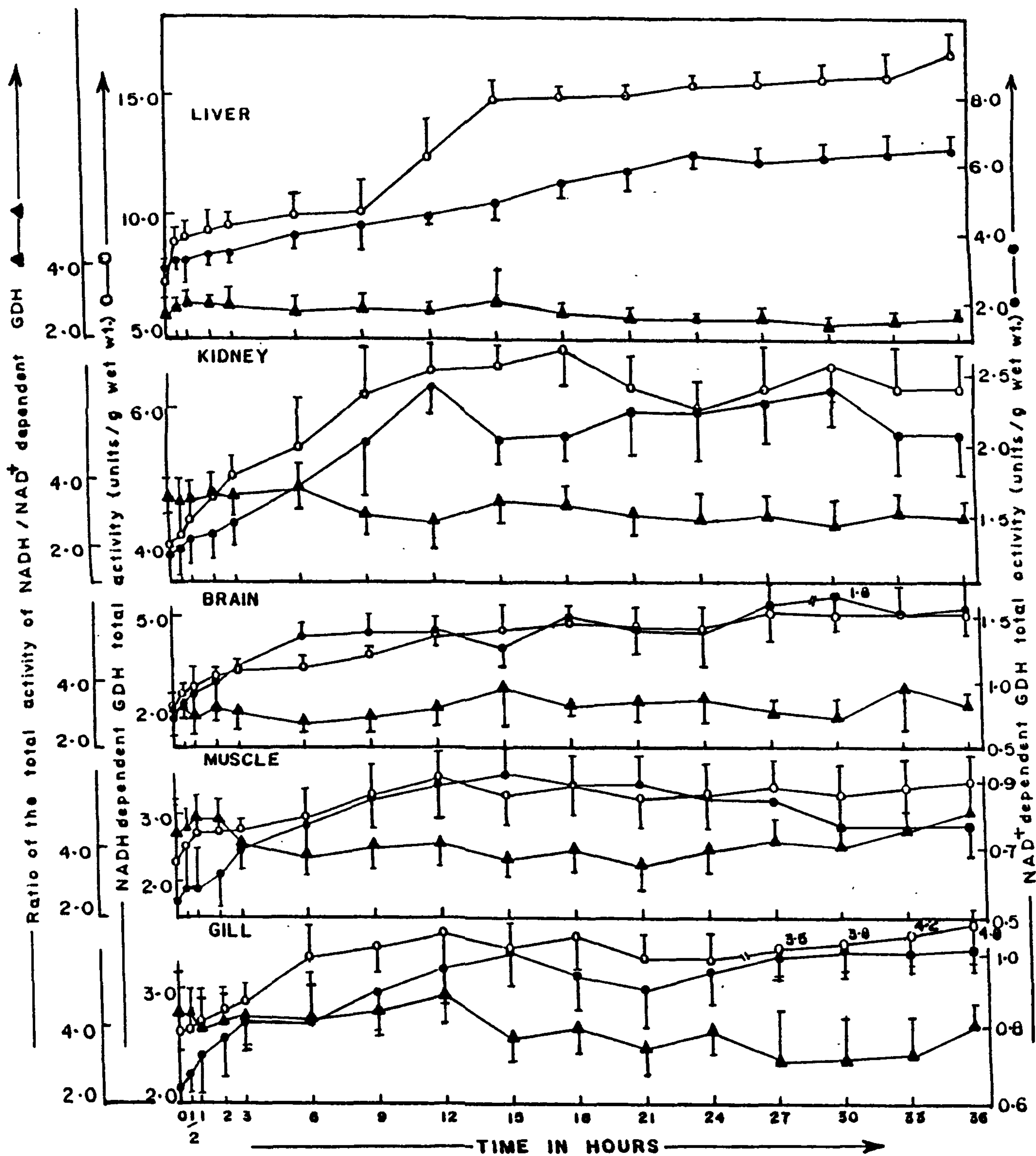


Fig.10. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) and their ratio ($NADH/NAD^+$) in various tissues of H. fossilis during water deprivation.

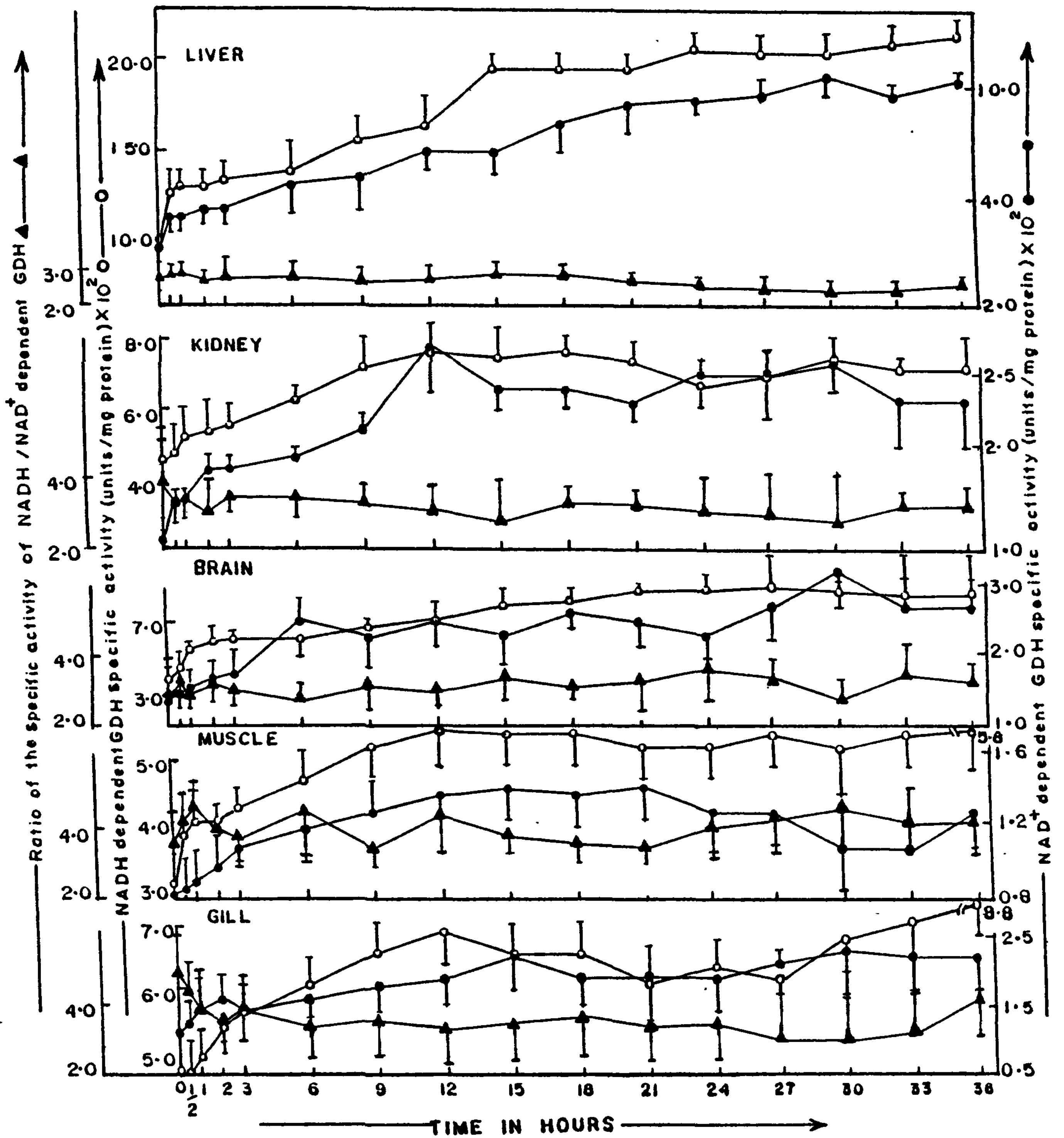


Fig.11. Alterations in the specific activity (units/mg protein)x10² of GDH (NADH and NAD+ dependent) and their ratio (NADH/NAD+) in various tissues of H. fossilis during water deprivation.

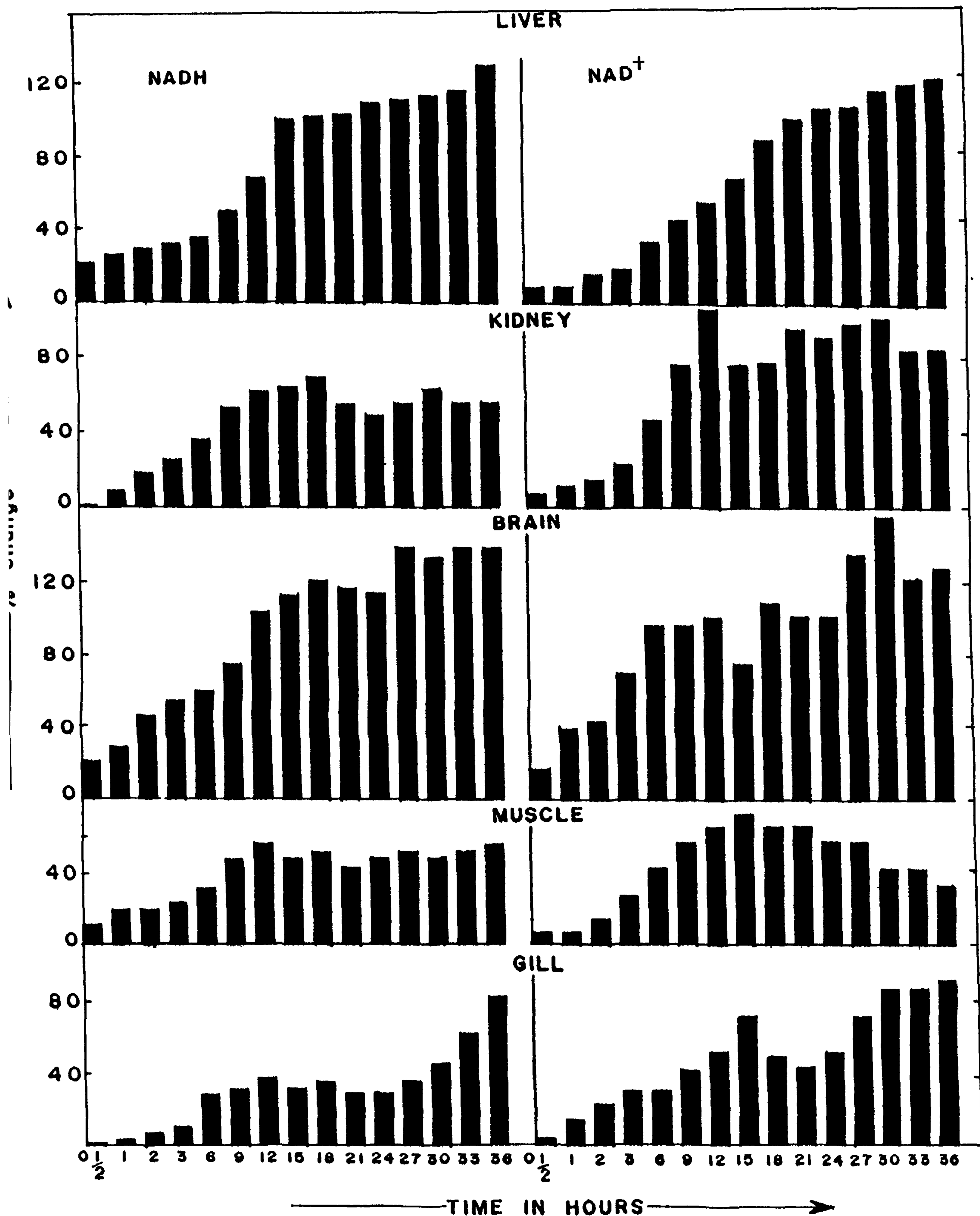


Fig.12. Percent(%) change in the total activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during water deprivation.

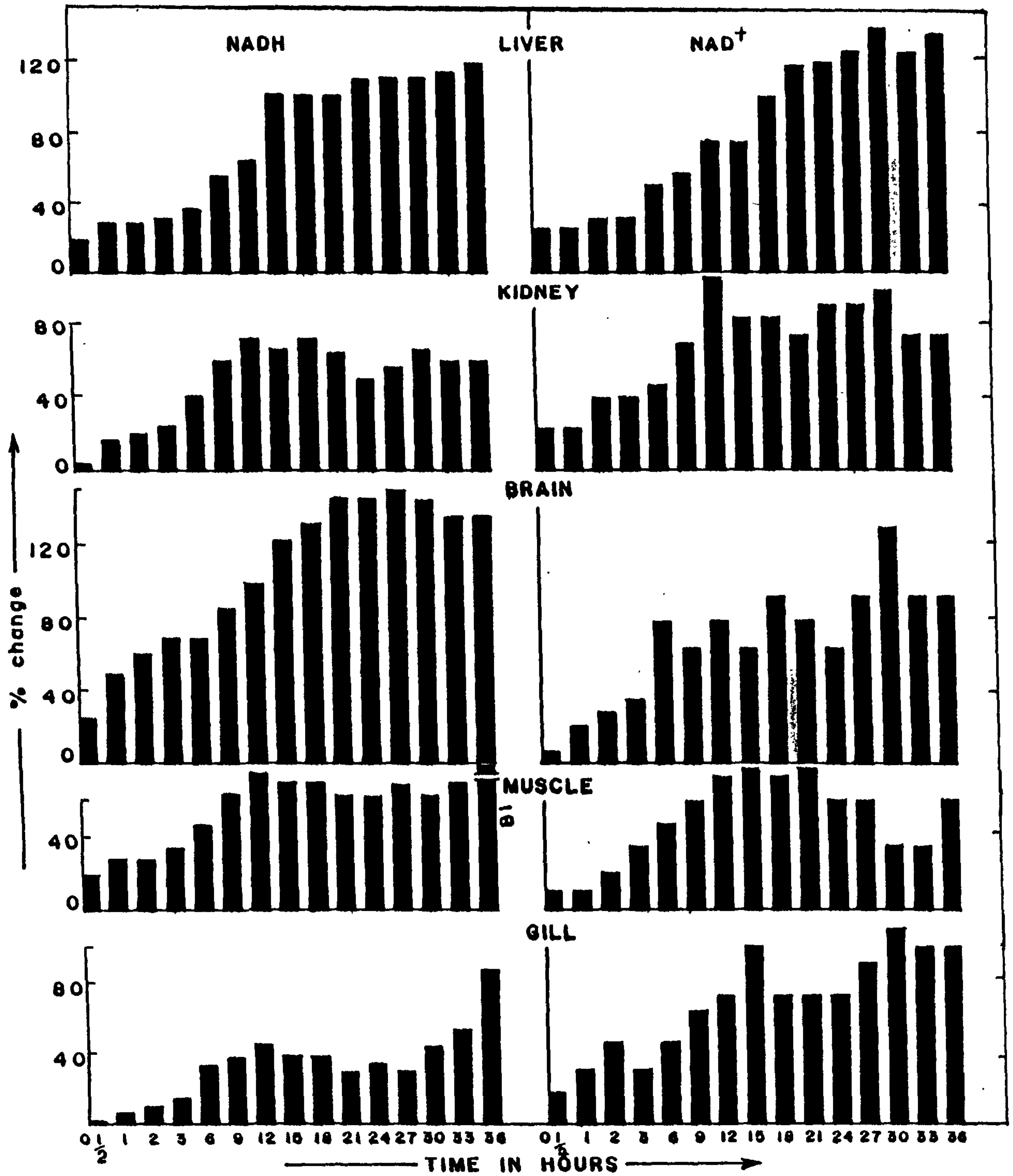


Fig.13. Percent(%) change in the specific activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during water deprivation.

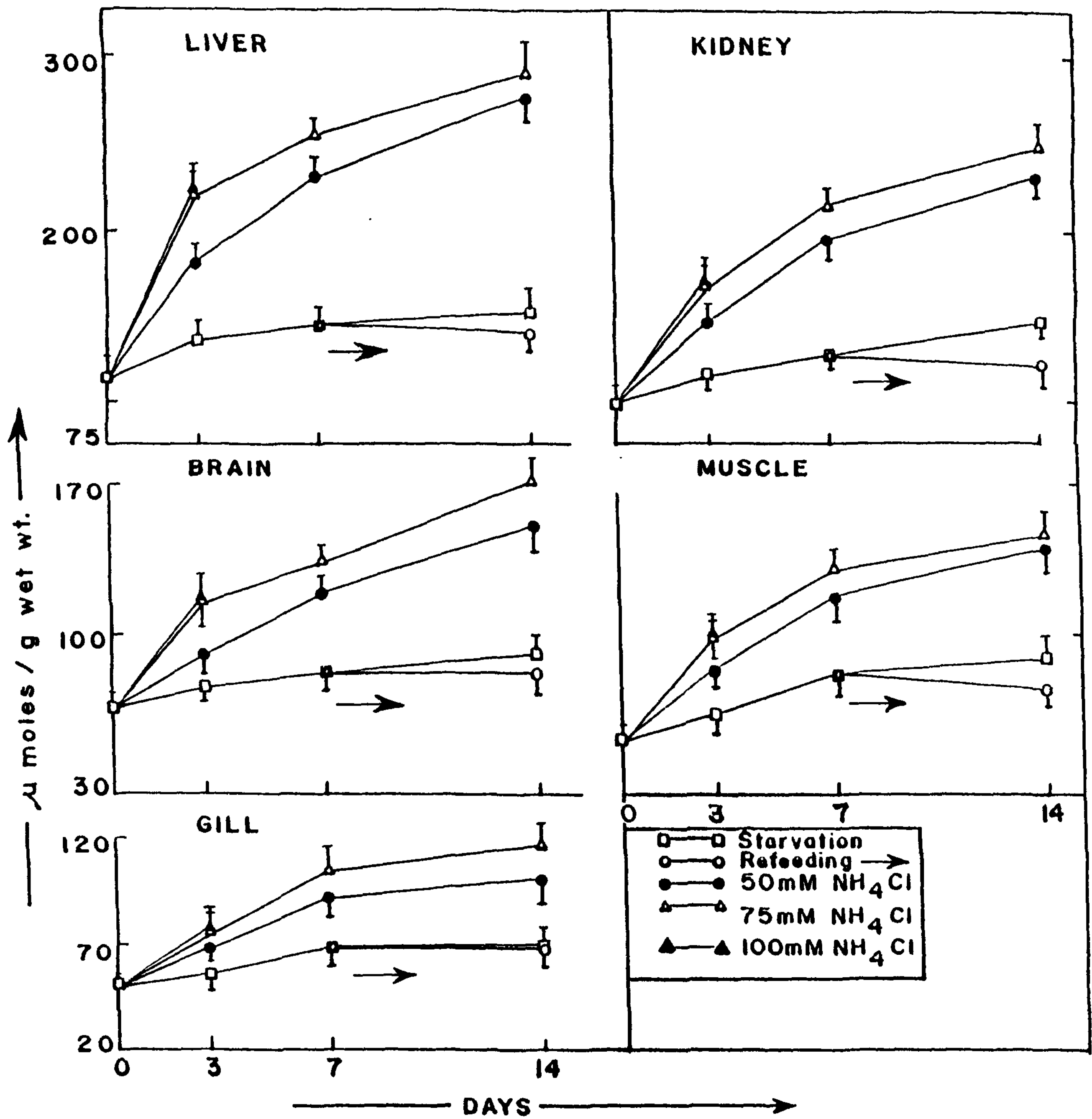


Fig.14. Alterations in the total free amino acids (μ moles/g wet wt.) in various tissues of H. fossilis during starvation, refeeding and hyper-ammonia stress.

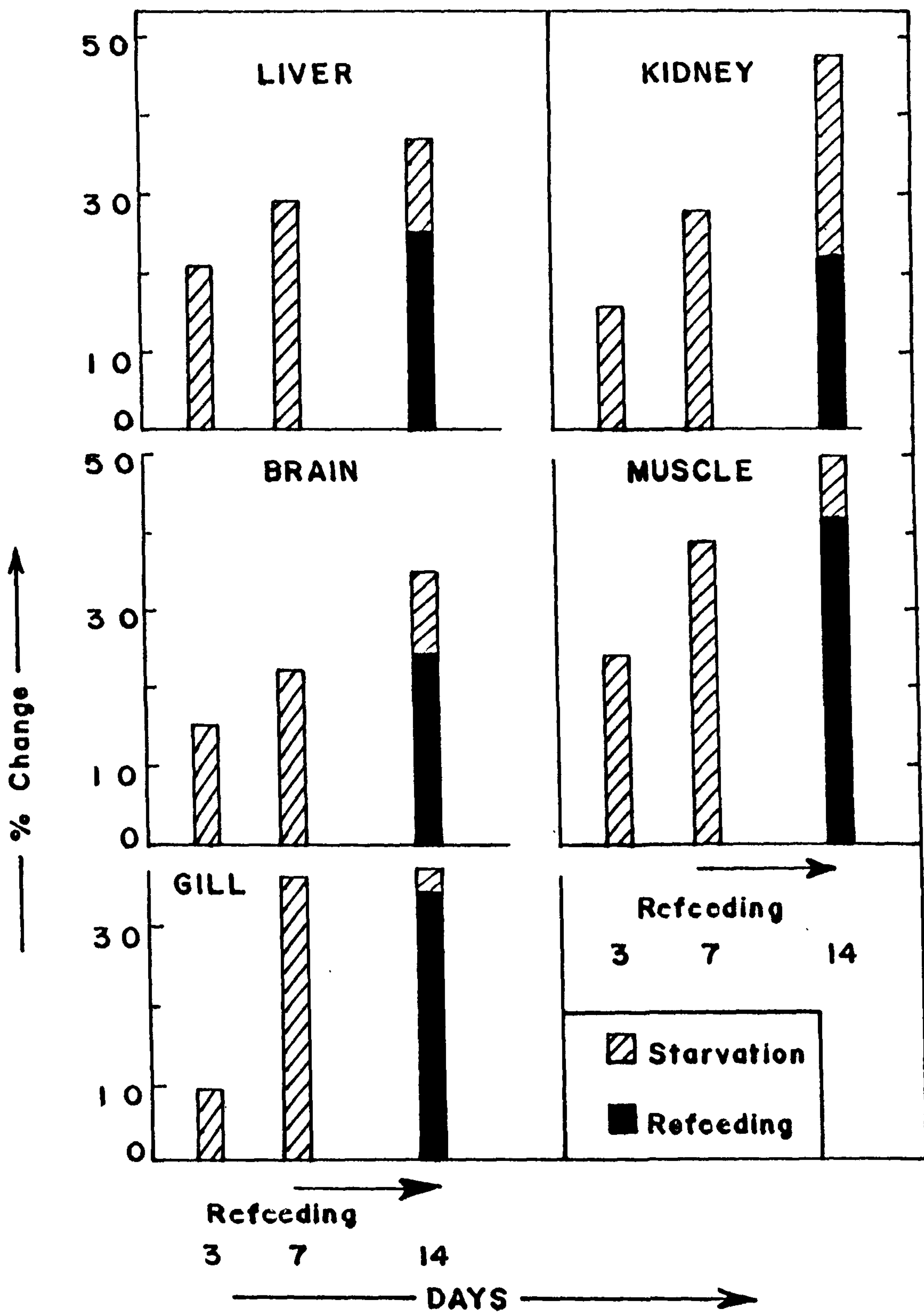


Fig.15. Percent (%) change in the total free amino acids in various tissues of *H. fossilis* during starvation and refeeding.

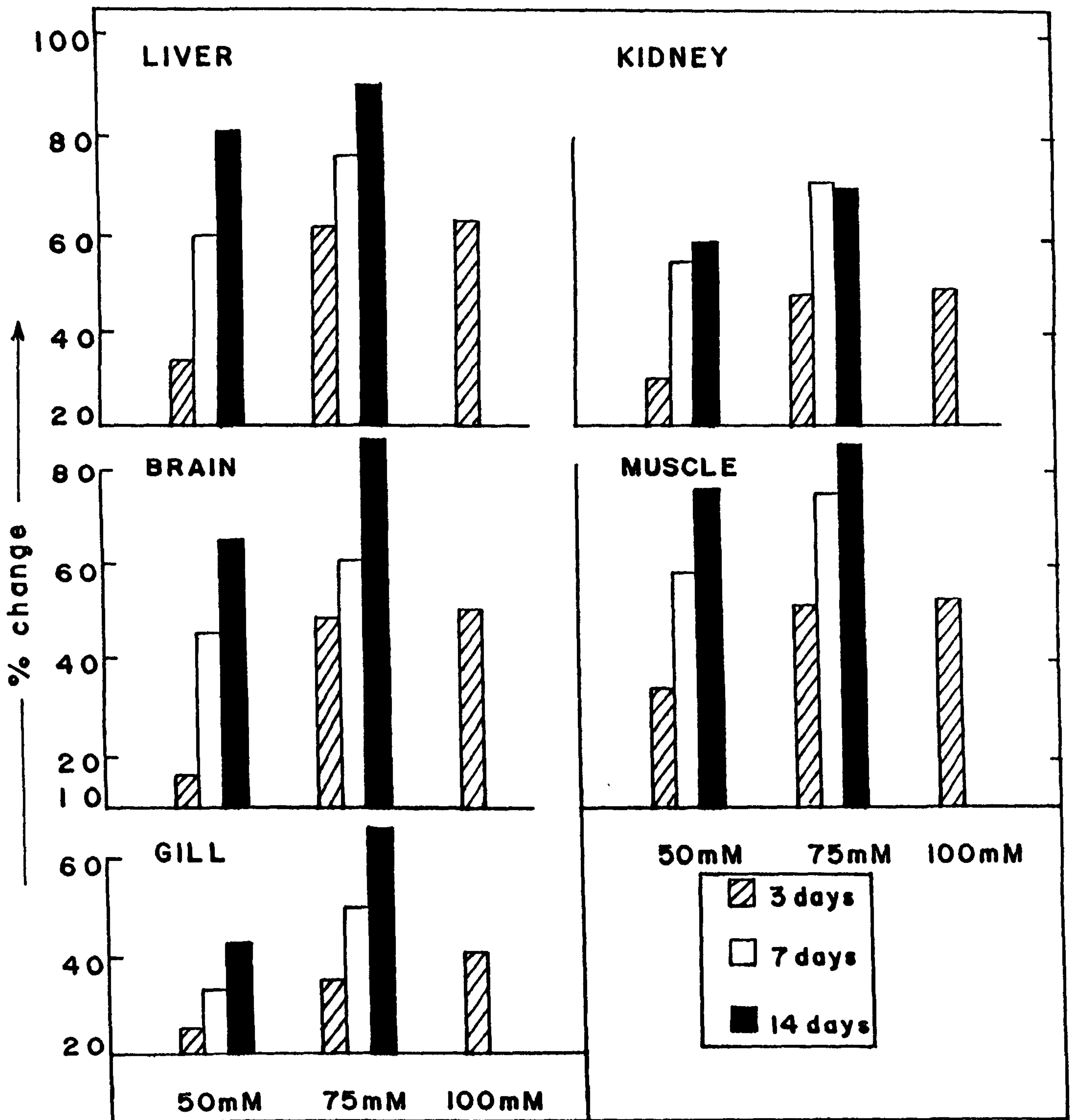


Fig.16. Percent (%) change in the total free amino acids in various tissues of H. fossilis during hyper-ammonia stress.

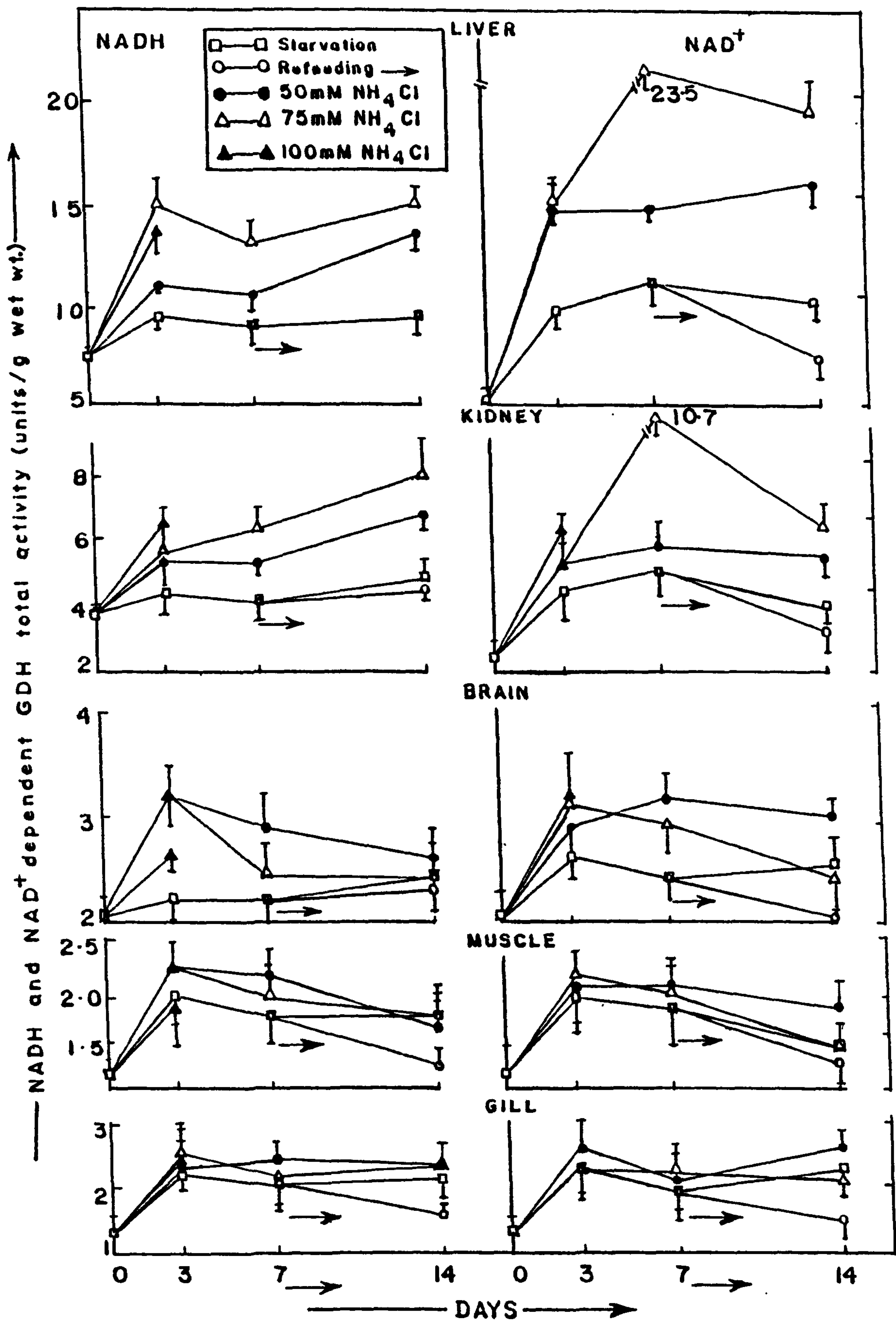


Fig.17. Alterations in the total activity (units/g wet wt.) of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during starvation, refeeding and hyper-ammonia stress.

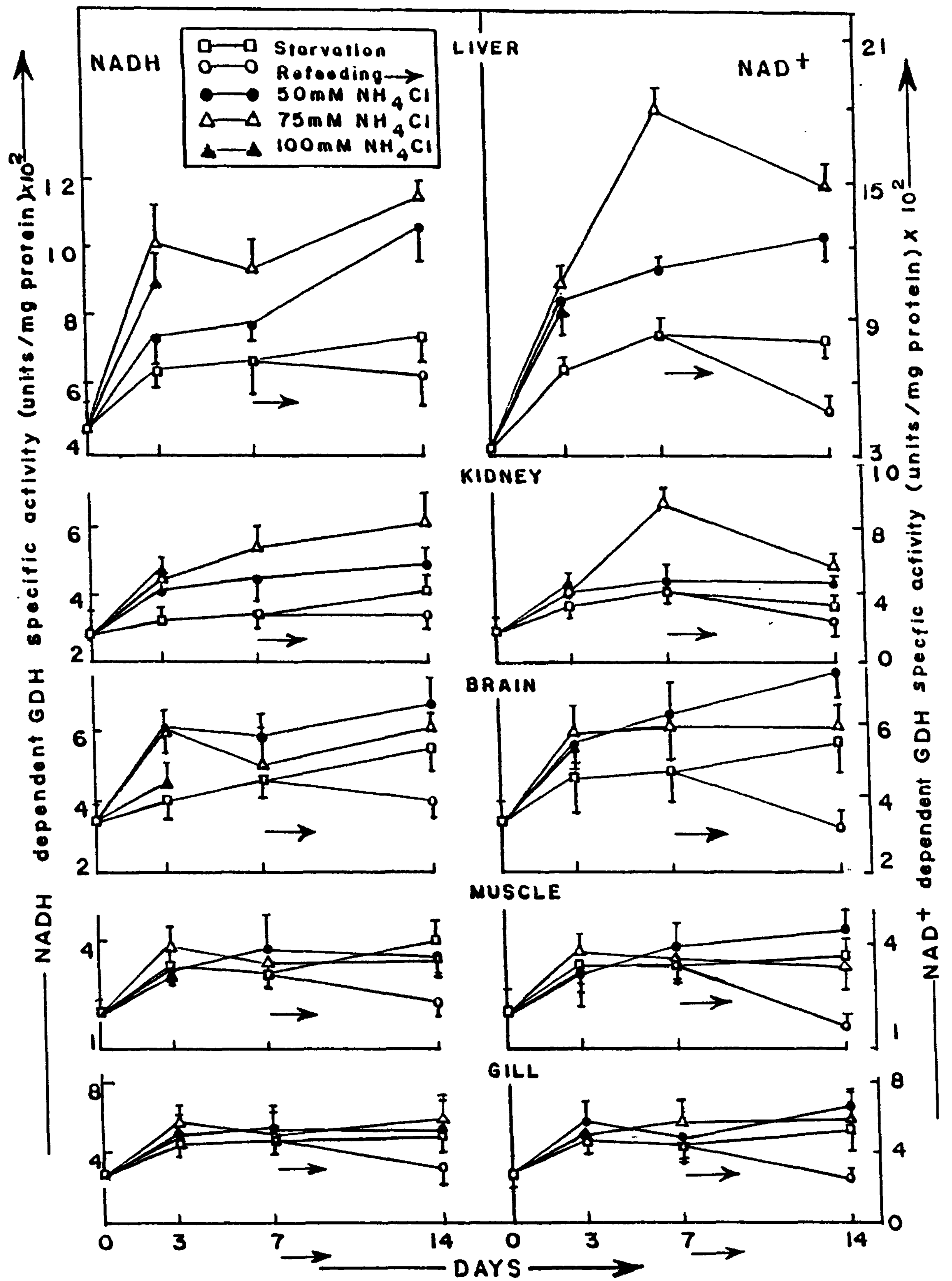


Fig.18. Alterations in the specific activity (units/mg protein)x10^2 of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during starvation, refeeding and hyper-ammonia stress.

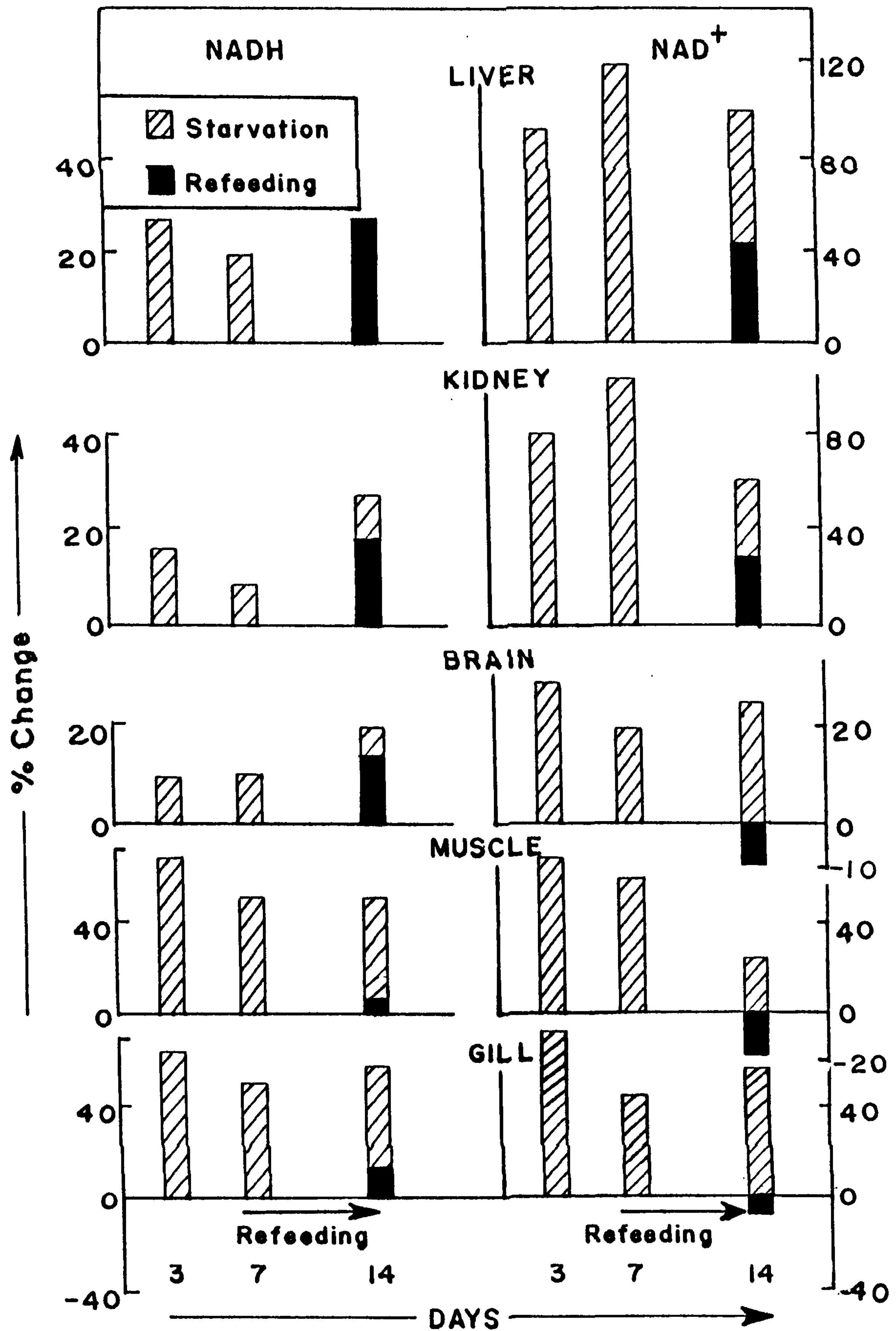


Fig.19. Percent (%) change in the total activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during starvation and refeeding.

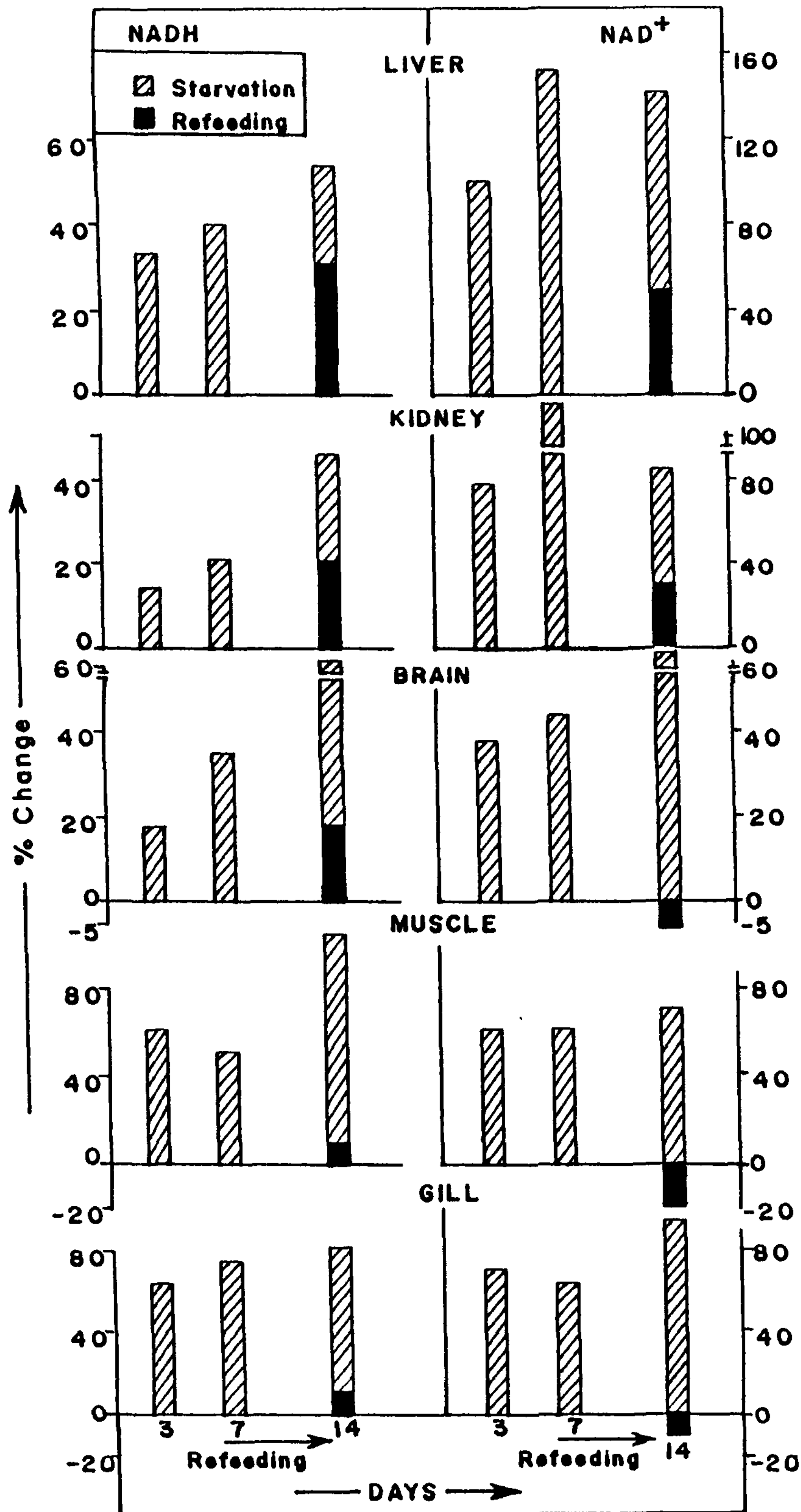


Fig.20. Percent (%) change in the specific activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during starvation and refeeding.

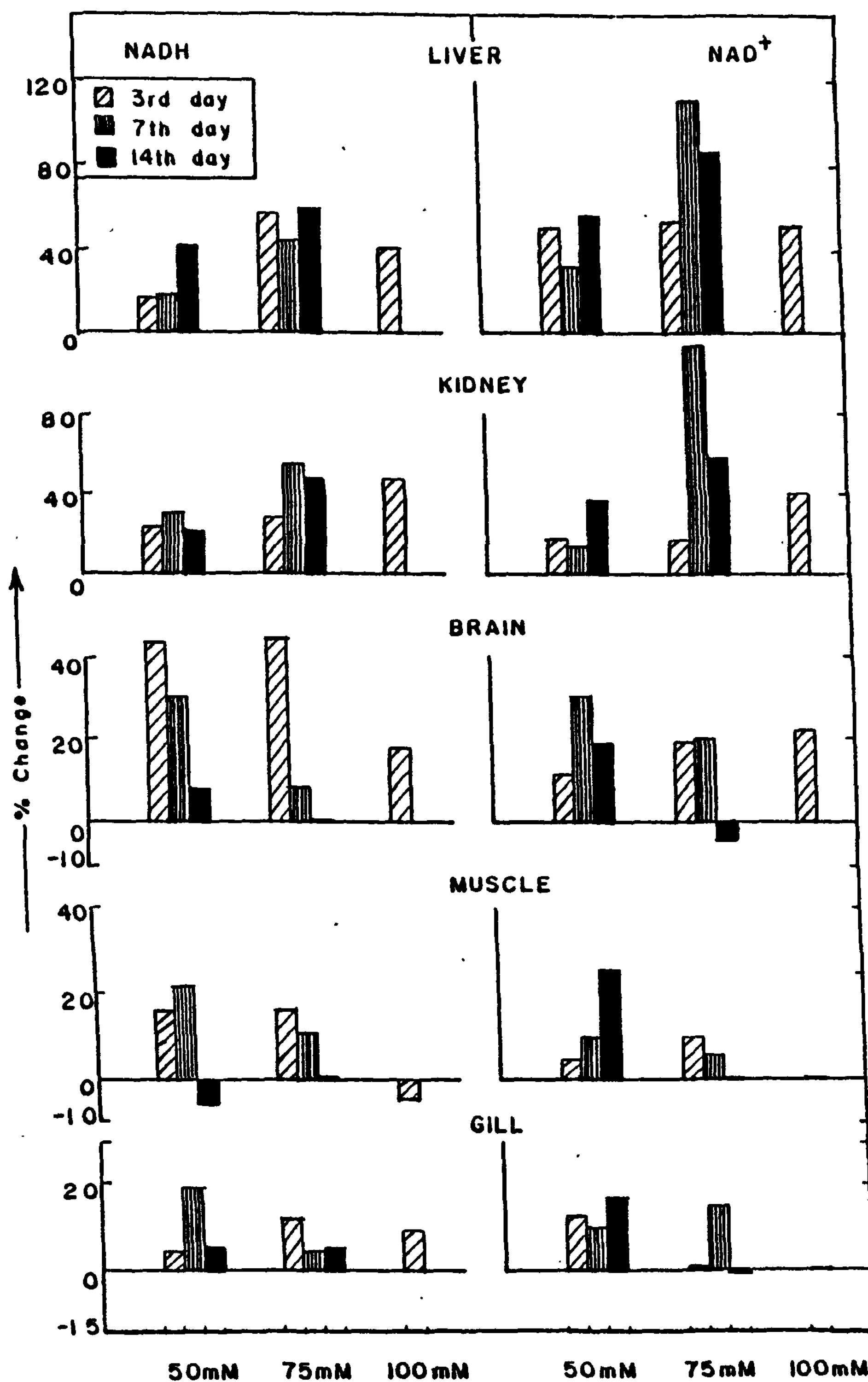


Fig.21. Percent (%) change in the total activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during hyper-ammonia stress.

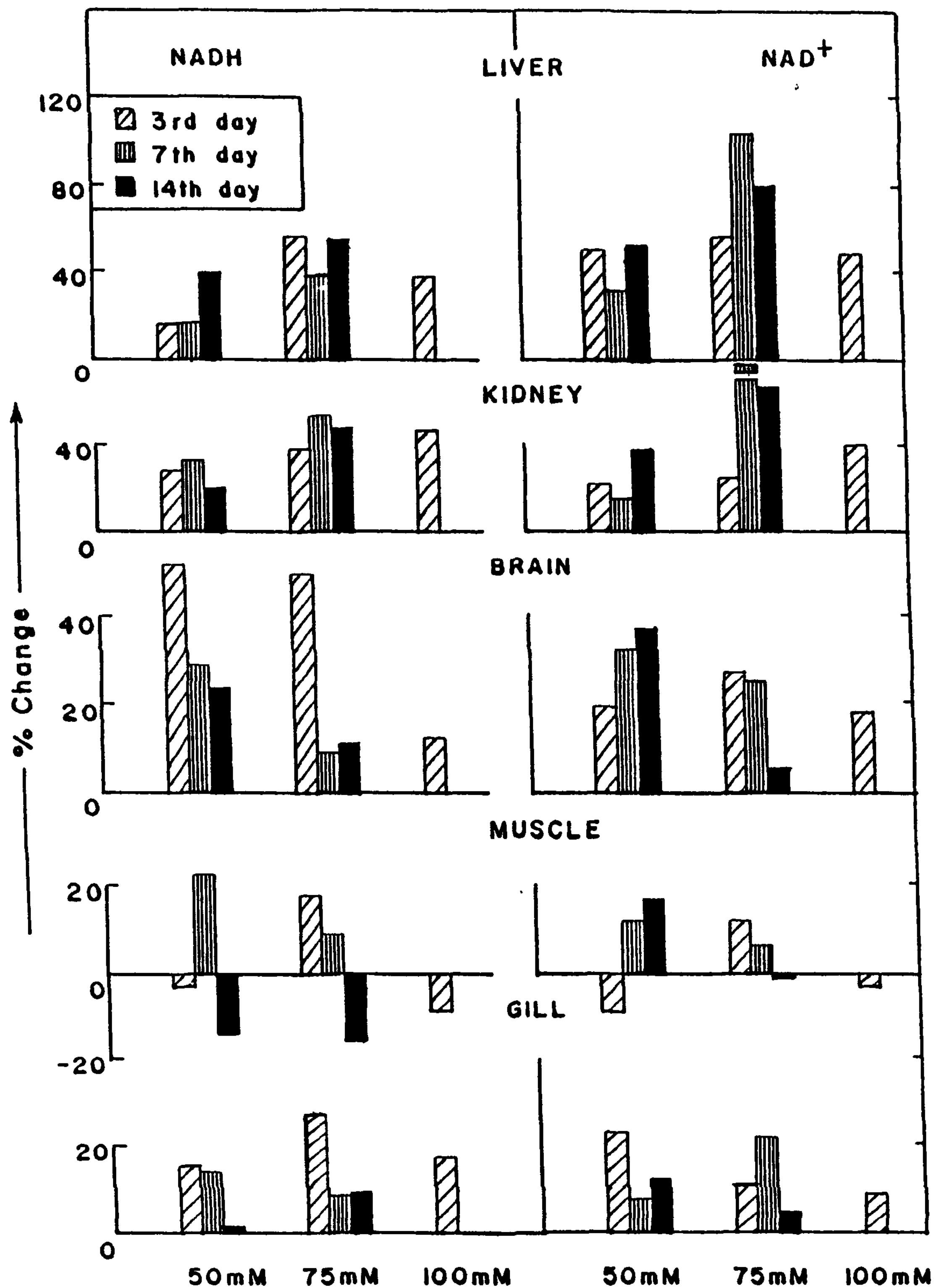


Fig.22. Percent (%) change in the specific activity of GDH (NADH and NAD^+ dependent) in various tissues of H. fossilis during hyper-ammonia stress.

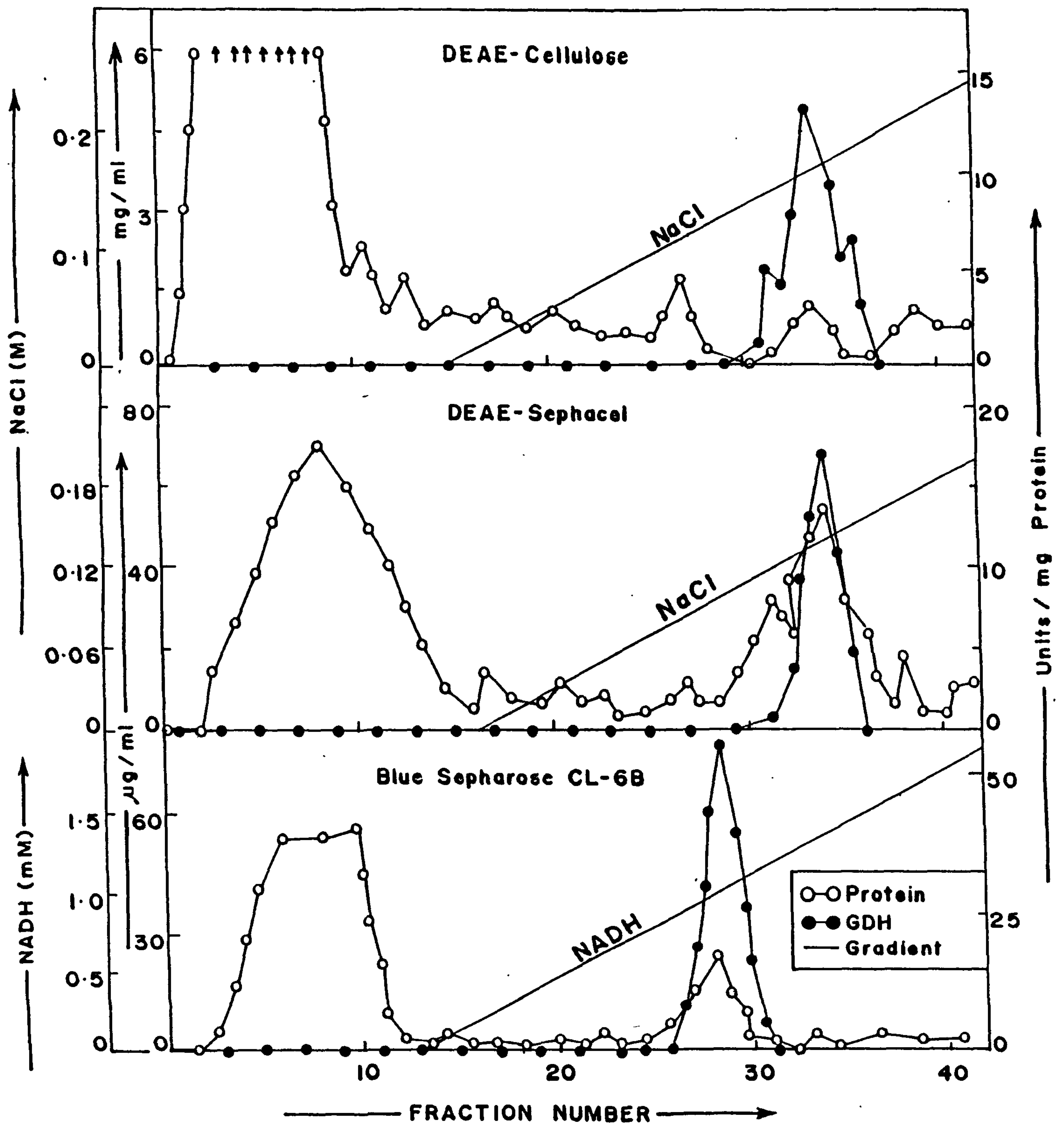


Fig.23. Elution pattern of GDH from DEAE-Cellulose, DEAE-Sephacel and Blue Sepharose CL-6B columns during purification from liver of H. fossilis.

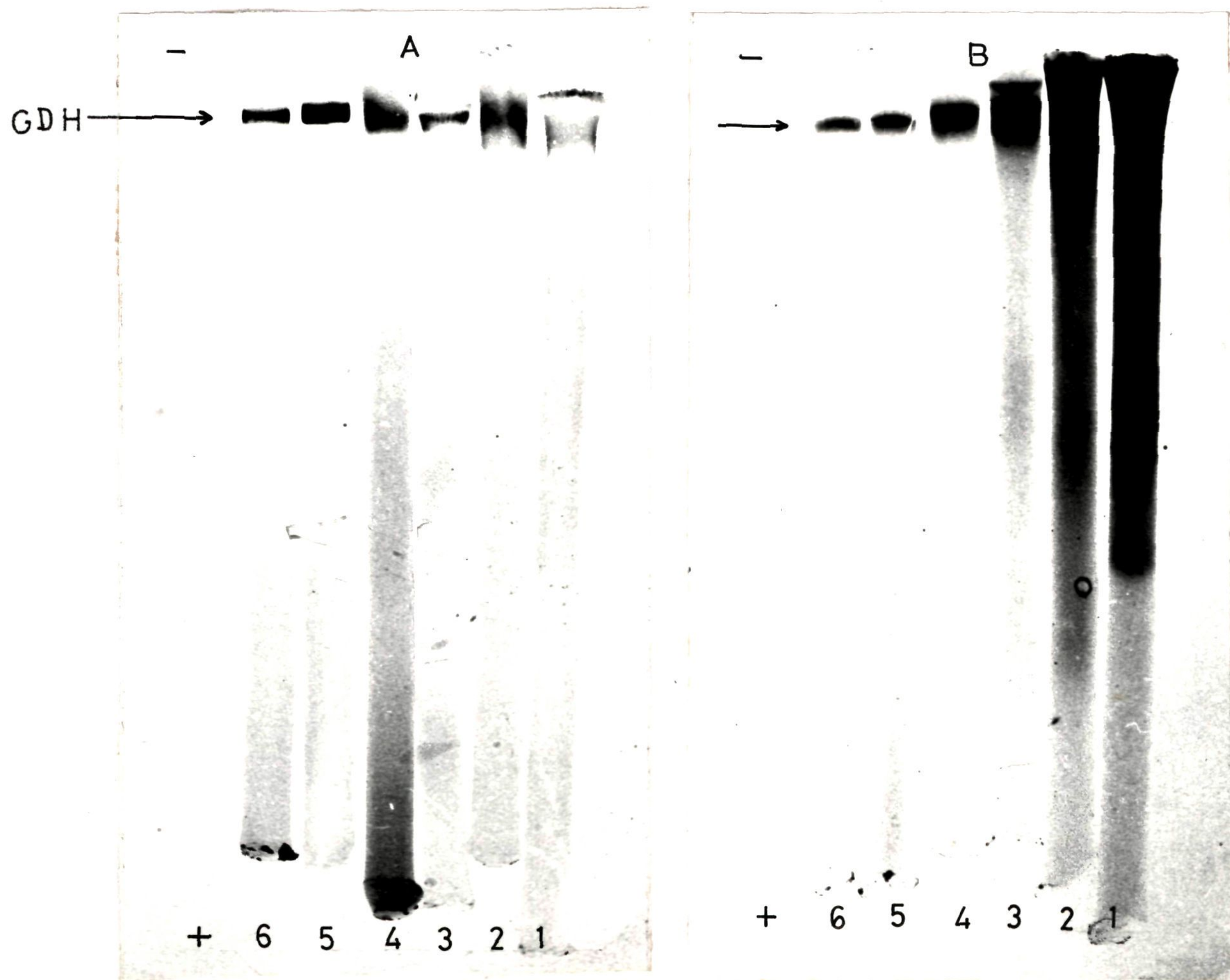


Fig.24(a) GDH specific staining (A) and protein staining (Coomassie blue) (B) on polyacrylamide gel (PAGE) of samples from different stages of purification of GDH, 1-Crude extract, 2-Ammonium sulphate, 3-Sephadex G-25, 4-DEAE-Cellulose, 5-DEAE-Sephacel, 6-Blue Sepharose CL-6B from liver of H. fossilis during Summer.

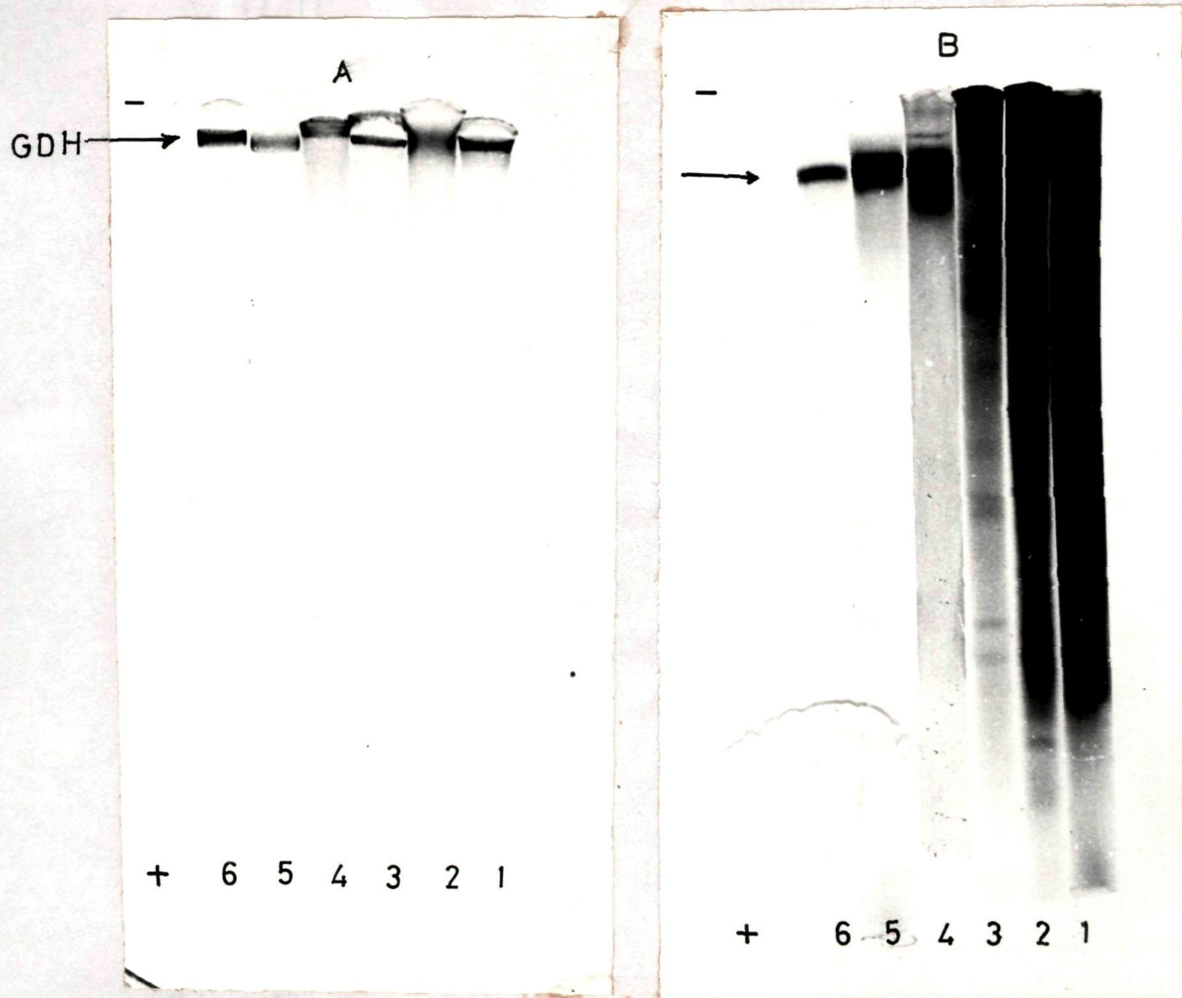


Fig.24(b) GDH specific staining (A) and protein staining (Coomassie blue) (B) on polyacrylamide gel (PAGE) of samples from different stages of purification of GDH, 1-Crude extract, 2-Ammonium sulphate, 3-Sephadex G-25, 4-DEAE-Cellulose, 5-DEAE-Sephacel, 6-Blue Sepharose CL-6B from liver of H. fossilis during Winter.

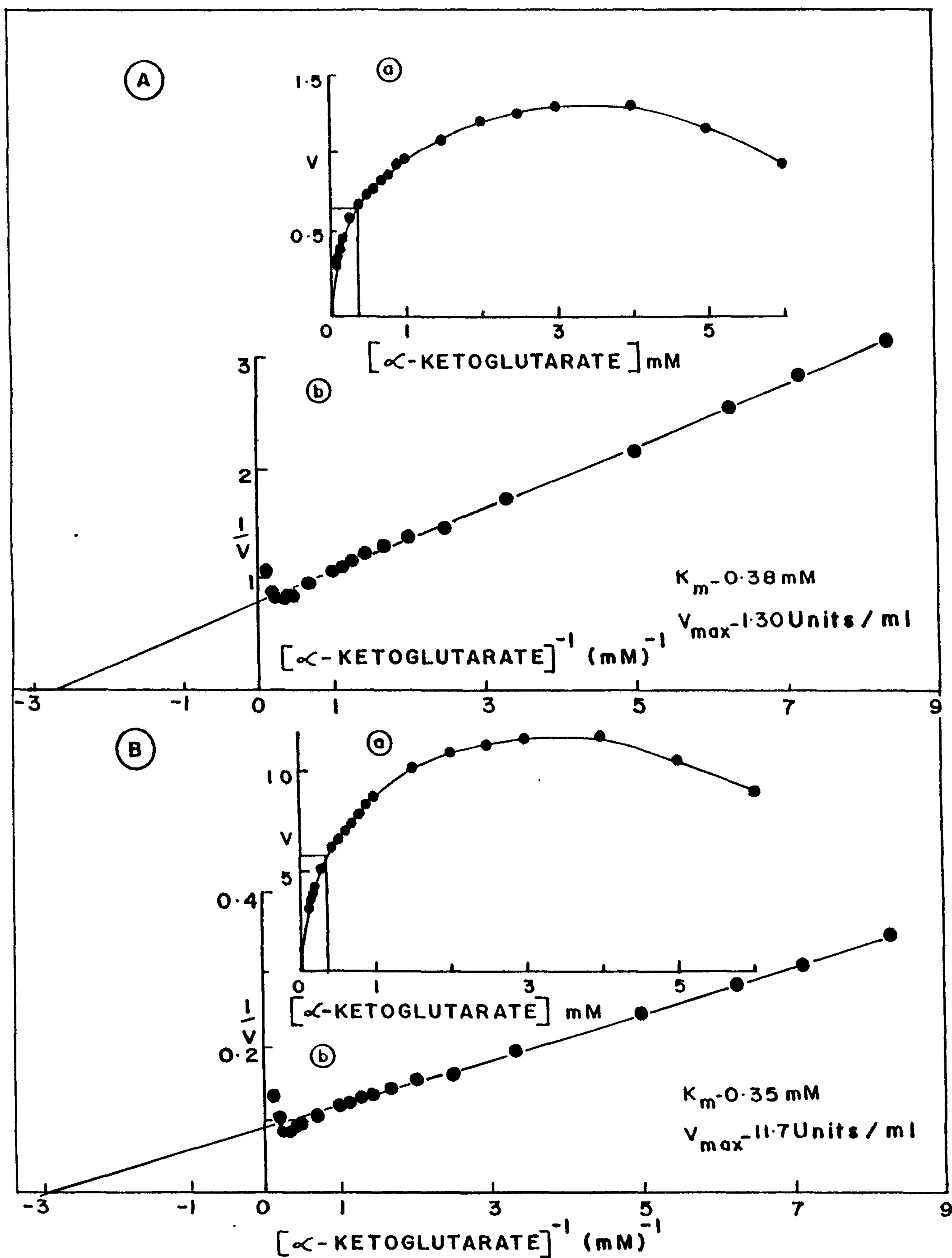


Fig.25. Michaelis-Menten (a) Lineweaver-Burk (b) plot for determination of K_m and V_{max} of purified GDH from liver of *H. fossilis* for α-ketoglutarate without ADP(A) and with ADP(B).

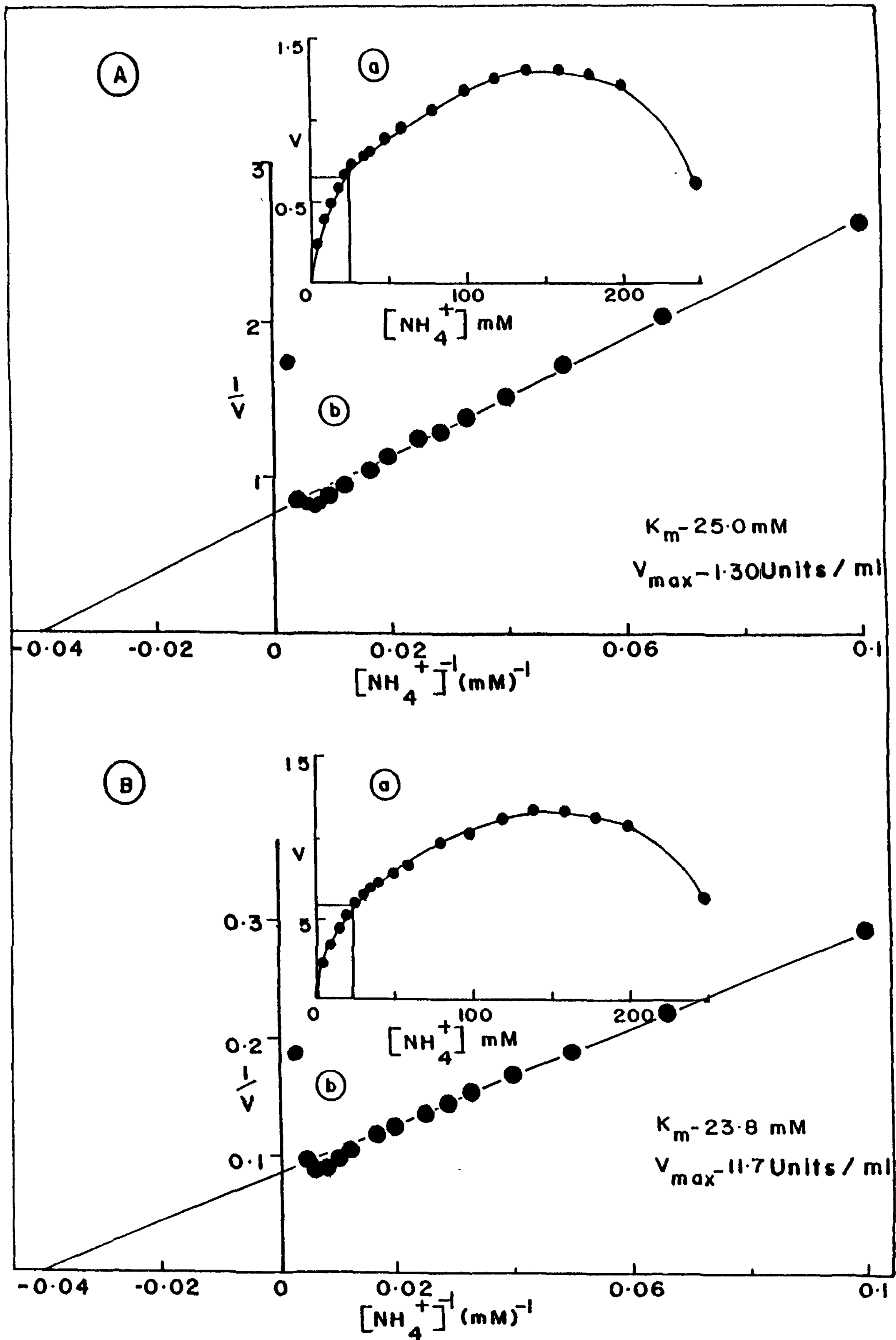


Fig.26. Michaelis-Menten(a) Lineweaver-Burk(b) plot for determination of K_m and V_{max} of GDH purified from liver of H. fossilis for NH_4^+ without ADP(A) and with ADP(B).

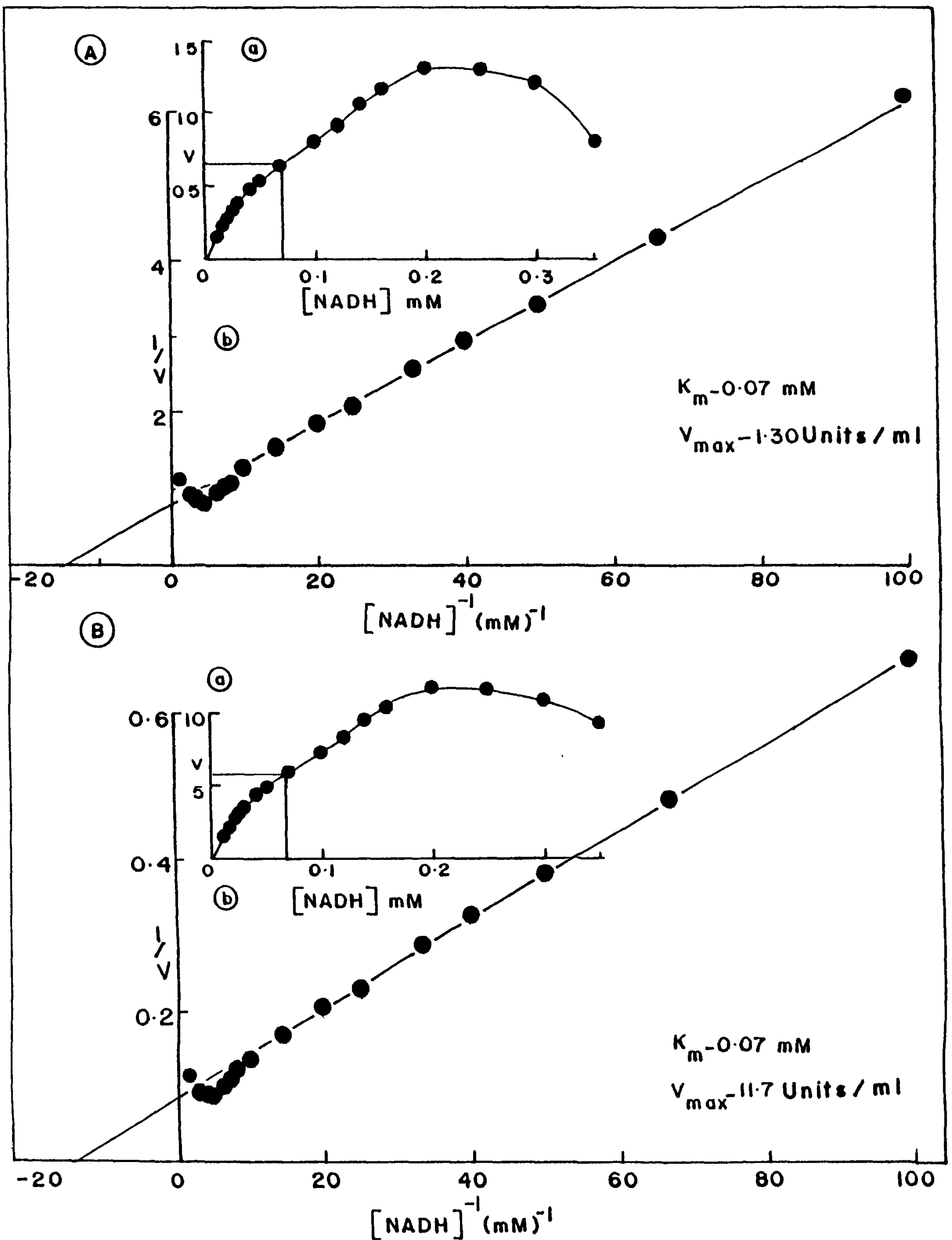


Fig.27. Michaelis-Menten(a) Lineweaver-Burk(b) plot for determination of K_m and V_{max} of GDH purified from liver of H. fossilis for NADH without ADP (A) and with ADP(B).

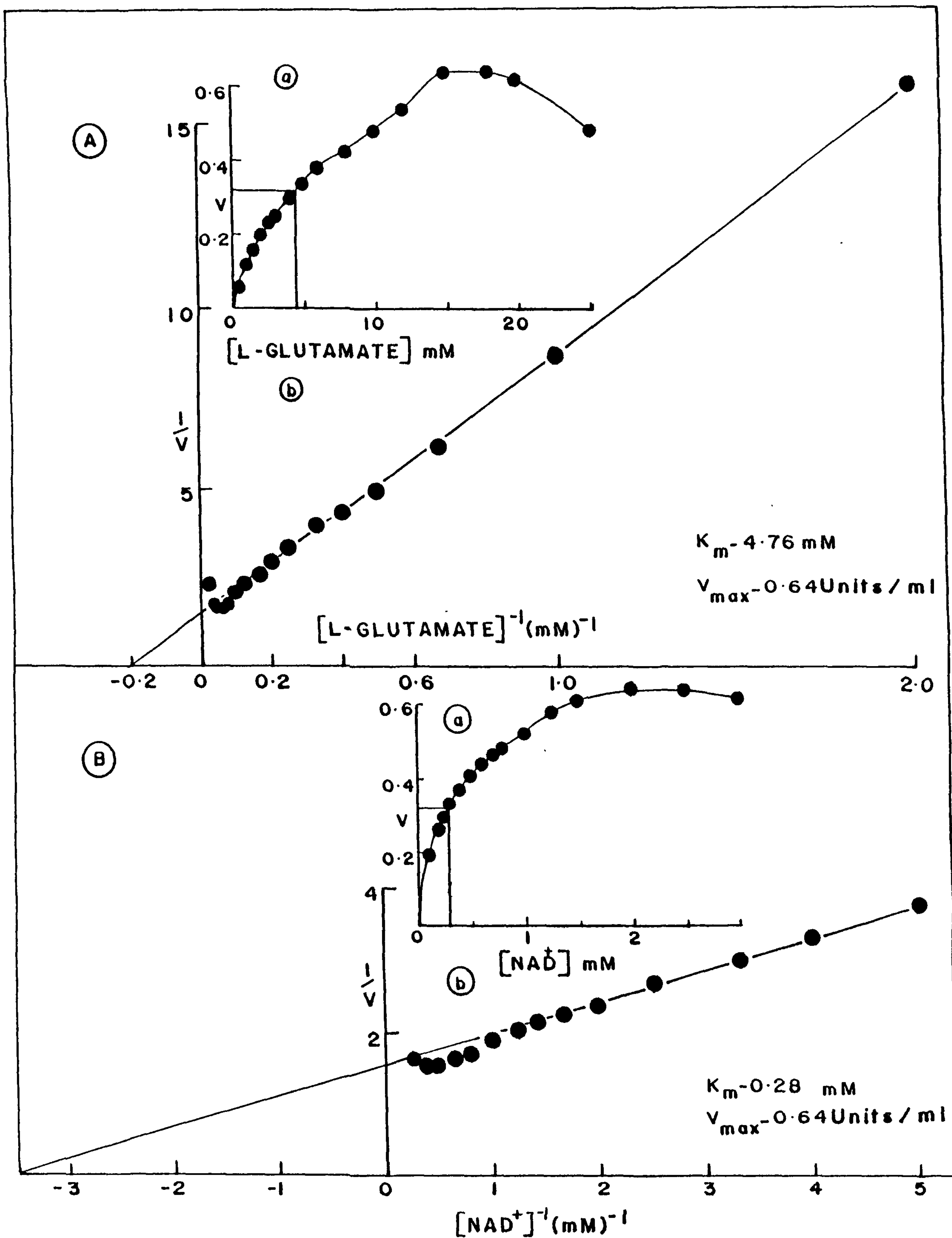


Fig.28. Michaelis-Menten (a) Lineweaver-Burk(b) plot for determination of K_m and V_{max} of purified GDH from liver of H. fossilis for L-glutamate (A) and NAD^+(B).

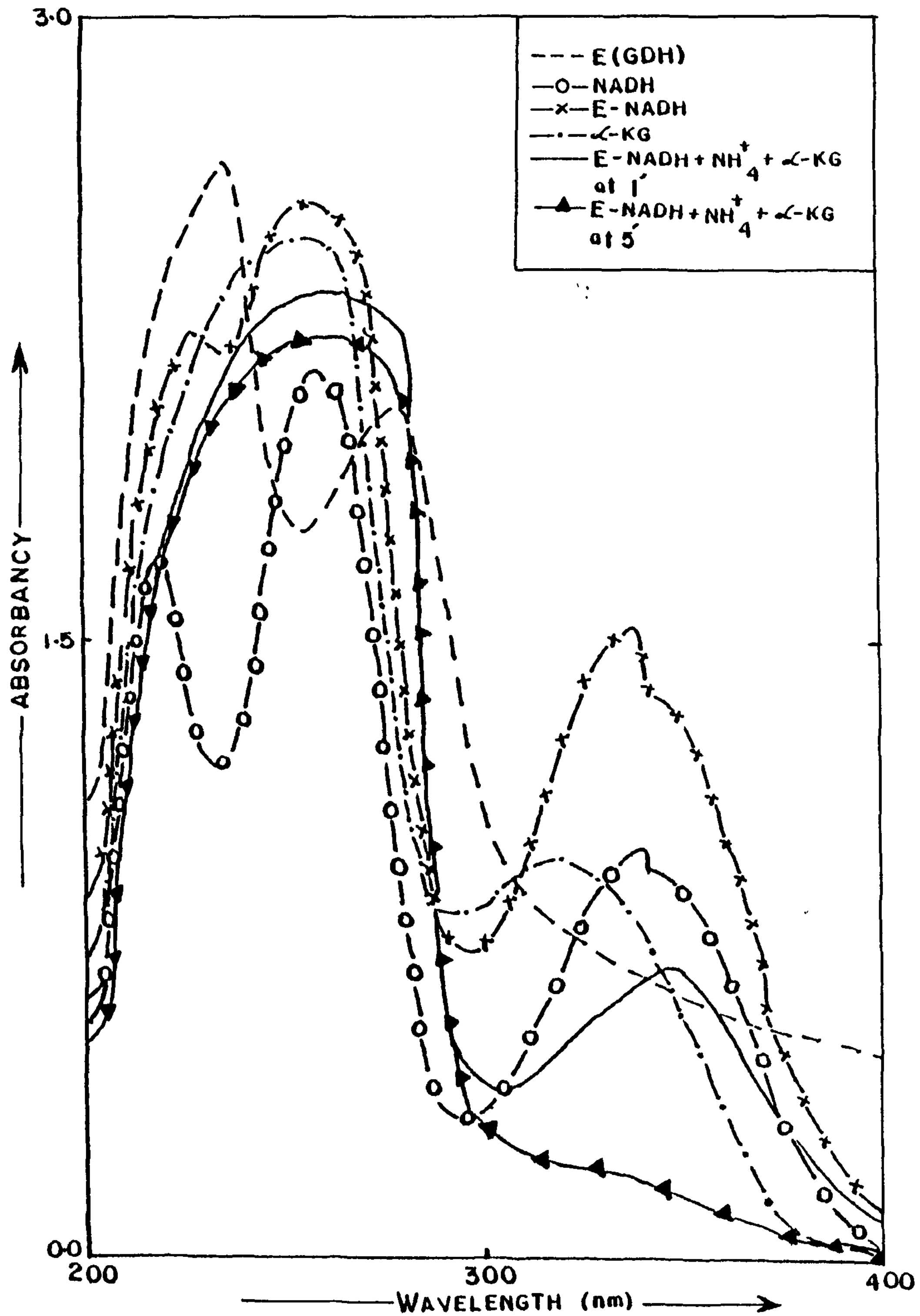


Fig.29. U.V. Absorption spectra of GDH purified from liver of H. fossilis in presence of various components of the reaction mixture (NADH dependent reaction).

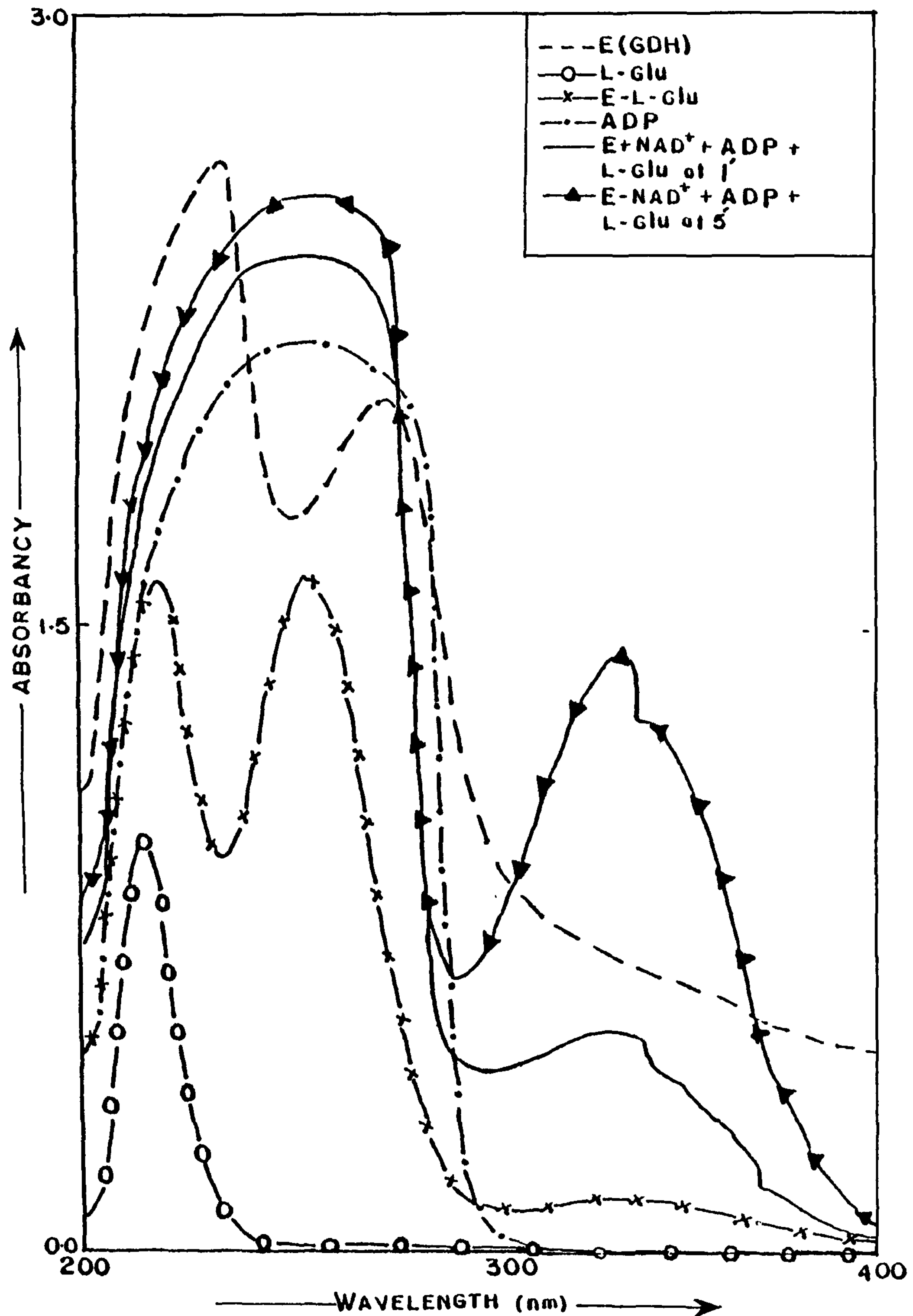


Fig.30. U.V. Absorption spectra of GDH purified from liver of H. fossilis in presence of various components for the reaction mixture (NAD^+ dependent reaction).

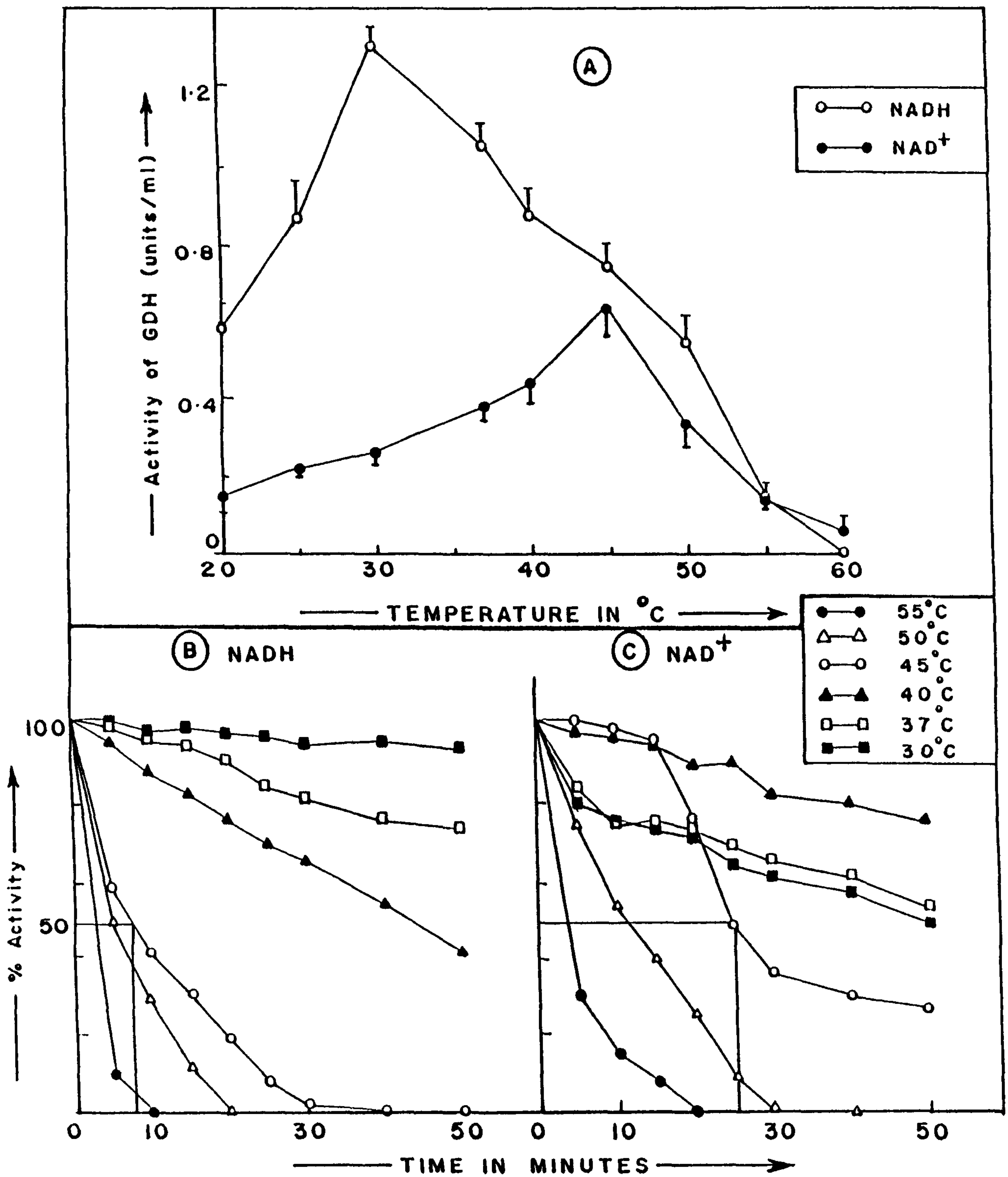


Fig.31. Effect of incubation temperature on GDH activity (A) and stability of the purified GDH from liver of H. fossilis at different temperature [NADH dependent (B) and NAD^+ dependent (C)].

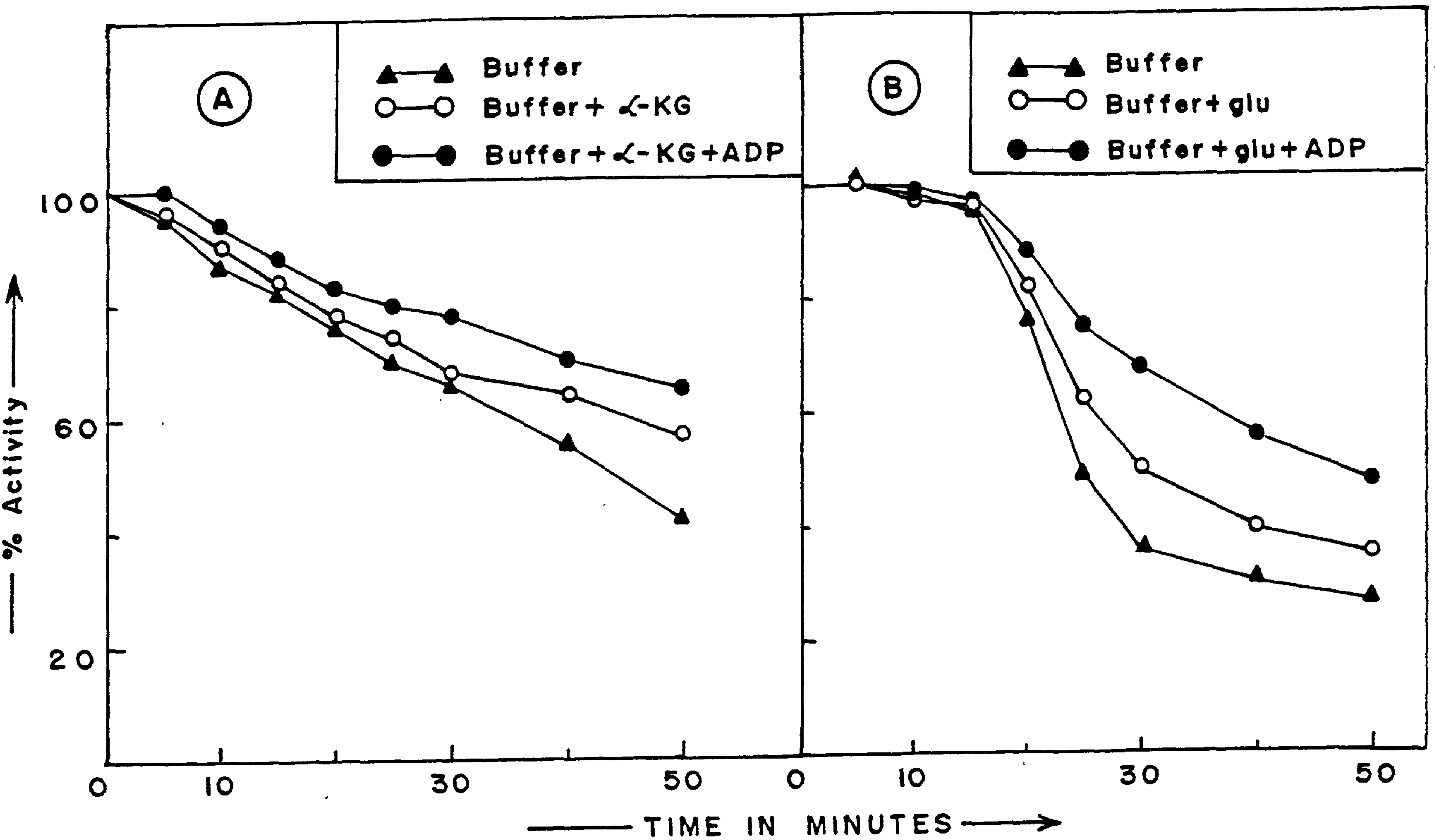


Fig.32. Thermal stability and effect of substrates and ADP on NADH (A) and NAD^+ (B) dependent GDH activity at 40° and 45°C respectively of the enzyme purified from liver of H. fossilis.

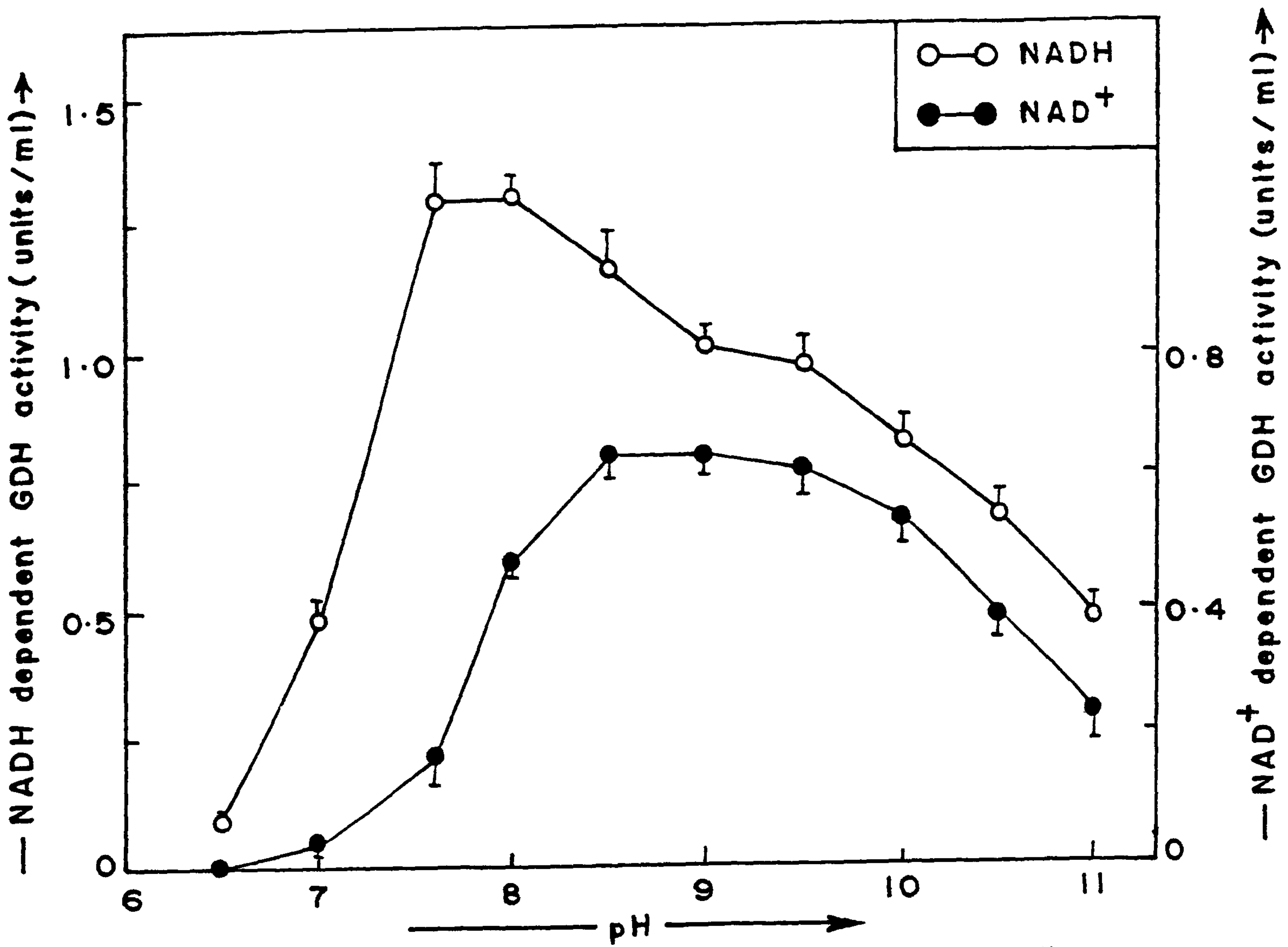


Fig.33. Effect of pH on purified GDH activity from liver of *H. fossilis*.

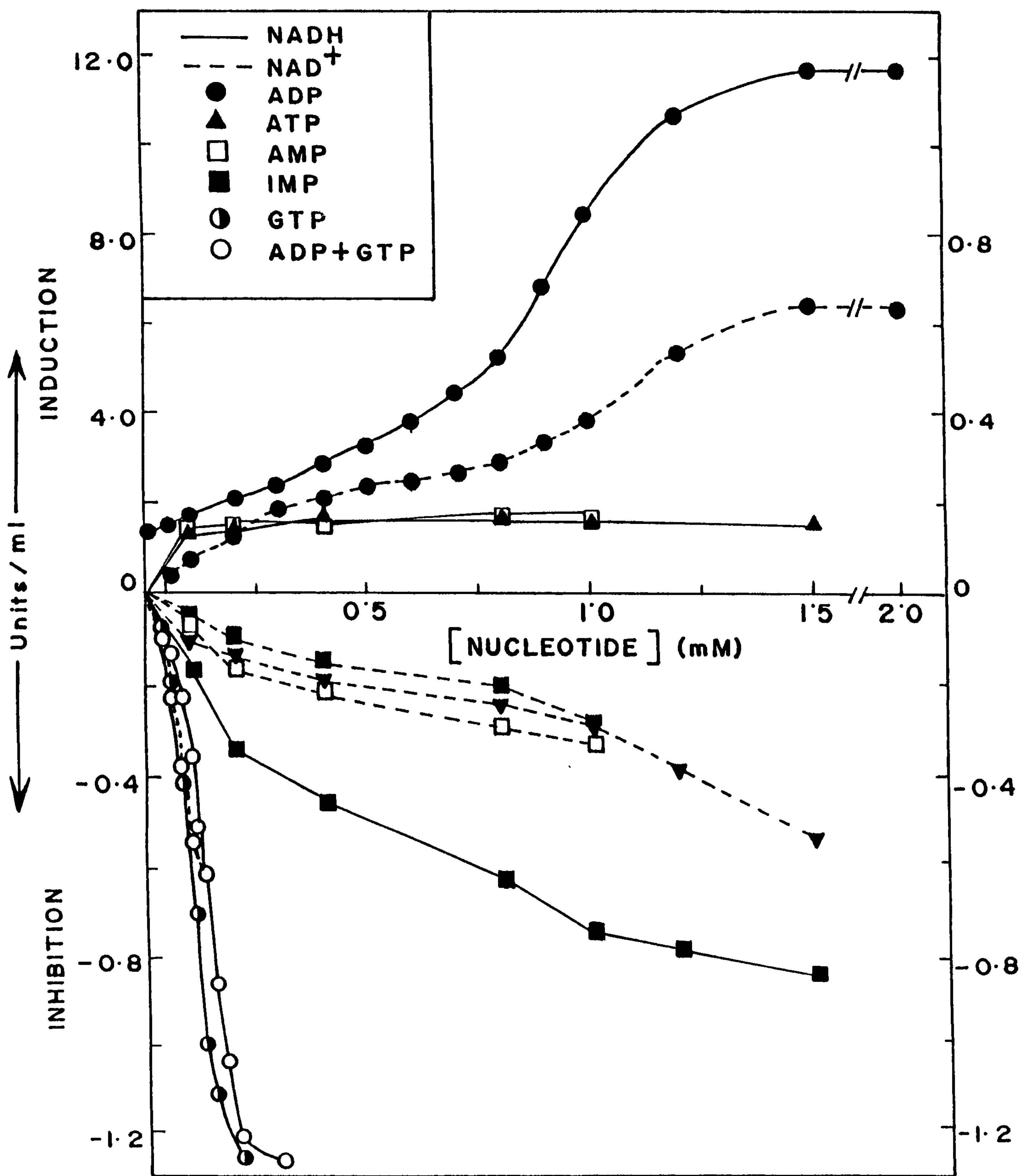


Fig.34. Modulation of GDH activity purified from liver of H. fossilis by various nucleotides.

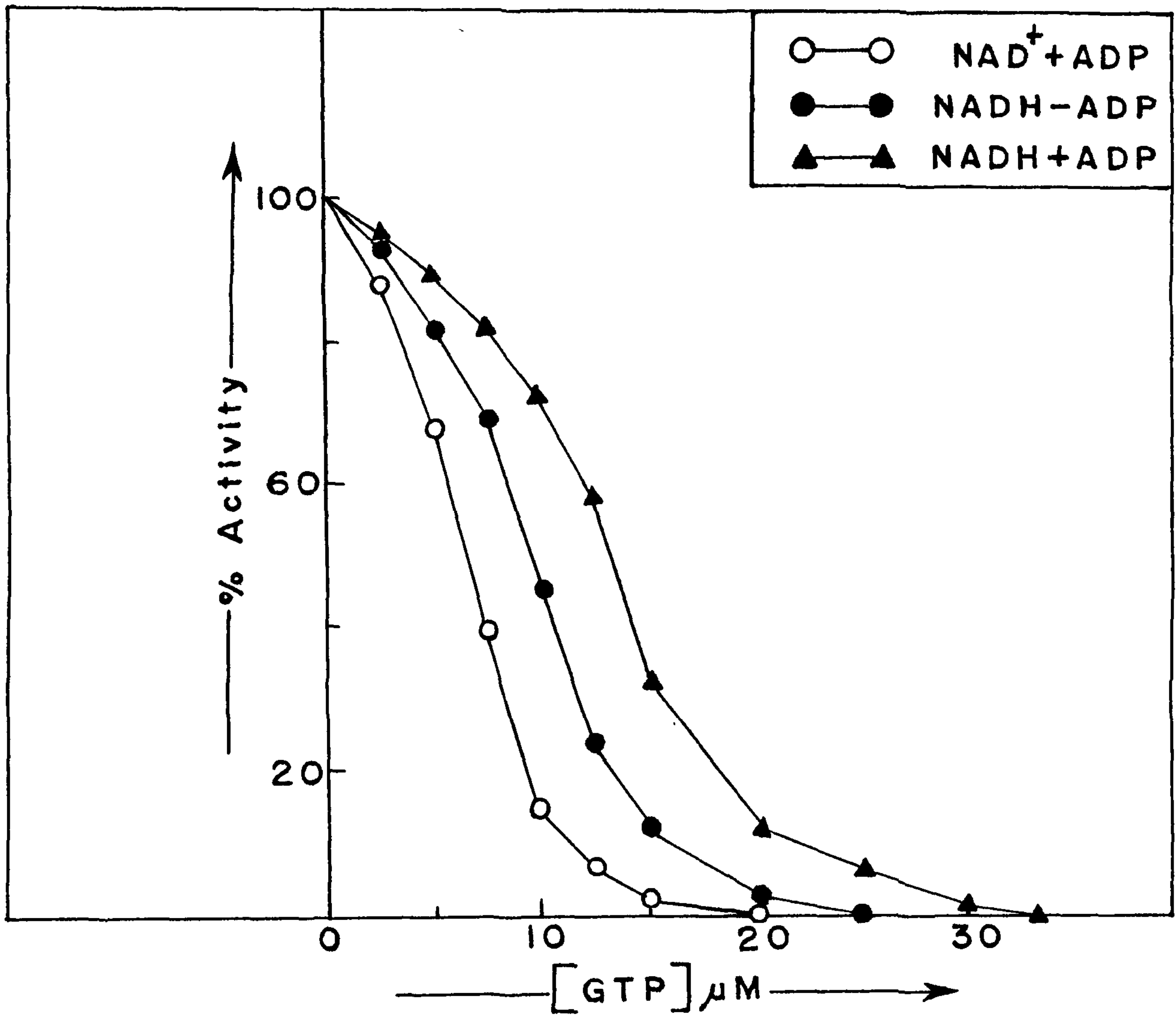


Fig .35. Pattern of inhibition by GTP of purified GDH activity from liver of H. fossilis.

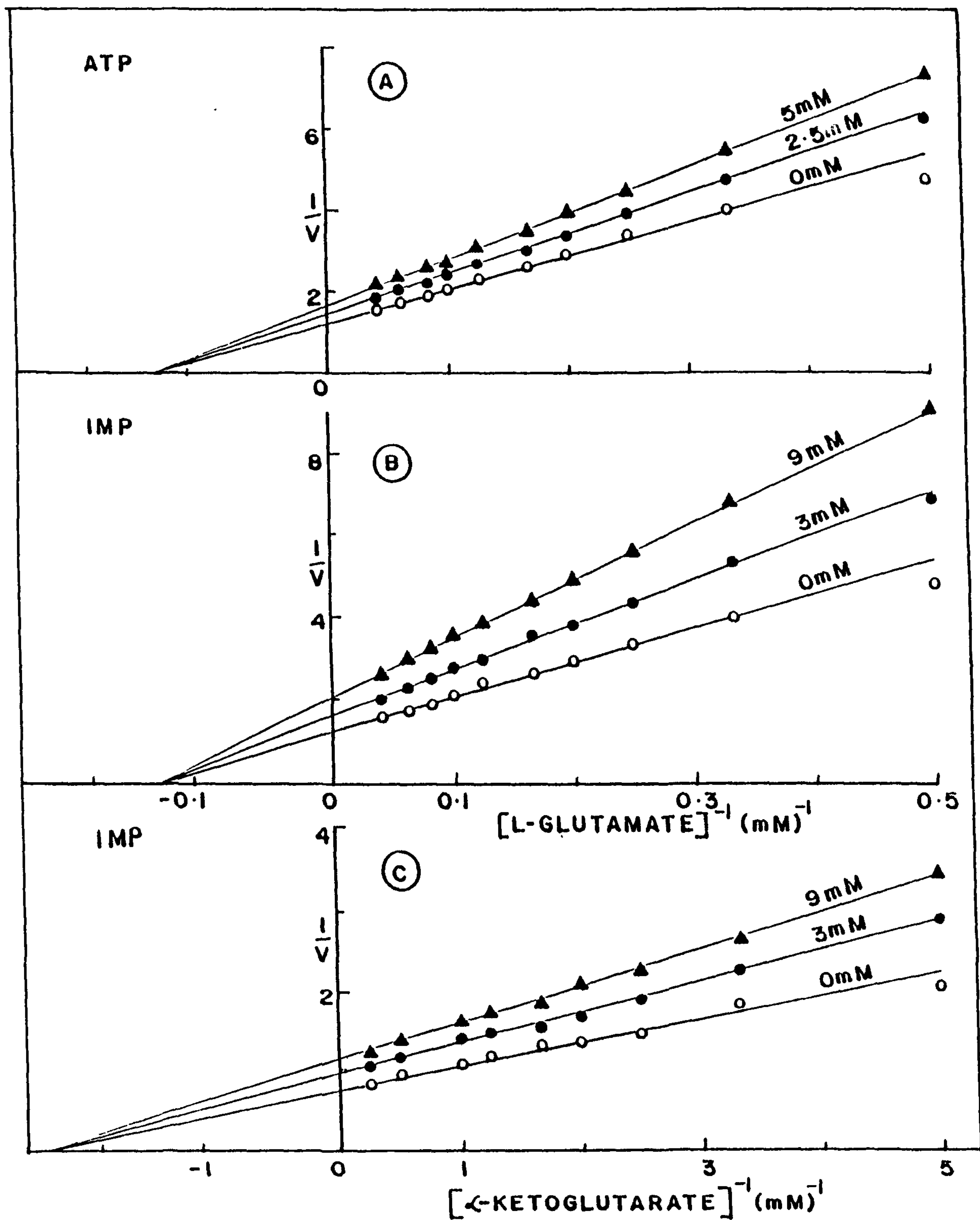


Fig.36. Lineweaver-Burk plot for determination of nature of inhibition of ATP(A) and IMP(B) on NAD^+ dependent activity and IMP(C) on NADH dependent activity of GDH purified from liver of *H. fossilis*.

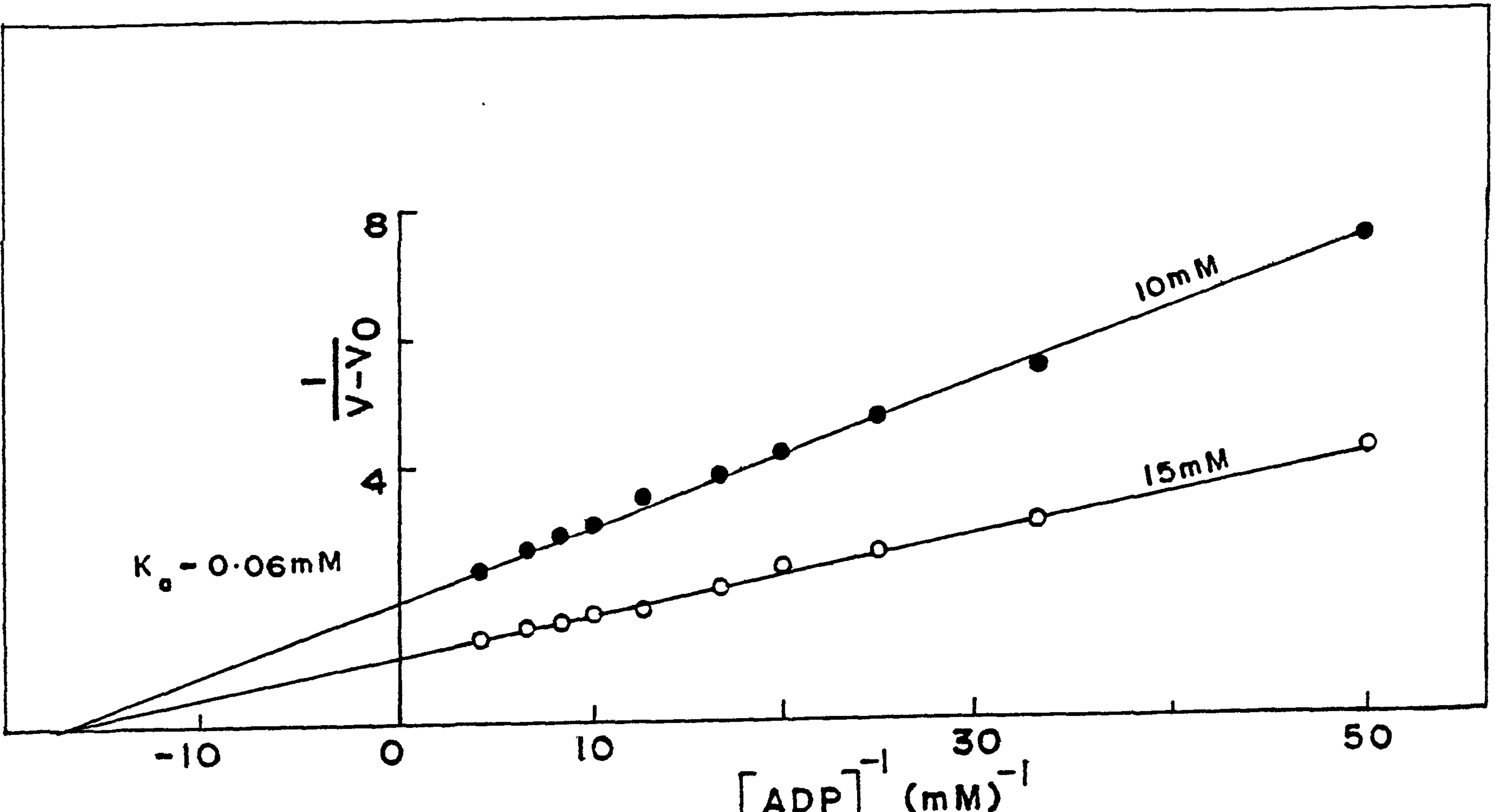


Fig.37. Hill plot for determination of K_a of ADP for purified GDH (NADH dependent) activity from liver of *H. fossilis*.

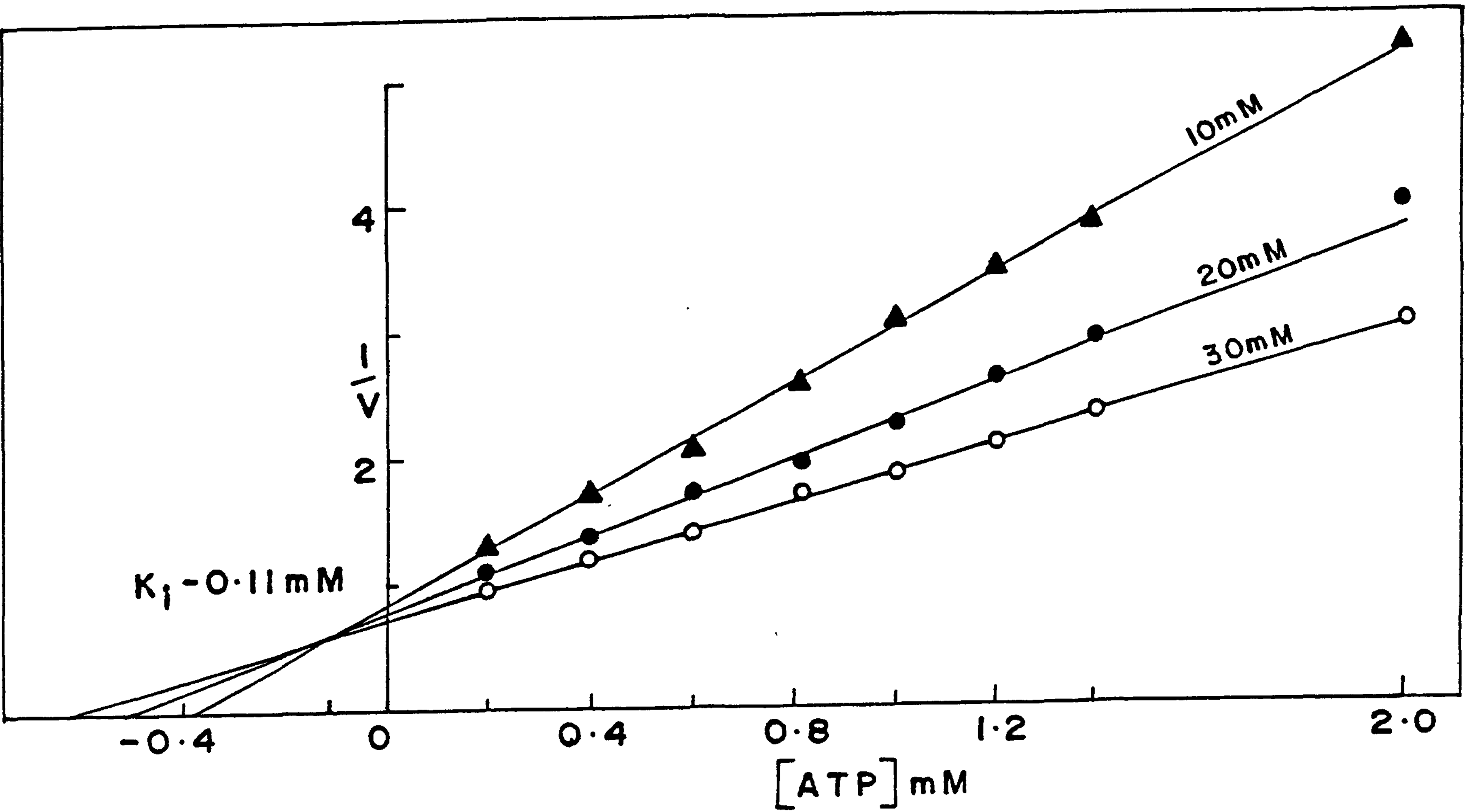


Fig.38. Dixon plot for determination of K_i of ATP for purified GDH (NAD^+ dependent) activity from liver of H. fossilis.

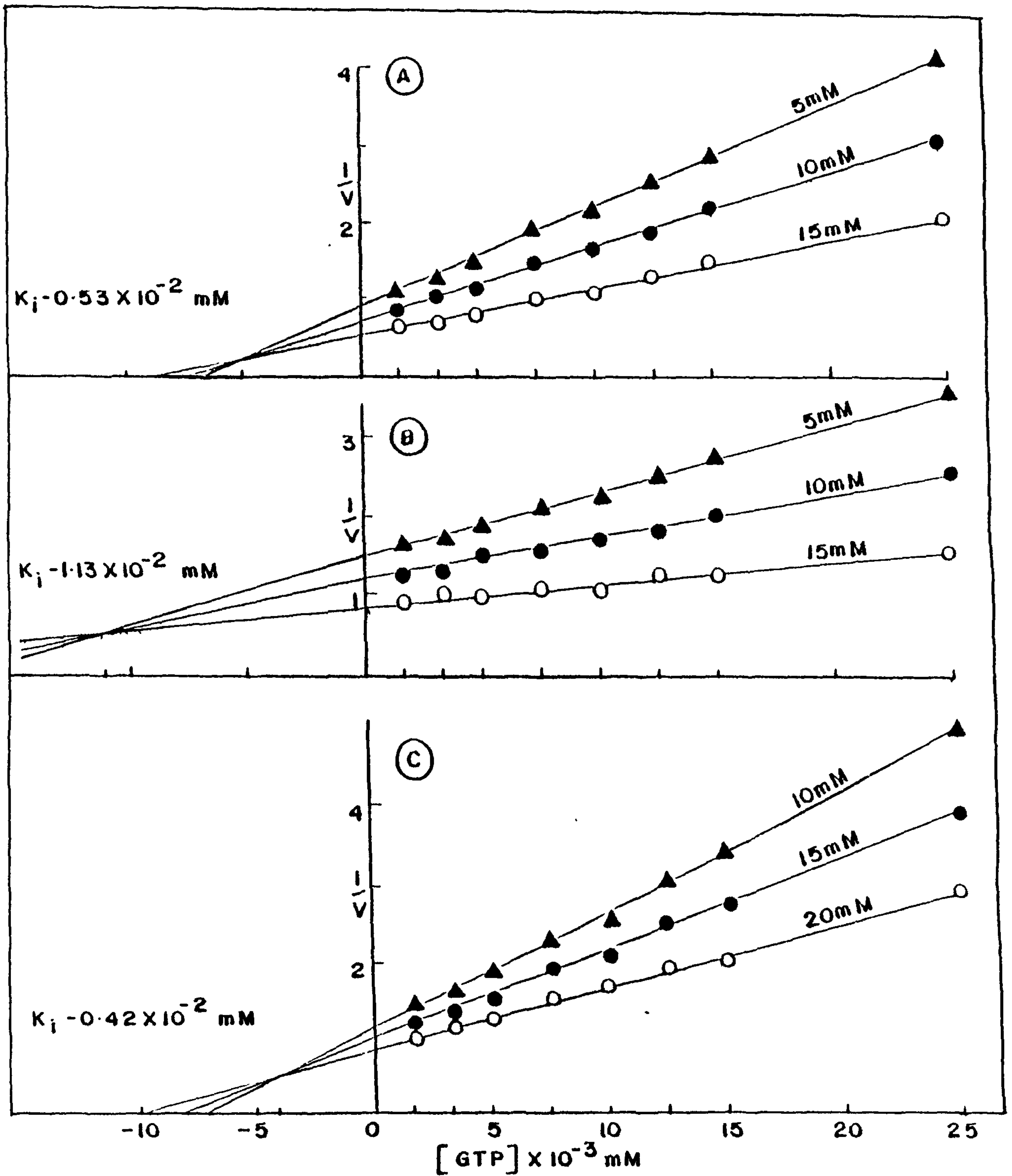


Fig.39. Dixon plot for determination of K_i of GTP for purified GDH activity from liver of H. fossilis, NADH dependent in absence and presence of ADP (A) & (B) NAD^+ dependent in presence of ADP (C).

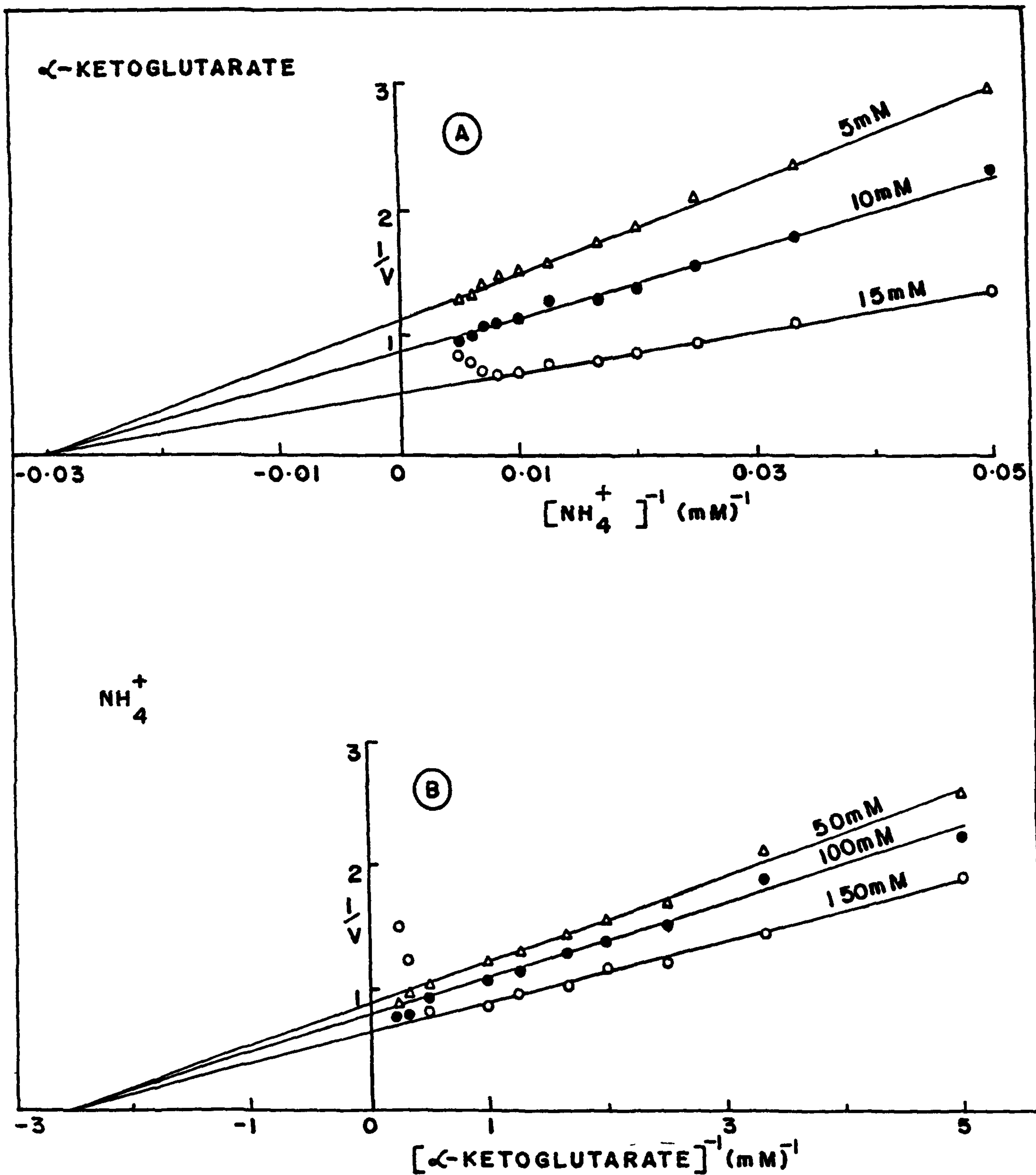


Fig.40. Double reciprocal plots of velocity (NADH dependent) against substrate concentration, (A) variation of NH_4^+ concentration and at several fixed concentrations of α-Ketoglutarate, (B) variation of α-ketoglurate and at several fixed concentrations of NH_4^+.

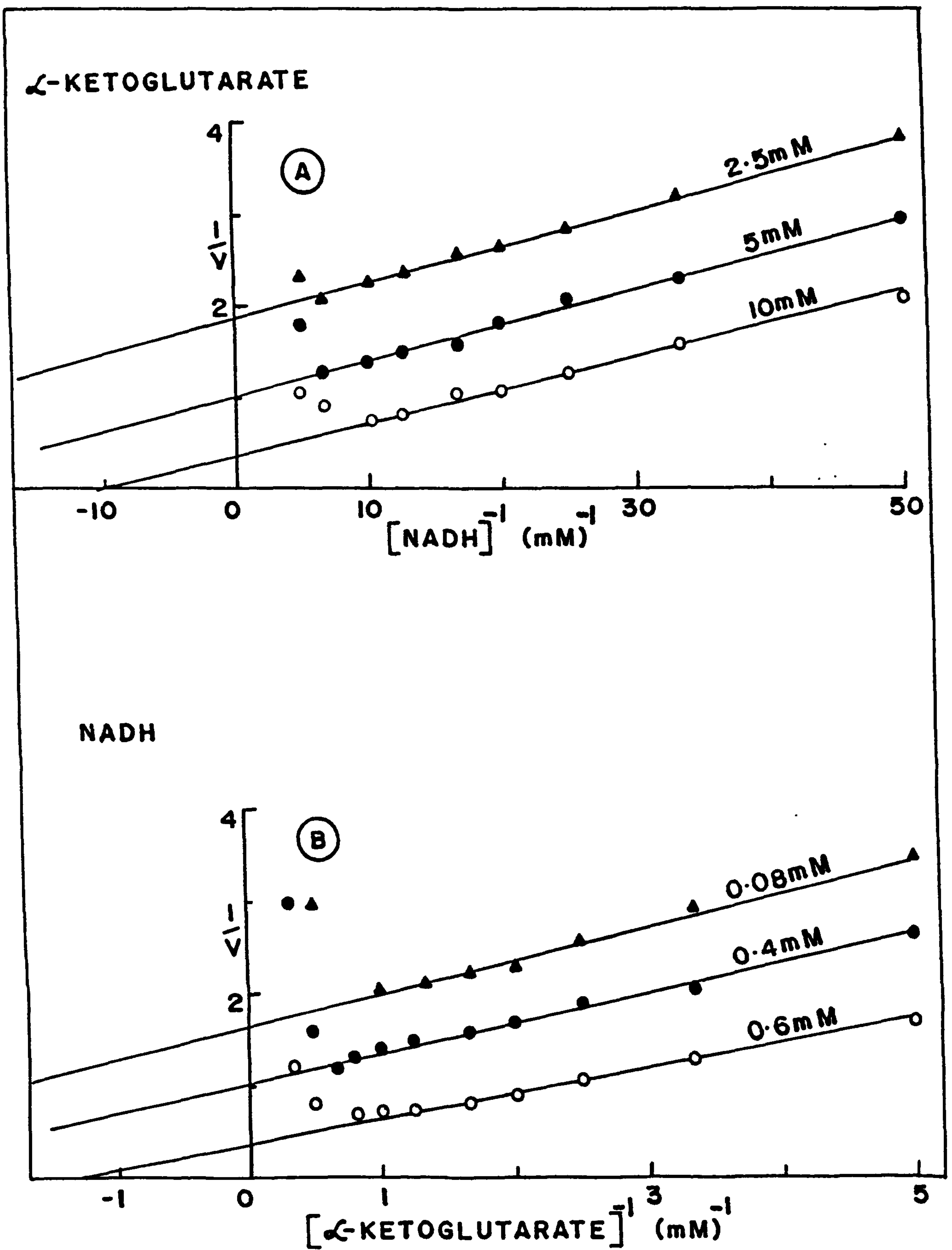


Fig.41. Double reciprocal plots of velocity (NADH dependent) against substrate concentration, (A) variation of NADH concentration and at several fixed concentrations of α-ketoglutarate, (B) variation of α-ketoglutarate and at several fixed concentrations of NADH.

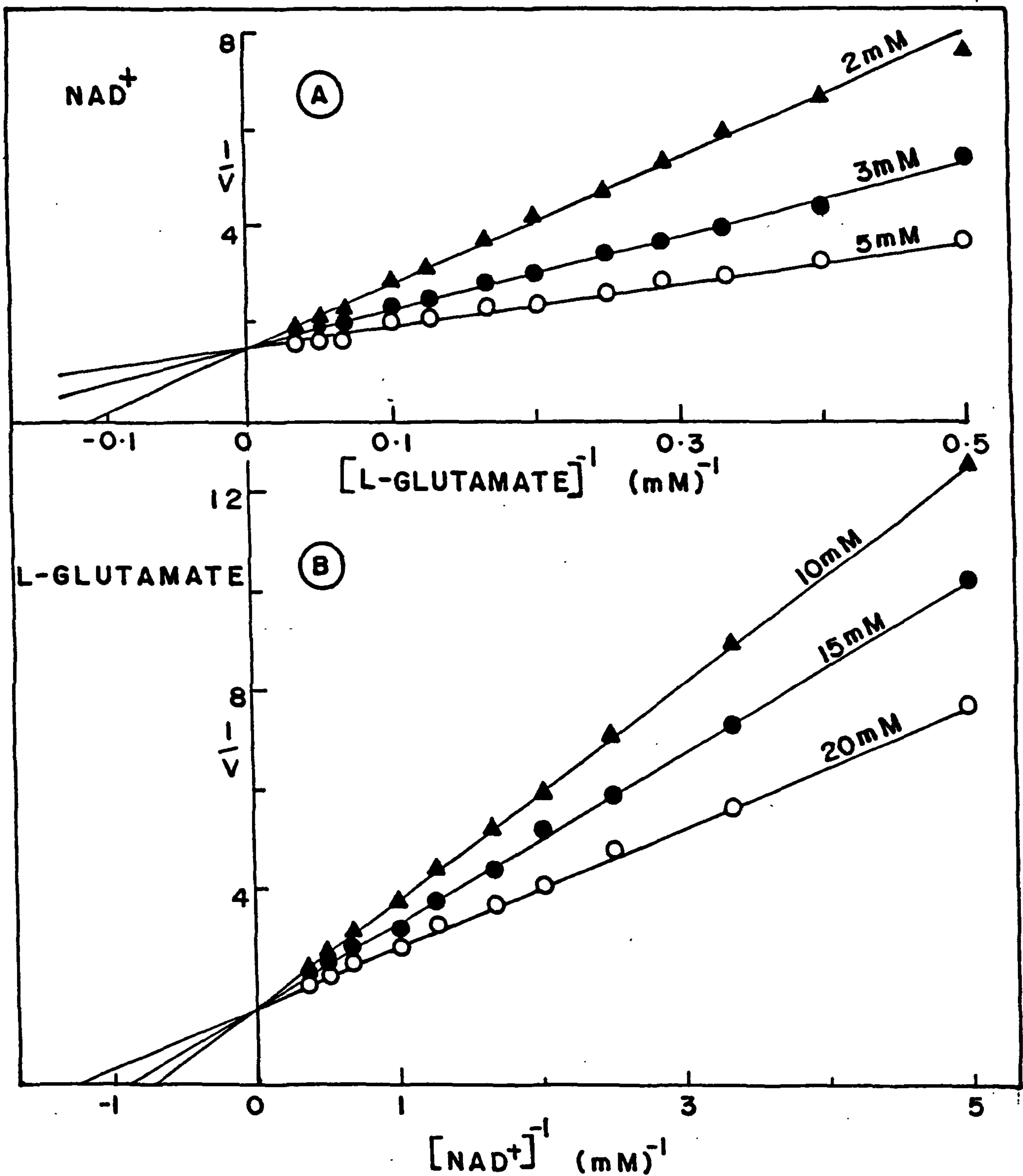


Fig.42. Double reciprocal plots of velocity (NAD^+ dependent) against substrate concentration, (A) variation of L-glutamate and at several fixed concentrations of NAD^+ (B) variation of NAD^+ and at several fixed concentrations of L-glutamate.

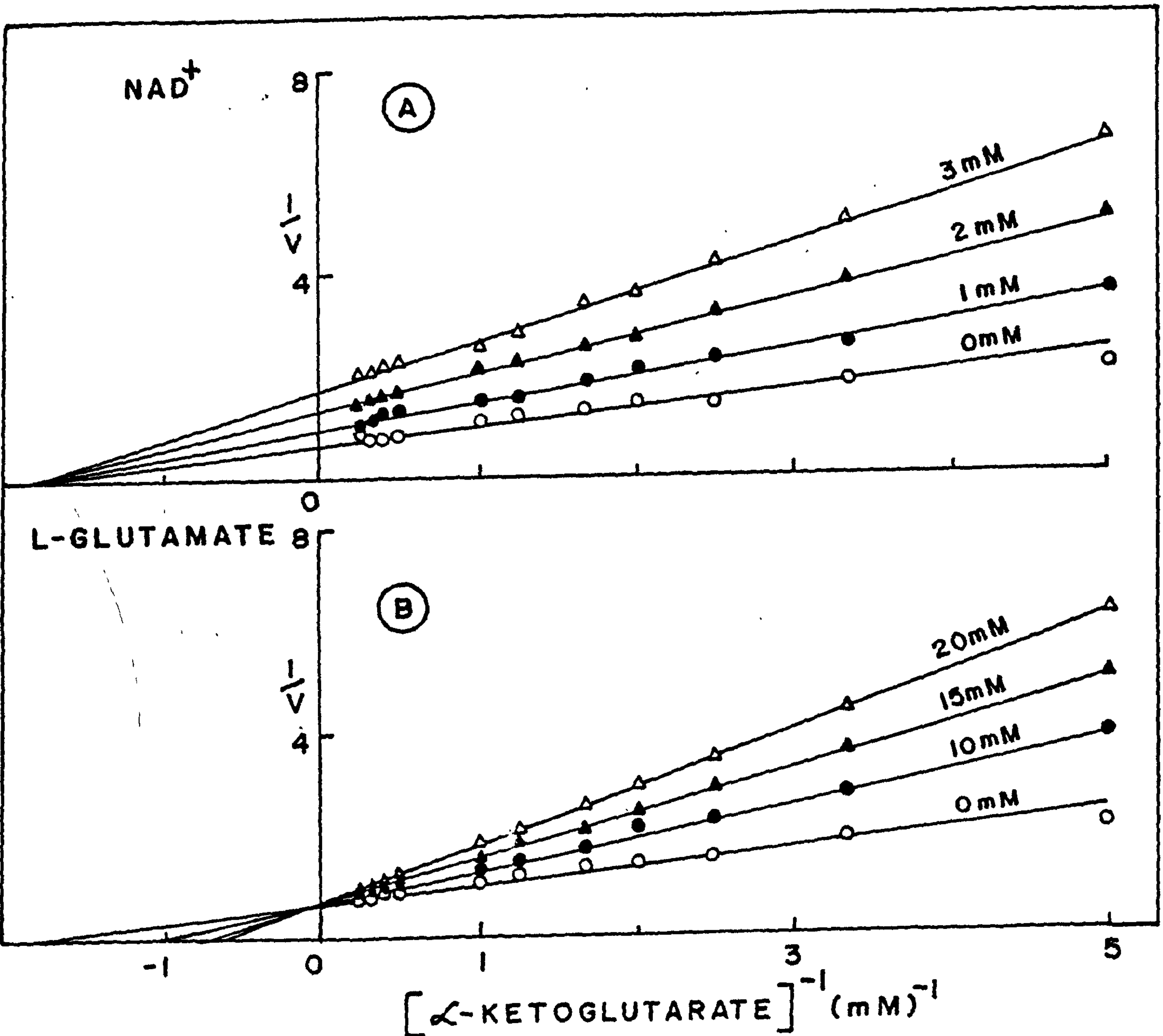


Fig.43. Lineweaver-Burk plot for determination of nature of inhibition of the products NAD^+(A) and L-glutamate(B) with relation to α -ketoglutarate as the substrate on the GDH (NADH dependent) activity purified from liver of <u>H. fossilis</u>.

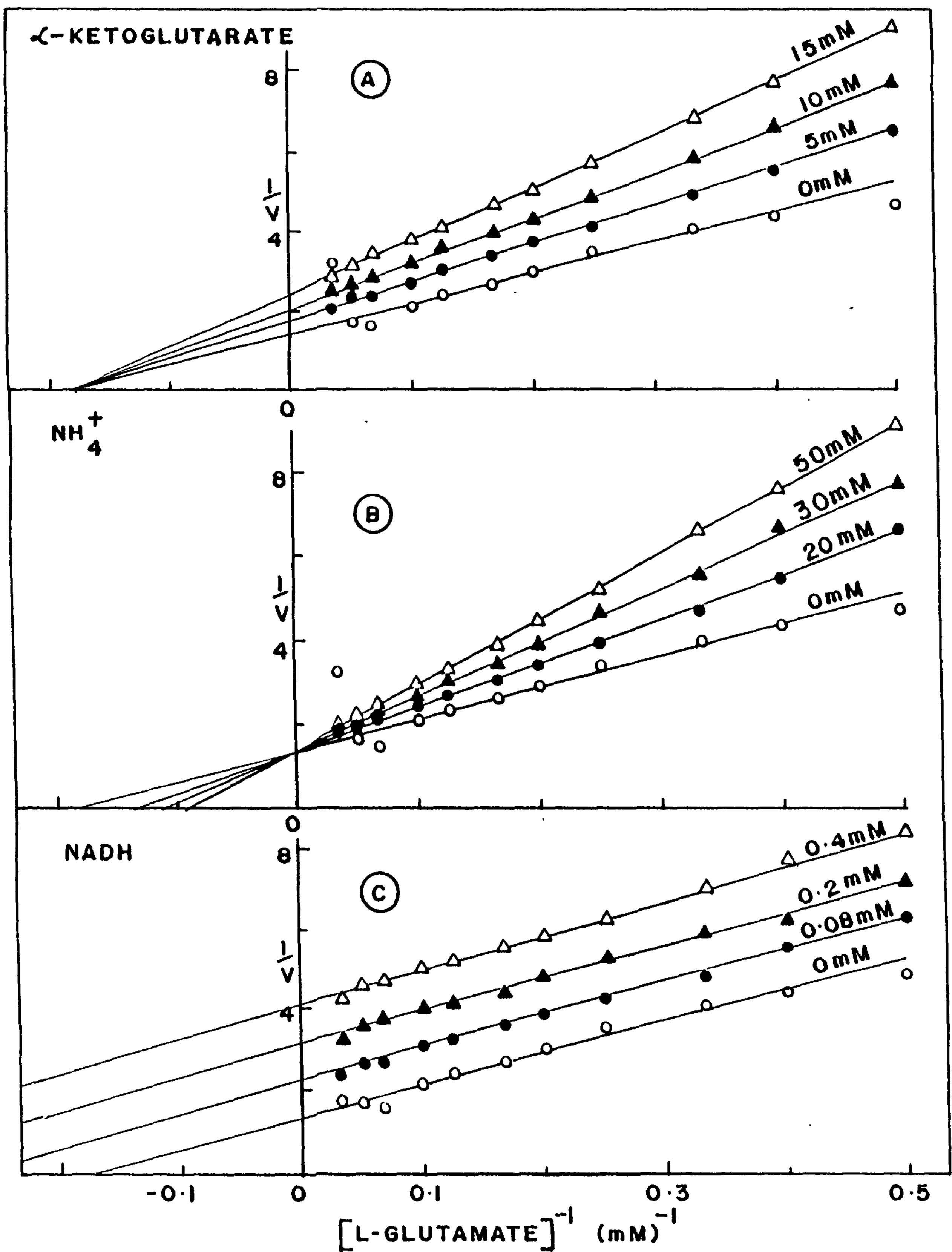


Fig.44. Lineweaver-Burk plot for determination of nature of inhibition of the products α -ketoglutarate (A) NH_4^+ (B) and NADH (C) with relation to L-glutamate as the substrate on the GDH (NAD^+ dependent) activity purified from liver of H. fossilis.

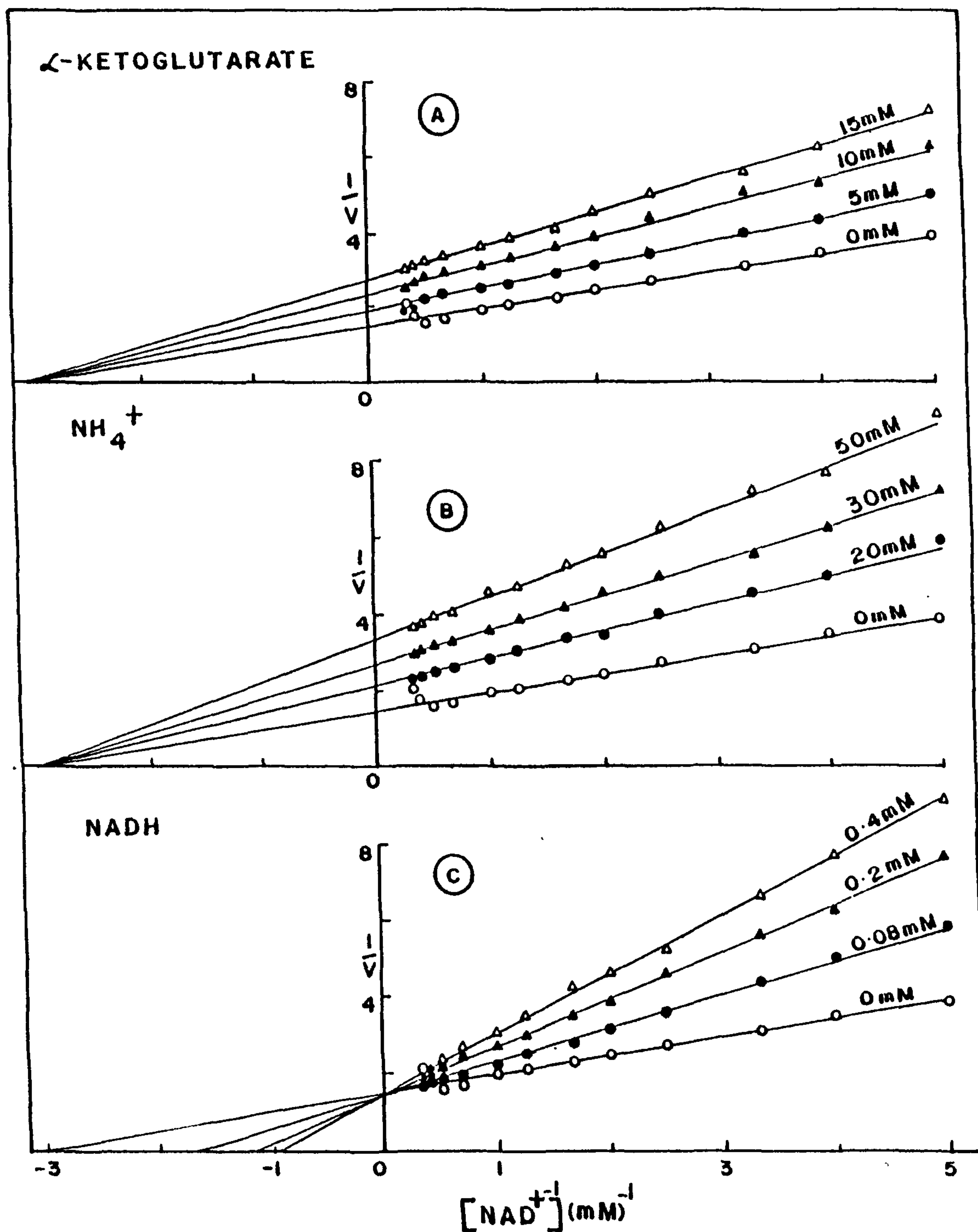


Fig.45. Lineweaver-Burk plot for determination of nature of inhibition of the products α -ketoglutarate (A) NH_4^+ (B) and NADH(C) with relation to NAD^+ as the substrate on the GDH (NAD^+ dependent) activity purified from liver of H. fossilis.

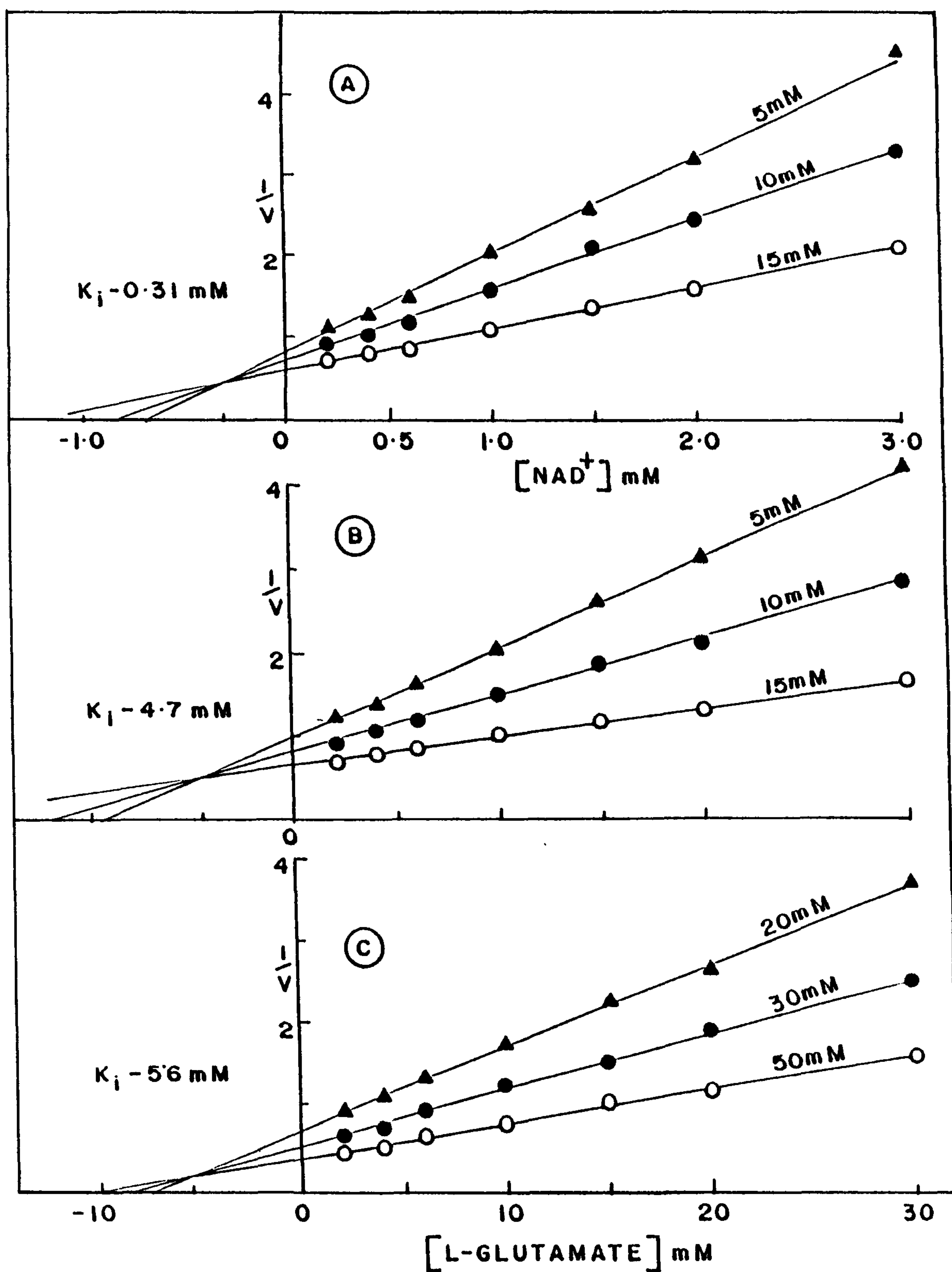


Fig.46. Dixon plot for determination of K_i of the products NAD^+ (A) and L-glutamate (B) with relation to α-ketoglutarate as the substrate and L-glutamate (C) with relation to NH_4^+ as the substrate for purified GDH (NADH dependent) activity from liver of H. fossilis.

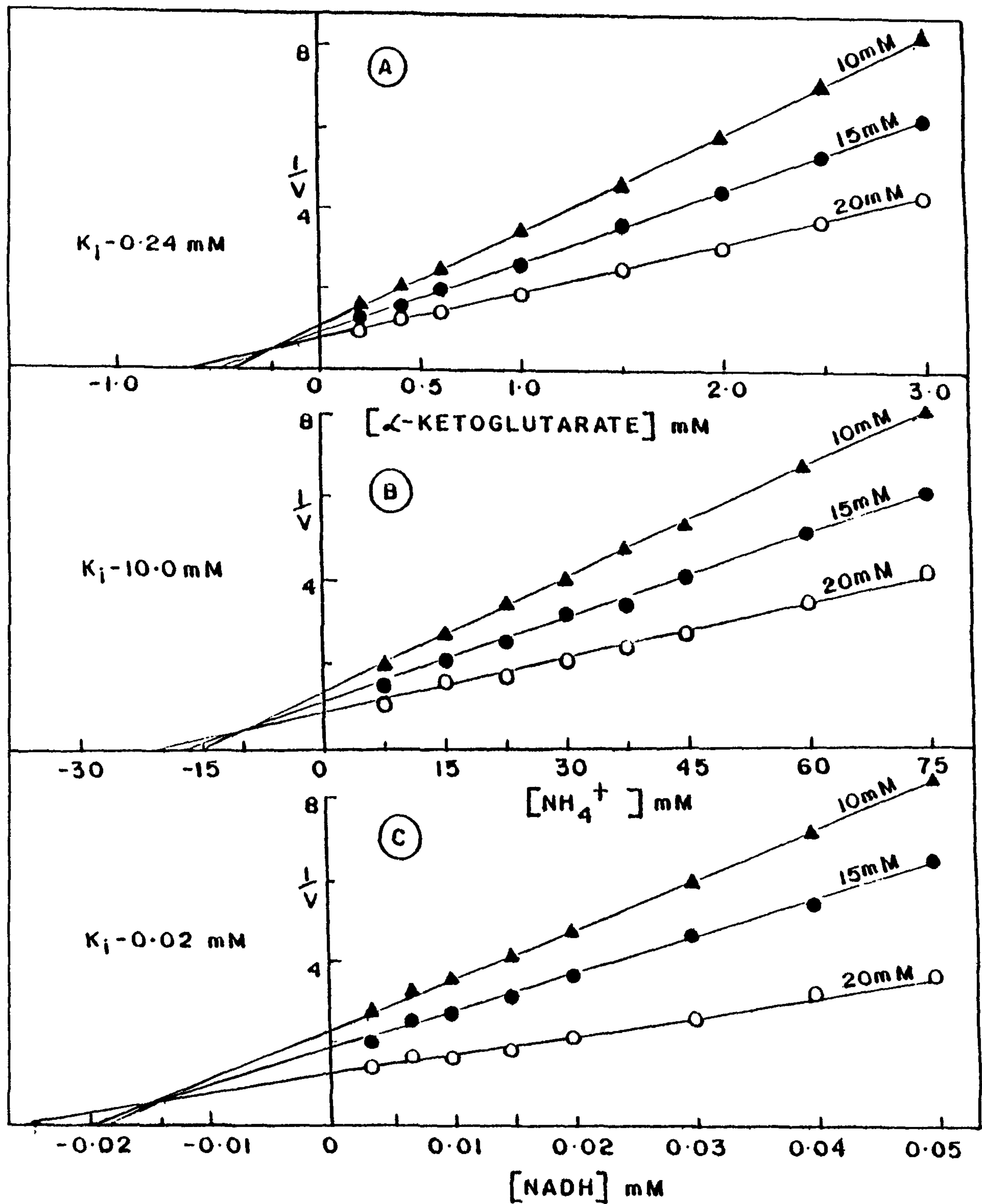


Fig.47. Dixon plot for determination of K_i of the products α-ketoglutarate(A) NH_4^+(B) and NADH(C) with relation to L-glutamate as the substrate for purified GDH (NAD^+ dependent) activity from liver of H. fossilis.

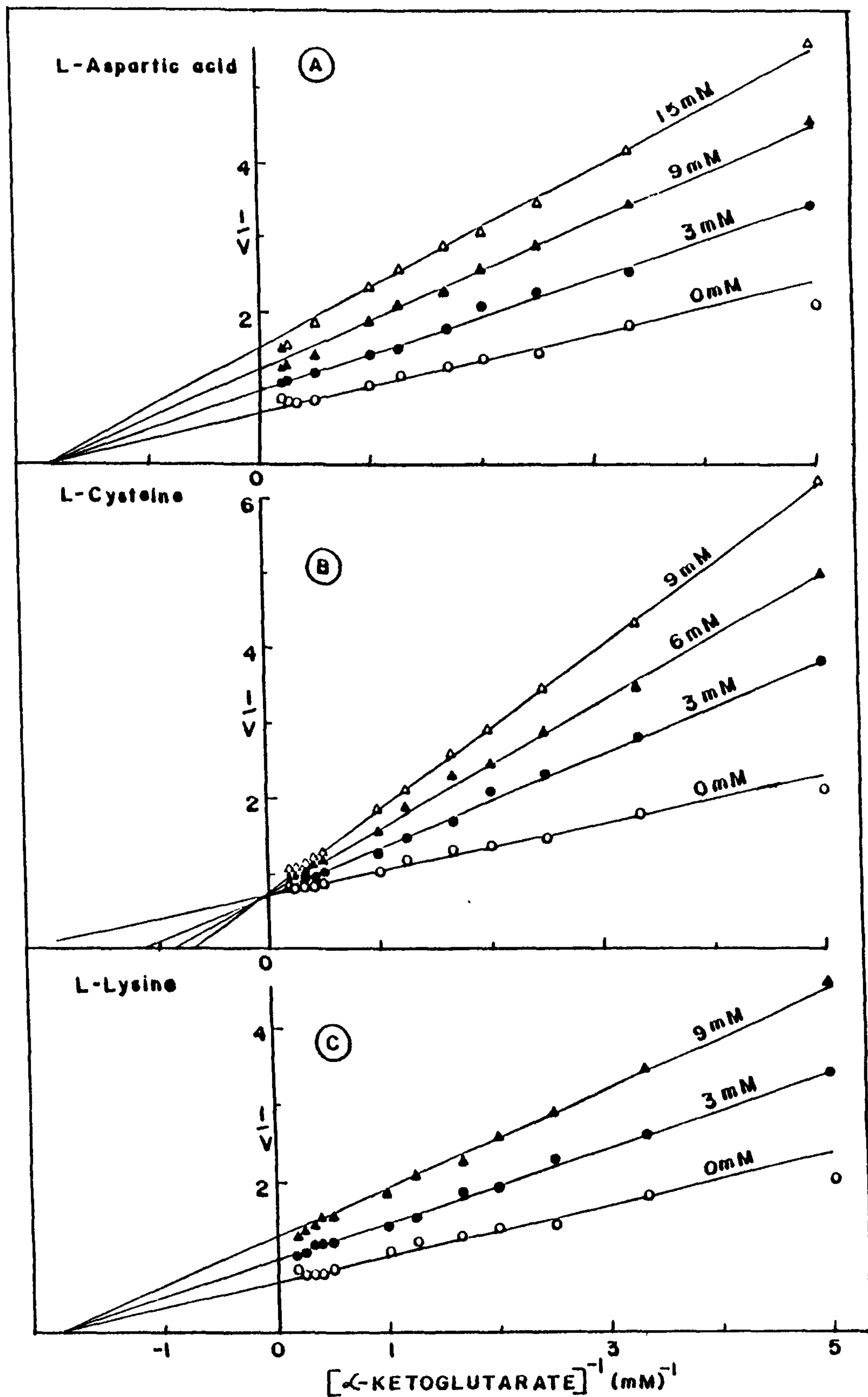


Fig.48. Lineweaver-Burk plot for determination of nature of inhibition by amino acids-Aspartic acid (A), Cysteine(B) and Lysine (C) on the GDH (NADH dependent) activity purified from liver of H. fossilis.

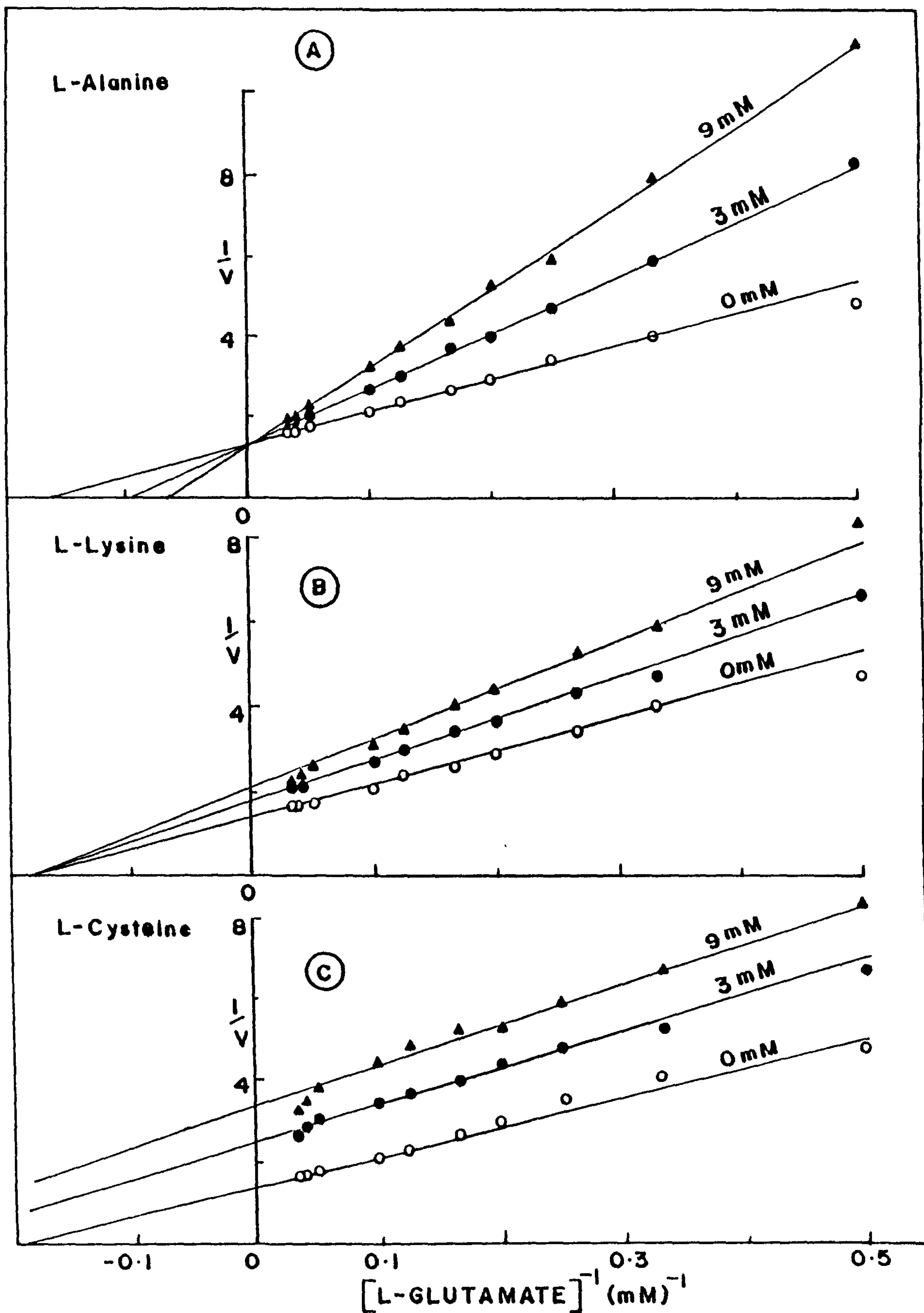


Fig.49. Lineweaver-Burk plot for determination of nature of inhibition by amino acids - Alanine (A), Lysine (B) and Cysteine (C) on the GDH (NAD^+ dependent) activity purified from liver of H. fossilis.

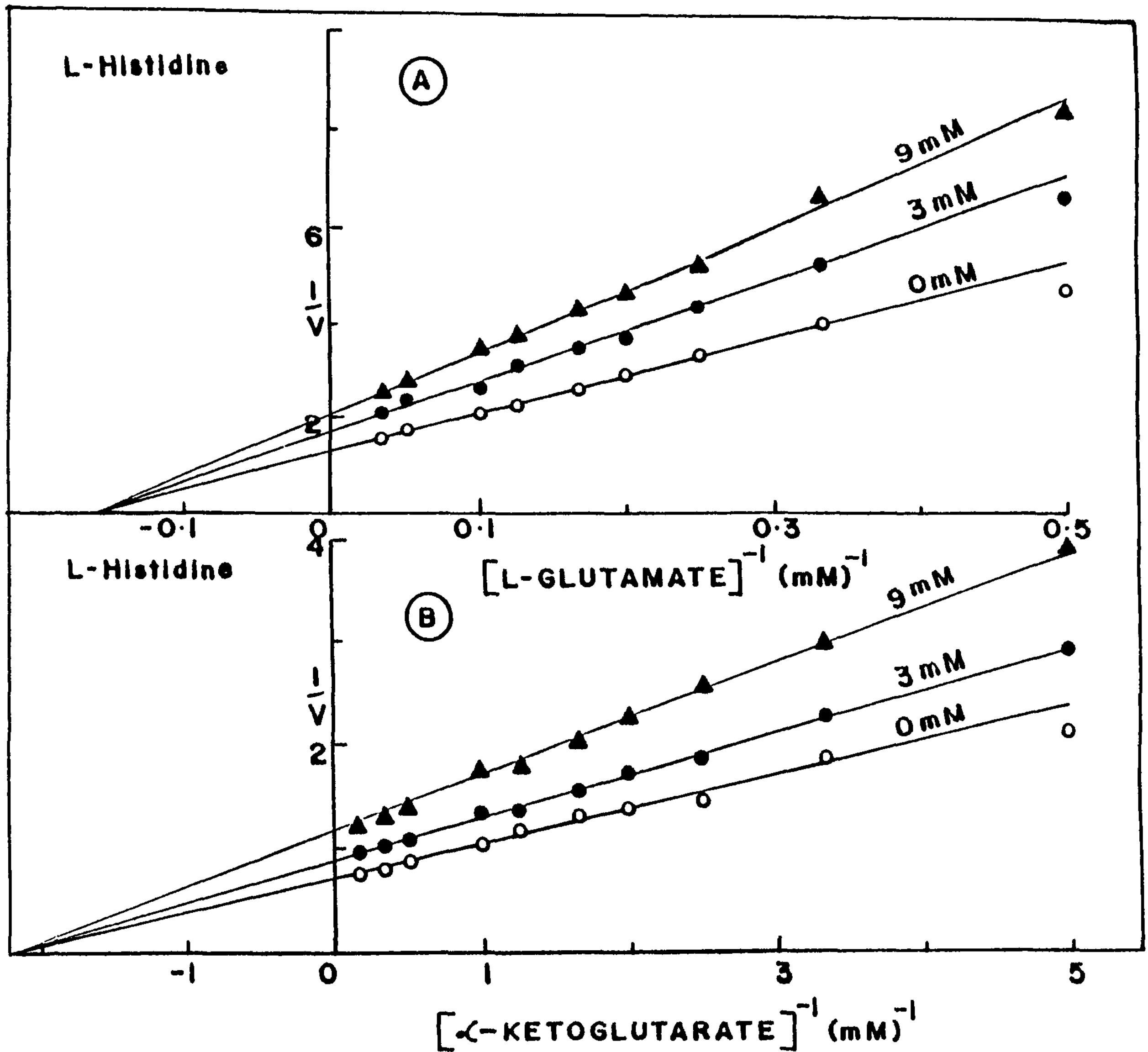


Fig.50. Lineweaver-Burk plot for determination of nature of inhibition by amino acid-Histidine on the GDH [NAD^+ dependent (A) and NADH dependent (B)] activity purified from liver of H. fossilis.

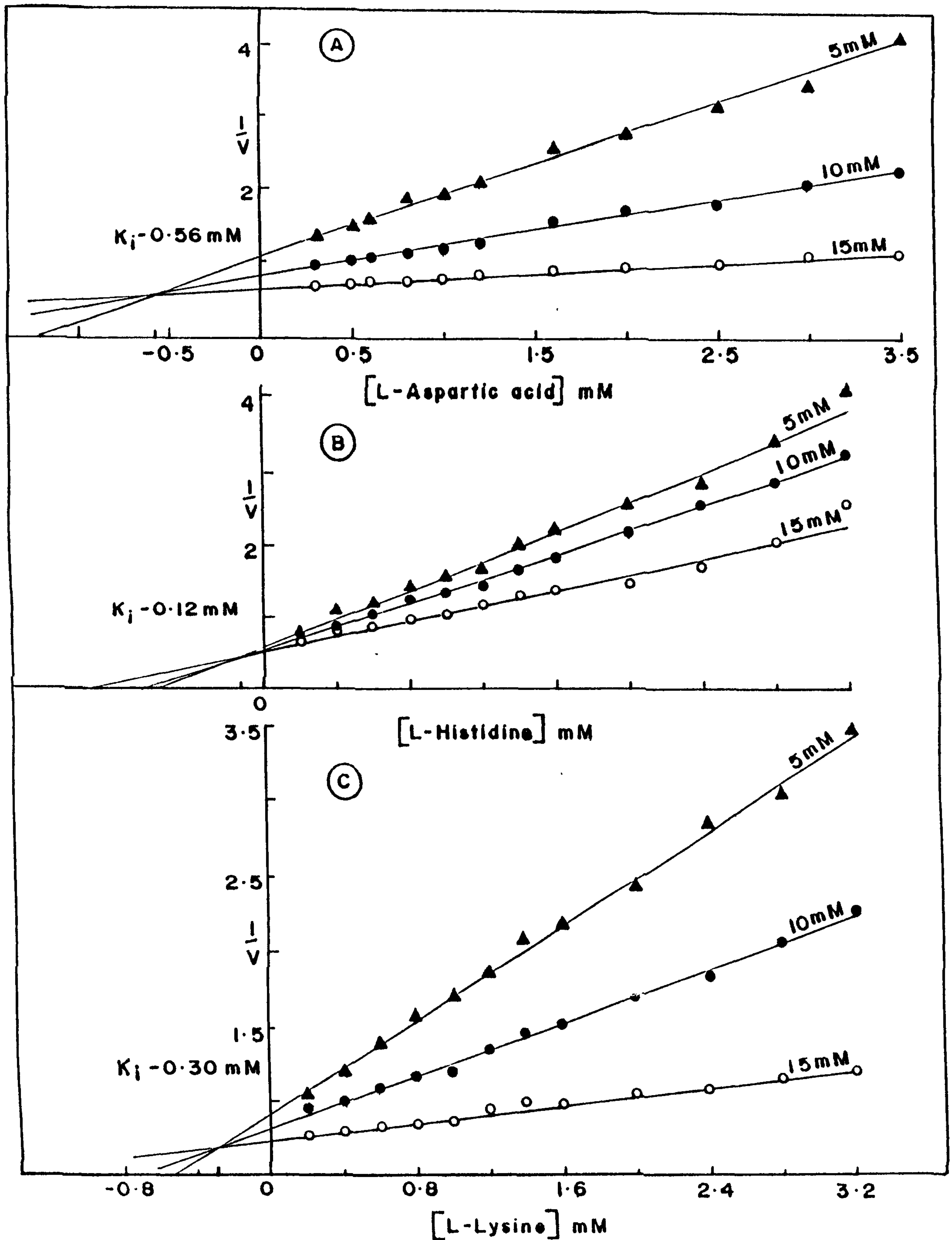


Fig.51. Dixon plot for determination of K_i of amino acids - Aspartic acid (A), Histidine (B) and Lysine (C) for purified GDH (NADH dependent) activity from liver of H. fossilis.

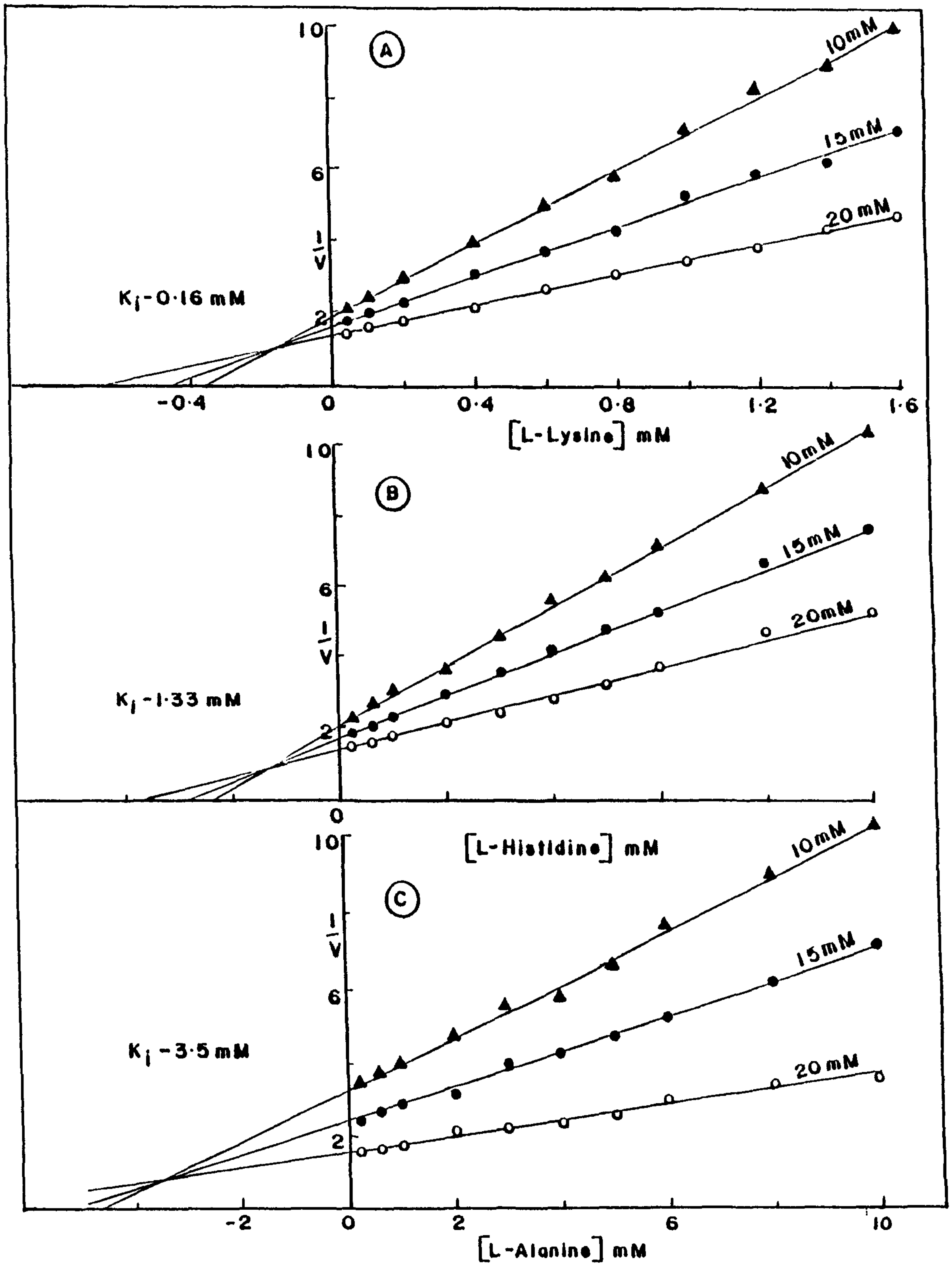


Fig.52. Dixon plot for determination of K_i of amino acids-Lysine (A), Histidine (B) and Alanine (C) for purified GDH (NAD^+ dependent) activity from liver of H. fossilis.

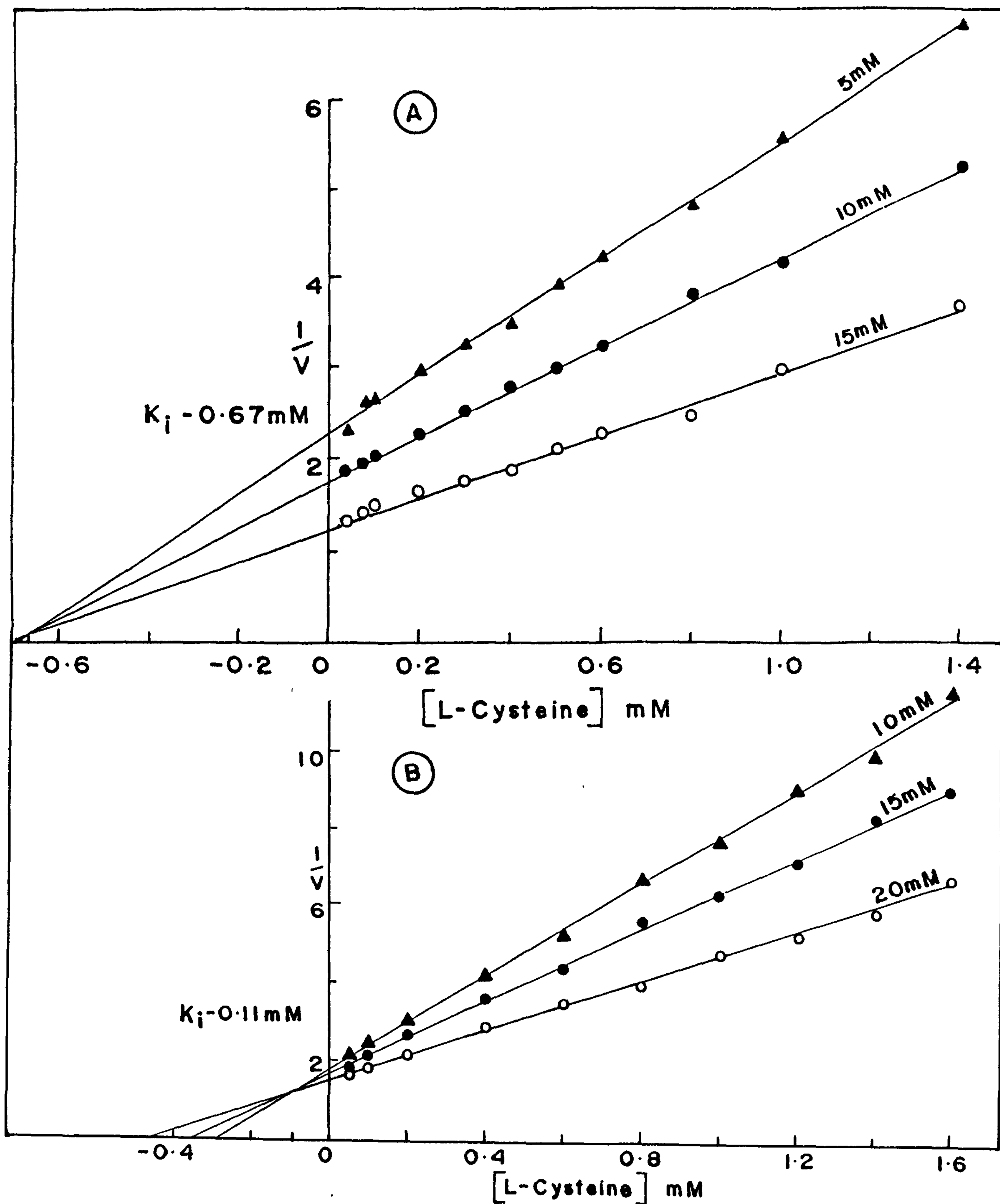


Fig.53. Dixon plot for determination of K_i of amino acid-Cysteine for purified GDH [NADH dependent (A) and NAD^+ dependent (B)] activity purified from liver of H. fossilis.

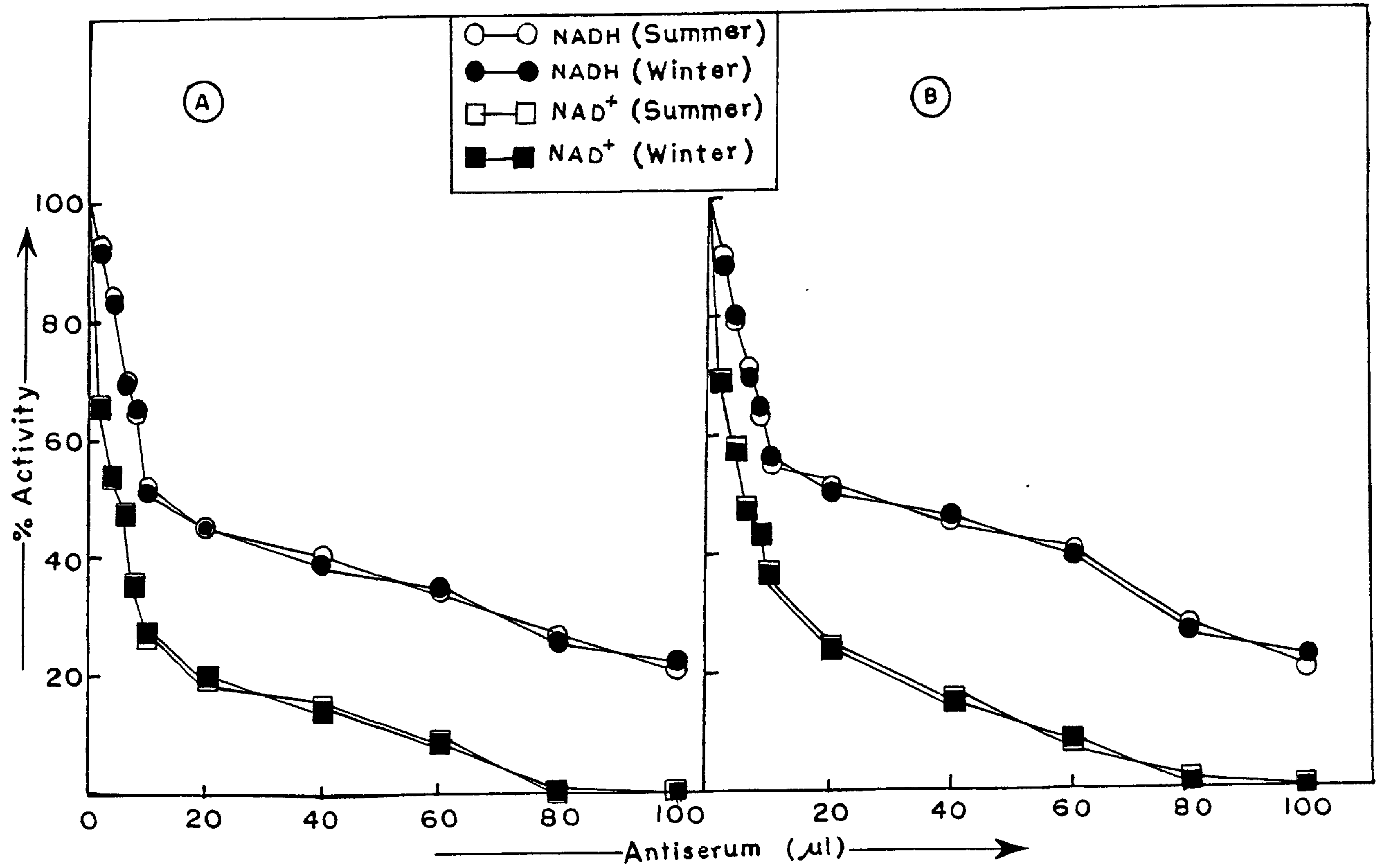


Fig.54. Inhibition of GDH (NADH and NAD^+ dependent) activity purified from liver of H. fossilis in Summer and Winter by antiserum raised against Summer (A) and Winter (B) purified GDH.

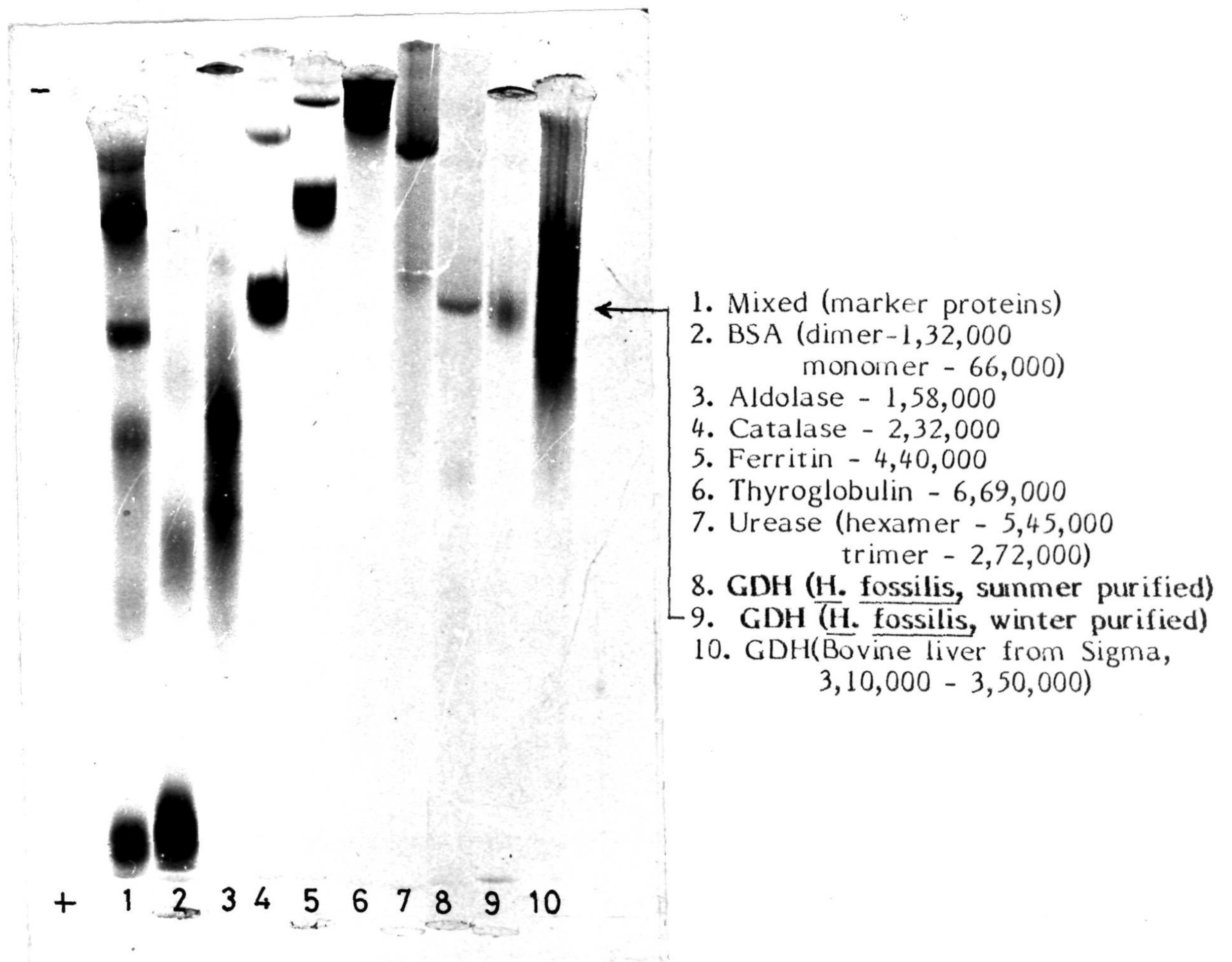
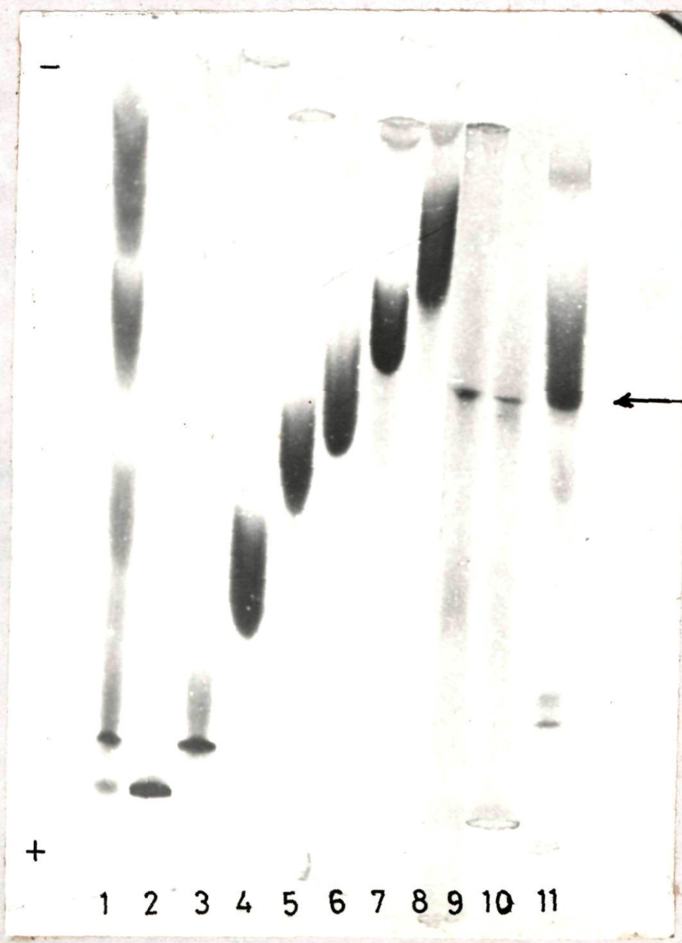


Fig.55(a) PAGE of native GDH purified from liver of H. fossilis for molecular weight determination along with standard marker proteins.



1. Mixed (marker proteins)
2. Ribonuclease A - 13,700
3. Chymotrypsingen A - 25,000
4. Carbonic anhydrase - 29,000
5. Aldolase - 38,994
6. Ovalbumin - 43,000
7. Catalase - 57,500
8. BSA - 67,000
9. **GDH (H. fossilis, summer purified)**
10. **GDH (H. fossilis, winter purified)**
11. GDH(Bovine liver from Sigma, 53,000 - 57,000)

Fig.55(b) SDS-PAGE of GDH (subunit) purified from liver of H. fossilis for molecular weight determination along with standard marker proteins.

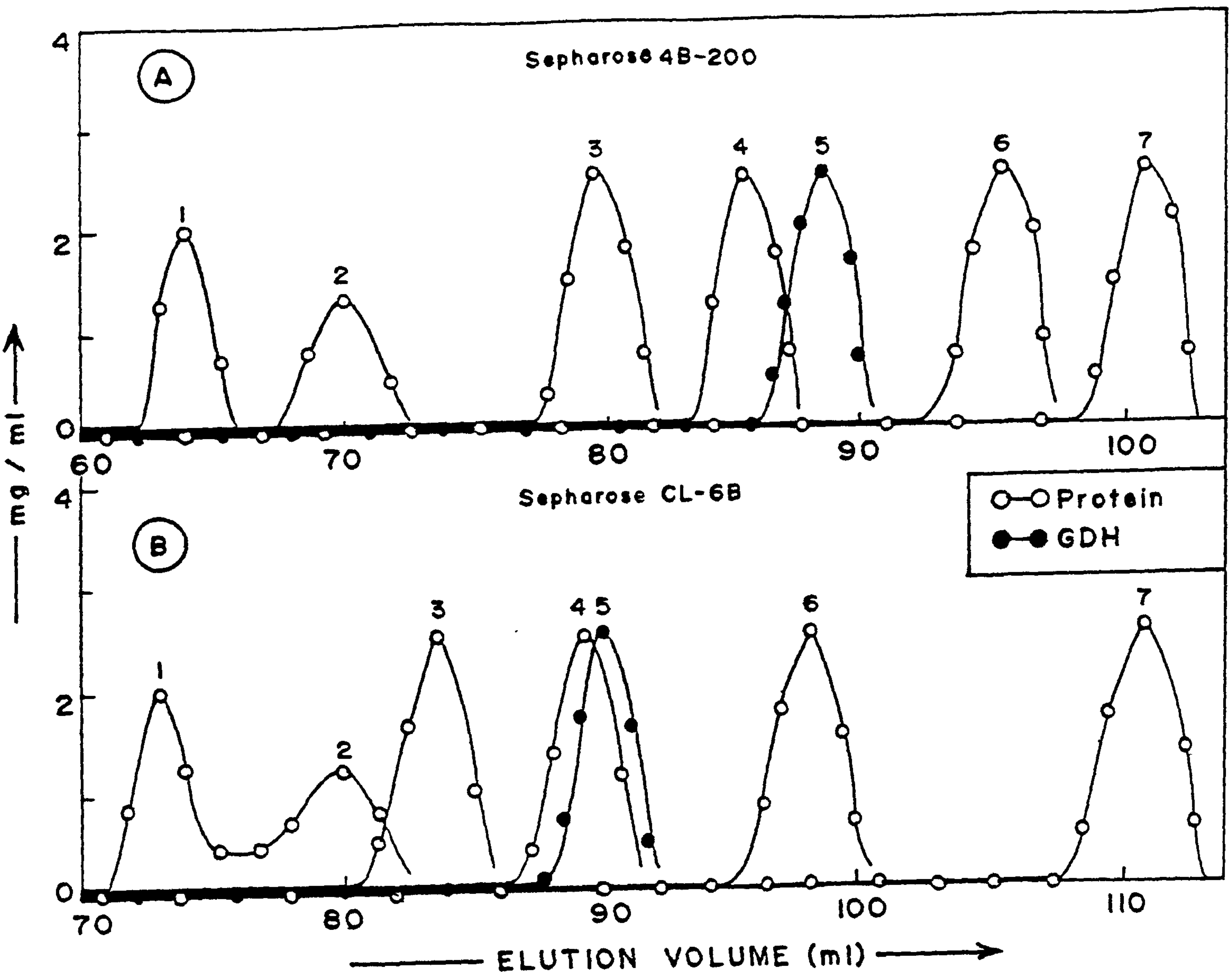


Fig.56. Elution pattern on Sepharose 4B-200 (A) Blue Dextran-2000 (1,2), thyroglobulin (3), ferritin(4), GDH (H. fossilis) (5), catalase (6) and aldolase (7) and on Sepharose CL-6B in presence of 8M urea (B) Blue Dextra-2000 (1,2), BSA(3), catalase(4), GDH (H. fossilis) (5), ovalbumin (6) and carbonic anhydrase (7) for molecular weight determination.

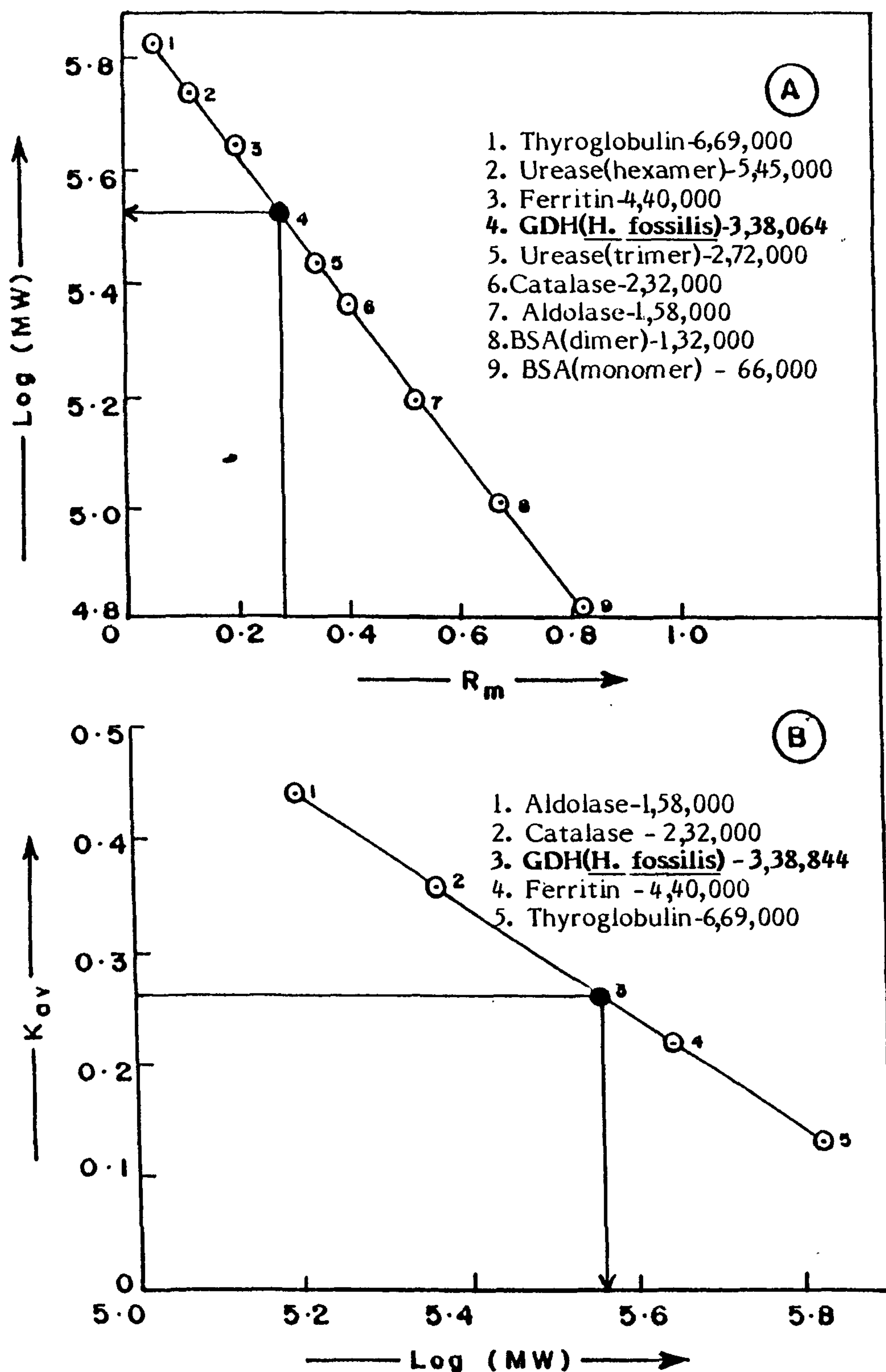


Fig.57. Determination of molecular weight of purified GDH from liver of H. fossilis by polyacrylamide gel electrophoresis (PAGE)(A) and exclusion chromatography on Sepharose 4B-200(B).

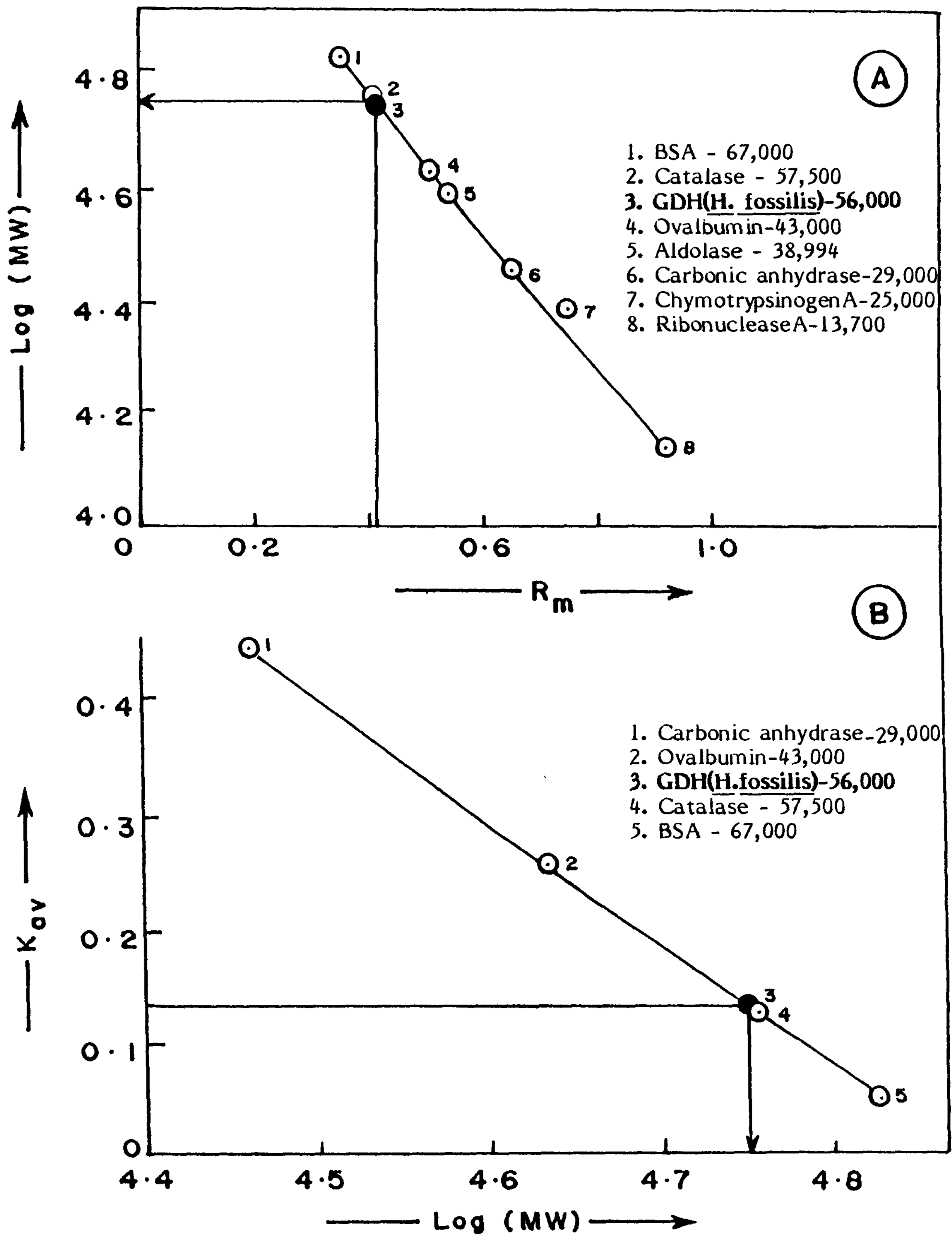


Fig.58. Determination of the subunit molecular weight of purified GDH from liver of H. fossilis by SDS-PAGE(A) and exclusion chromatography on Sepharose CL-6B in presence of 8M urea(B).

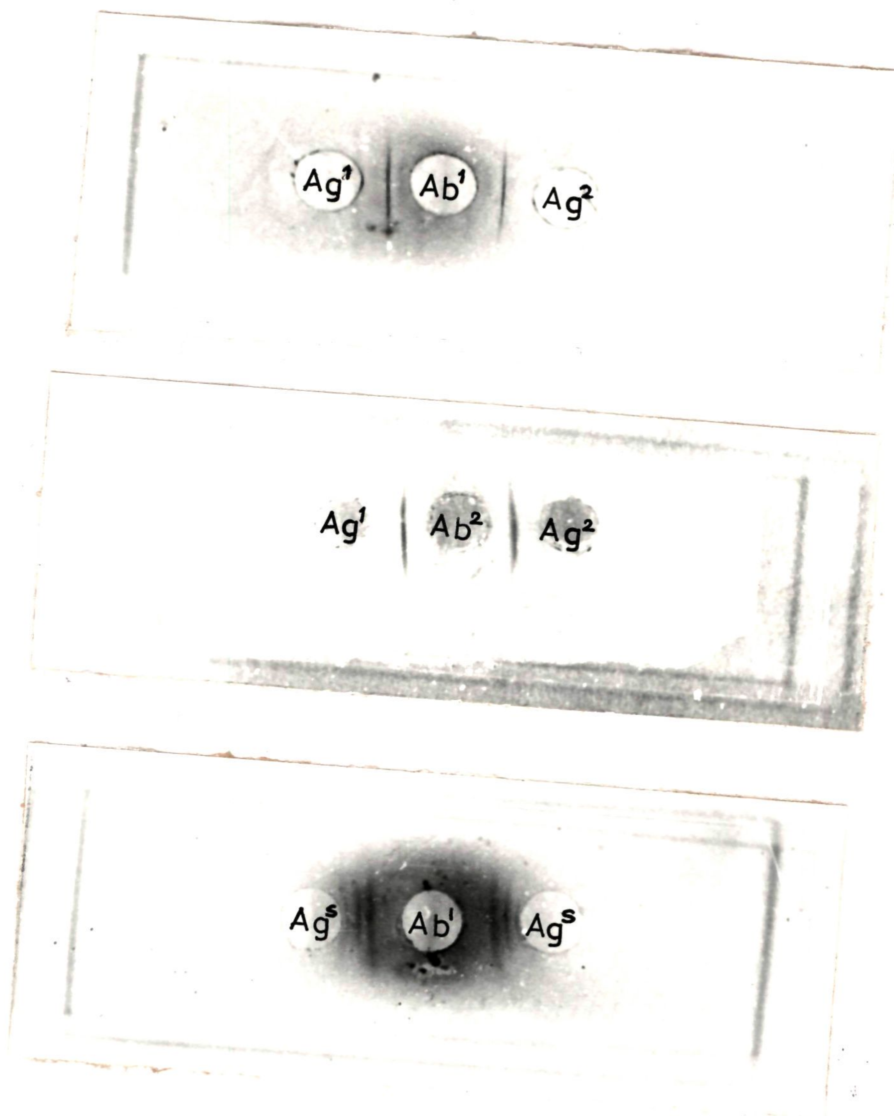


Fig.59. Immunodiffusion plate. Central well has antiserum and adjacent wells have antigen.

Ag^1 - Hepatic GDH purified from H. fossilis in Summer.

Ag^2 - Hepatic GDH purified from H. fossilis in Winter.

Ab^1 - Antiserum developed against summer purified GDH.

Ab^2 - Antiserum developed against winter purified GDH.

Ag^s - Bovine liver GDH from Sigma, Type-II

DISCUSSION

The reversible catalytic functions of GDH in ammoniogenesis and ammonia detoxification/utilization have made the role of GDH unique in nitrogen metabolism. Modulation of GDH activity by various physico-chemical factors and several metabolites has helped the organisms to use this enzyme in biochemical adaptations. The results obtained during the present study have elucidated the importance of GDH activity in Heteropneustes fossilis with special reference to its role in ammonia management in vivo.

Effect of buffer on stability of GDH activity: (Fig.1)

Hepatic GDH (NADH and NAD^+ dependent) activity in H. fossilis was more stable in potassium phosphate buffer than Tris-HCl buffer (Das et al., 1991). Phosphate has been shown to maintain the stability of GDH activity from various sources (Olson & Anfinsen, 1953; di Prisco, 1967; di Prisco & Strecker, 1970; Fahien & Cohen, 1970; Corman & Inamder, 1970; di Prisco & Garofano, 1975; di Matteo et al., 1976; Fisher, 1985). Hepatic GDH of H. fossilis was, therefore, similar to GDH from other sources with relation to ion requirement for maintaining enzyme activity in homogenates.

ADP and nicotinamide coenzyme requirement: (Table 1; Fig.2)

The oxidative deamination of GDH in H. fossilis showed absolute requirement for ADP as cofactor for its catalytic activity (Table 1) (Das et al., 1991) like most other earlier reports in freshwater teleosts (McBean

et al., 1966; Walton & Cowey, 1977) and in mollusca (Storey et al., 1978b; Ruano et al., 1985). ADP has been shown to maintain the three dimensional structure and to promote the binding of the coenzyme to the GDH molecular (Goldin & Frieden 1971). The reductive amination activity was not absolutely dependent on ADP. However, ADP acted as a positive modulator by increasing the reductive amination (NADH dependent) activity by 8 to 10-fold. GDH from animal sources, in general, were activated by ADP and the degree of activation differed from organism to organism. The increase in hepatic GDH activity in presence of ADP was 8-fold in the Modiolus demissus (Reiss et al., 1977) and 100-fold in squid Loligo pealeii (Storey et al., 1978b) muscle and 4-fold in bovine, 10-fold in chicken (Goldin & Frieden, 1971), 30-fold in trout (French et al., 1981) and 27 to 40-fold in catfish, Ictalurus punctatus (Casey et al., 1983). Increase in GDH (NADH dependent) activity by 8 to 10-fold in presence of ADP observed could help H. fossilis detoxify ammonia efficiently even at low energy level. Fishes which use primarily amino acids for energy production should tend to favour oxidative deamination reaction when the level of ATP was low and ADP high in vivo. However, high ADP level could modulate the GDH activity to facilitate amination reaction with its positive modulator function and thereby maintaining a physiological balance between the reverse reactions.

NADH and NADPH served equally well as coenzymes for reductive amination reaction of GDH in all the tissues studied in H. fossilis (Table 1) (Das et al., 1991). However, in the oxidative deamination reaction the enzyme showed specificity for NAD^+ as coenzyme in all the tissues studied. GDH has been reported to use only NAD^+ and not $NADP^+$ for its activity in different

fishes such as dogfish (Corman & Kaplan, 1967;Corman et al., 1967; Electricwala & Dickinson, 1979), lungfish (Janssens & Cohen, 1968), tuna (Veronese et al., 1976), trout (Walton & Cowey, 1977), goldfish (van Waarde, 1981), osteoglossids (Fields et al., 1978; Storey et al., 1978a) and mudskippers (Iwata et al., 1981; Iwata & Kakuta, 1983) and some marine invertebrates (Storey et al., 1978b; Batrel & Gal, 1984; Batreli & Regnault, 1985) and amphibians (Fahien et al., 1965a,b; Wiggert & Cohen, 1966; Lee & Balinsky, 1974). In several mammalian liver NAD(H) and NADP(H) have been shown to be equally good coenzymes for GDH activity (Frieden, 1965; Fisher, 1973; Gonzâlez et al., 1976). NADPH has been associated with the process of biosynthesis of lipid whereas NADH in oxidative energy production. However, the lipid level in H. fossilis have been very low compared to most other freshwater teleosts and mammals. Reduced production and effective use of NADPH in amino acid metabolism might have helped H. fossilis to maintain low level of lipids in the body. However, no definite conclusion can be drawn on this point.

Tissue distribution: (Table 2; Fig.3)

The tissue distribution of GDH activity (Table 2) (Das et al., 1991) was similar to those reported in other teleosts (McBean et al., 1966; Wilson, 1973a; Walton & Cowey, 1977; van Waarde, 1981; Casey et al., 1983; Iwata & Kakuta, 1983; Chew & Ip, 1987; Ip et al., 1990) and in mammals (Zinkl et al., 1971; Lowenstein, 1972) having the maximum activity in liver followed by kidney, gill, muscle and brain. The deamination reaction of GDH was, however, slightly higher in brain than muscle. The activities of GDH (both amination and deamination reaction) observed in various tissues of H. fossilis were higher than other freshwater and marine teleosts, and ureo-

osmotic elasmobranchs. This could be seen from the comparative table given below (Table A). High physiological level of GDH suggested its greater role in the maintenance of glutamate, α-ketoglutarate and ammonia balance in H. fossilis. GDH has been reported to be involved in ammoniogenesis (Pequin & Serfaty, 1963; Janssens, 1964; McBean et al., 1966; Pequin et al., 1970; Wilson, 1973a; Vellas & Serfaty, 1974; van Waarde, 1981; Casey et al., 1983; Chew & Ip, 1987; Ip et al., 1990). Ammonia thus produced gets immediately removed through the gills and body surface in freshwater teleosts to the aquatic medium (Smith, 1929; Wood, 1958; Morii et al., 1978; Wright et al., 1988) before it reaches toxic level in vivo. H. fossilis usually inhabits the benthic region of stagnant and slow-flowing shallow water bodies, swamps and sewage fed water bodies. It is also capable of living inside mud during drought conditions (Beavan, 1982; Jhingran, 1983), surviving temporary dehydration for more than 60 hrs (Saha & Ratha, 1989) and tolerating hyper ambient ammonia stress (Saha & Ratha, 1986,1990). The excretion of ammonia by diffusion from body through gills or body surface becomes difficult under the environmental condition of high ambient ammonia. Therefore, high physiological level of GDH activity with 2.5-5 times greater reductive amination activity than the oxidative deamination activity in all the tissues studied could be a biochemical adaptation in H. fossilis to detoxify ammonia to synthesize glutamate more efficiently.

The ratio of NADH/NAD^+ dependent GDH activity was observed highest in muscle followed by gill, kidney, brain and liver. The glutamate produced from ammonia in the muscle might be converted to other amino acids by transamination such as alanine in presence of alanine aminotransferase (AAT). Alanine being a neutral amino acid easily escapes into the blood

TABLE A: Comparison of total activity (units/g wet wt) of GDH (NADH and NAD^+ dependent) in various tissues of different groups of fish.

Species	Enzyme	Liver	Kidney	Brain	Muscle	Gill	Reference
Elasmobranch (marine)							
Pollachius virens (American pollock)	NAD^+	1.52	-	-	-	-	McBean et al. (1966)
Myoxocephalus scorpius (Southern sculpin)	NAD^+	4.29	-	-	-	-	"
Raja ocellata (Big skate)	NAD^+	1.38	-	-	-	-	"
Squalus acanthias (Spiny dogfish)	NAD^+	0.93	-	-	-	-	"
Teleosts							
Freshwater							
Cyprinus carpio (carp)	NADH				0.01 - 0.3		Pequin et al. (1970)
Salmo gairdneri (rainbow trout)	NAD^+	0.95±0.17	0.78±0.13	-	-	0.21 ± 0.06	Walton and Cowey(1977)
Carassius auratus (goldfish)	NAD^+	2.30±1.23	-	-	0.31±0.17	-	van Waarde (1981)
Ictalurus punctatus(catfish)	NADH	3.80±0.49	2.67±0.27	BLD	-	4.30±0.33	Wilson (1973a)
Air-breathing							
Marine							
Boleophthalmus boddaerti (mudskipper)	NADH	2.703±1.3	-	-	0.039±0.030	0.421±0.075	Chew and Ip (1987)
	NAD^+	0.165±0.106	-	-	0.0012±0.0009	0.023±0.004	"
Periophthalmodon schlosseri (mudskipper)	NADH	2.203±0.509	-	-	0.044±0.015	0.28±0.009	"
	NAD^+	0.097±0.015	-	-	0.002±0.001	0.011±0.001	"
Freshwater							
Anguilla rostrata (eel)	NAD^+	3.50	-	-	-	-	McBean et al.(1966)
H. fossilis (catfish)	**NADH**	**5.82-14.47**	**2.31-6.56**	**1.56-3.28**	**1.45-2.60**	**1.45-2.79**	**Present work.**
	NAD^+	**2.74-5.71**	**1.43-2.51**	**0.85-1.01**	**0.62-0.89**	**0.73-0.93**	

and is carried to the liver for further metabolism. Glutamate could be converted to pyruvate in presence of GPT which could be utilized in gluconeogenesis like other teleosts (Suarez & Mommsen, 1987). Glutamate could also be converted to glutamine accepting another molecule of ammonia. Both glutamate and glutamine could release the ammonia in target tissues either for excretion or conversion to urea via ornithine urea cycle. The ratio was lesser in liver and kidney which are known to be the ammoniogenic tissues. A functional o-u cycle has been reported in liver and kidney of H. fossilis to use the ammonia produced (Saha & Ratha, 1987). High activity of glutamine synthetase has been reported in various tissues of H. fossilis (Chakravorty et al., 1989) indicating the efficient ammonia utilization via glutamate → glutamine pathway.

Specific staining of GDH activity on polyacrylamide gel after electrophoresis showed a single band for each tissue of H. fossilis. However, the migration of the GDH band was different in different tissues (Fig.3). There might exist inter-tissue variations in GDH species to meet specific metabolic needs. Isoenzymes of GDH have not been reported in animal tissues (Frieden, 1963; 1976). However, in higher plants isoenzymes of NAD^+ dependent GDH have been well established (Thurman et al., 1965; Yue, 1969; Errel et al., 1973; Lee, 1973; Ratajczak et al., 1977).

Sub-cellular distribution: (Tables 3-6)

GDH (NADH and NAD^+ dependent) activity was found to be primarily localized in the mitochondria in all the tissues of H. fossilis (Das et al., 1991). Mitochondrial localization of GDH has been reported in mammals (Snoke, 1956; Salganicoff & deRobertis, 1965; Arnold & Maier, 1971; Addink et al., 1972; Matlib & O'Brien, 1975; Schoolwerth et al., 1978), amphibians

(Fahien et al., 1965a; Fahien & Cohen, 1970; King & Cohen, 1975; Petrucci et al., 1980) and fishes (Wainwright et al., 1967; Corman & Inamder, 1970; Wilson, 1973a; Casey & Anderson, 1982, 1985; Chew & Ip, 1987) specifically in the matrix compartment. Immunohistochemical studies showed GDH in two forms [soluble and particulate (membrane associated)] inside mitochondria of mammalian brain (Plaitakis et al., 1984; Knecht et al., 1986; Aoki et al., 1987a,b; Kaneko et al., 1987; Madl et al., 1988). This differential distribution has been suggested to serve different metabolic functions - one helping in glutamate synthesis and the other in glutamate oxidation in brain (Colon et al., 1986). Besides being mitochondrial, GDH has been reported to be localized in nuclei of mammalian liver (diPrisco & Strecker, 1970; Franke et al., 1970; Herzfeld et al., 1973; diPrisco & Casola, 1975) and brain (Kato & Lowry, 1973; Lai et al., 1985, 1986), and in cytosolic fraction of cestoda (Mustafa et al., 1978), parasitic protozoa (Singh et al., 1981; Hellebust & Larochelle, 1988) and micro-organisms (Doherty, 1970; Osmani & Scrutten, 1983). The mitochondrial GDH might have facilitated the availability of sufficient glutamate for the mitochondrial glutamine synthetase reported in the liver and kidney of H. fossilis (Chakravorty et al., 1989).

Circadian (24 hr) Cycle: (Table 7-10; Figs.4-7)

GDH (NADH and NAD^+ dependent) activity did not show any significant variation during 24 hr cycle in various tissues of H. fossilis studied during Summer and Winter. There has been no report of any variation of GDH activity during 24 hr cycle in any animal tissues. Absence of circadian variation suggests GDH as a general metabolic enzyme in H. fossilis whose activity continued at the same rate throughout the 24 hr cycle in various tissues.

Annual cycle: (Tables11&12; Figs. 8&9)

H. fossilis maintained under controlled temperature (20±2°C) in the laboratory showed seasonal variation in GDH (NADH and NAD^+ dependent) activity. This generally coincided with the reproductive cycle of the fish. The fishes probably retained their internal clock, even after acclimatization to laboratory conditions. The gonadal maturation occured during Summer (May-August) when GDH (reductive amination) activity was high with the peak in June in all the tissues. On the onset of Winter reductive amination activity of GDH decreased and oxidative deamination activity increased. The ratio of $NADH/NAD^+$ dependent activity was the lowest during Winter. This coincided with the resting phase of the gonad. The variations were more prominent in the general metabolic tissues such as liver and kidney and less prominent in brain, muscle and gill. The requirement of enough amino acids and proteins for vitellogenesis in liver reported during pre-spawning phase in fishes (Schmidt et al., 1965; Campbell & Jalabert, 1979; deVlaming et al., 1980; Wiegand, 1982; Wallace, 1985) might have been the guiding factor for enhanced reductive amination reaction to increase glutamate synthesis. Glutamate could be transaminated to other amino acids easily. Seasonal variation in the NAD^+ dependent GDH activity in shrimp (Crangon crangon) was reported by Regnault and Batrel (1987). Alterations in the environmental factors and variation in the metabolic level due to moulting have been correlated with the variation of GDH (oxidative deamination) activity.

Regulation of GDH activity by different hormones, including steroid hormones have been reported (Caughely et al., 1957; Yielding & Tomkins,

1960, 1964; Wolff, 1962; Hillar, 1974; Fahien *et al.*, 1988). The level of steroid hormones *in vivo* alter during various phases of reproduction in fish during the year and are high during the pre-spawning and spawning periods (Sundararaj, 1959; Liley, 1969; Sundararaj & Goswami, 1969). This hormonal flux might have also influenced the seasonal variation in GDH activity in *H. fossilis.*

Effect of temperature: (Tables 13&14)

Exposure of the fish to 10°C higher and lower temperatures than the acclimated temperature (20°C) during Summer (June) and Winter (December) showed interesting results. During Summer (June), when NADH dependent reductive amination reaction was higher than NAD^+ dependent oxidative deamination reaction of GDH, exposure to higher temperature (30°C) apparently had no effect. However, exposure to lower temperature (10°C), generally, induced NAD^+ dependent activity and inhibited NADH dependent GDH activity. This was more prominent in liver and kidney indicating greater glutamate utilization probably for higher energy production to keep up the high metabolic level required for the fish during spawning season.

However, in Winter (December) both lower (10°C) and higher (30°C) temperature shocks induced the lower level of reductive (NADH dependent) amination activity and inhibited the higher level of oxidative (NAD^+ dependent) deamination activity of GDH. The effect was more pronounced on exposure to higher temperature which might have been a signal for the fish to start spawning or to shift to the Summer pattern of GDH activity. The NADH dependent GDH activity used to start increasing after its lowest level in November to reach the peak in May/June (Summer). However, similar results

of a lesser degree obtained during exposure to lower temperature (10ºC) in Winter were not clear except indicating enhanced glutamate synthesis than its utilization.

These findings indicate that the GDH activity in *H. fossilis* also gets influenced by the temperature changes during the annual cycle besides the internal physiological clock.

Effect of water deprivation: (Tables 15-20; Figs.10-13)

The ammoniotelic-ureogenic freshwater teleost, *H. fossilis* was reported to survive more than 60 hrs outside water (Saha & Ratha, 1989). GDH (both NADH and NAD^+ dependent) activity in various tissues of *H. fossilis* were significantly induced during the water deprivation for 36 hrs. Ammonia excretion decreased due to non-availability of water and accumulated significantly in different tissues of *H. fossilis* during aerial exposure (Saha, 1986). Enhanced synthesis, accumulation and excretion of urea were reported suggesting the transition from ammoniotelism to ureotelism as a physiological adaptation during water deprivation in *H. fossilis* (Saha, 1986).

The induction of GDH (NADH dependent) activity might be an additional strategy for detoxification of accumulated ammonia *in vivo*. Some accumulated ammonia might have been converted to glutamate by reductive amination activity of GDH to reduce ammonia load *in vivo*. Glutamate accumulation observed in liver, kidney and brain of *H. fossilis* during water deprivation (Chakravarty, J. personal communication) supports this suggestion. The percentage of induction of GDH (NADH and NAD^+ dependent) activity was highest in brain (~150%) followed by liver (~130%), gill (~90%), kidney (70-

(70-100%) and muscle (60-80%). Quick and high induction of GDH might have helped the highly sensitive brain tissue to immediately convert accumulated ammonia to glutamate. Glutamate being a neurotransmitter (Hamberger et al., 1979a,b; Cotman et al., 1981; Watkins & Evans, 1981; Fonnum, 1984) might not accumulate beyond certain level. It might have been converted to glutamine using another molecule of ammonia. Induction of GS level and inhibition of glutaminase level has been observed in various tissues of H. fossilis during ammonia accumulation (Chakrovarty, J. personal communication). The results clearly show that the increased level of ammonia in vivo induced the GDH and GS activity to incorporate ammonia to synthesize first glutamate and then glutamine while maintaining glutamate-glutamine pool in various tissues. Glutamine is more efficient than glutamate to cross the membrane and serve as an ammonia carrier in vivo. Glutamate formed might also be converted to some other non-essential free amino acids (FAA). Significant accumulation of various FAA along with 4-5 fold increase in GDH activity in liver and kidney of mudskipper during aerial exposure was reported (Iwata et al., 1981).

Induced GDH activity in liver and kidney of H. fossilis might also help in inducing ureogenesis by converting the excess of glutamate formed while capturing free ammonia to aspartate which is the second nitrogen donor for urea synthesis (Cohen, 1966; Chamalaun & Tager, 1970). Induction of o-u cycle enzyme activities have also been reported in this fish during water deprivation (Saha, N. personal communication). Accumulation of some inhibitory free amino acids might have decreased the induced level of GDH (NAD^+ dependent) in muscle after the peak between 15-21 hrs of water

deprivation. Inhibition of GDH (NAD^+ dependent) activity by amino acids such as alanine and aspartate have been known (Wiggert & Cohen, 1965; Hillar, 1974; present study). However, a definite conclusion on this can be drawn after analysing the free amino acid accumulation profile during water deprivation. The ratio of NADH/NAD^+ dependent GDH activity which favoured reductive amination remained fairly constant during 36 hrs of water deprivation. This indicated that both the reactions were probably catalyzed by the same GDH molecule and the physiological state of accumulated ammonia favoured reductive amination reaction for its detoxification during water deprivation.

Effect of starvation, refeeding and hyper-ammonia stress: (Tables 21-29; Figs. 14-22)

Carbohydrate has been recognized as the main energy source in most animals (Bennett, 1978; Lehninger, 1987). After carbohydrates, lipids are used up for energy production during starvation in mammals (Cahill, 1986) and in birds (LeMaho et al., 1981). However, teleosts derive their metabolic energy primarily from proteins. Lipids and carbohydrates are next in priority (Cowey et al., 1977a,b; Pandian & Vivekanandan, 1985). The relative importance of lipid has also been species dependent in fish. In fat fishes such as eels, energy production is predominantly maintained by depletion of lipid reserves first from liver and then from muscle (Inui & Ohshima, 1966; Larsson & Lewander, 1973; Dave et al., 1975). In non-fatty fishes such as carp, pike and plaice, protein catabolism covers the energy need (Nagai & Ikeda, 1971; Johnston & Goldspink, 1973; Creach & Serfaty, 1974; Diana, 1982). Fishes are able to survive extended periods of starvation by consumption of their own proteins (Creach & Serfaty, 1974; Moon & Johnston,

1980; Moon, 1983). Considerable amount of decrease in total protein was observed in various tissues of H. fossilis during 14 days of starvation (unpublished observation).

There was significant accumulation of total free amino acids (FAA) in various tissues of H. fossilis which increased with increasing period of starvation (Table 21; Figs. 14&15). Hydrolysis of protein as reported in other teleosts (Jürss, 1980; Mommsen et al., 1980; Renaud & Moon, 1980b; French et al., 1981) might have caused this increase in FAA pool in various tissues. The importance of enhanced FAA pool during starvation has been recognised for gluconeogenesis. Alanine, serine and glycine were incorporated into glucose and glycogen in liver of rainbow trout (Cowey et al., 1977a; Walton & Cowey, 1979a,b; Mommsen & Suarez, 1984; Petersen et al., 1987), eel (Hayashi & Ooshiro, 1977b, 1979; Renaud & Moon, 1980a), sea raven (Foster & Moon, 1987) and tench (Mosse, 1980). Maximum increase in total FAA was observed in the muscle which is the protein rich tissue. The amino acids might have been either transported to the gluconeogenic tissues such as liver (Moon et al., 1985) and kidney (Jørgensen & Mustafa, 1980; Mommsen et al., 1985) for synthesis of glucose or used up for energy production in various tissues releasing ammonia. Besides proteolysis, some amino acids might have been produced by GDH (NADH dependent) activity and transamination reactions. Induction of several transaminases during prolonged starvation have been reported in freshwater teleosts (Storer, 1967; Larsson & Lewander, 1973; Creach & Serfaty, 1974; Whiting & Whiggs, 1977; Zébian & Creach, 1979). The induction of GDH (NADH dependent) activity during starvation was observed in the liver of rainbow trout (Malevski et al., 1974; French et al., 1981; Jürss et al., 1983). In the present study significant induction

of GDH (both NADH and NAD^+ dependent) activity was observed during starvation upto 14 days in H. fossilis. The rate of induction was found higher for oxidative deamination than the reductive amination activity of GDH resulting in reduction in the ratio of $NADH/NAD^+$ dependent GDH activity in liver, kidney and brain tissues. In muscle and gill the induction of both NADH and NAD^+ dependent activity were parallel causing no apparent alteration in the ratio during starvation. Higher rate of induction of oxidative deamination activity of GDH indicated enhanced amino acid utilization through glutamate for energy production during starvation. As GDH reverse reaction is regulated by substrates, products and various modulators, the exact rate of the two reactions in vivo cannot be decided on the basis of their in vitro assay. Ammonia level during the starvation did not alter significantly in different tissues (Chakravorty, J. personal communication). Enhanced reductive amination activity of GDH might have controlled the ammonia level which was expected to increase due to amino acid catabolism in various tissues during starvation. The rise in glutamate during starvation was also observed in different tissues of H. fossilis (Chakravorty, J. personal communication). The increase was minimum in brain probably to maintain the level of the neurotransmitter within physiological limit. The activity of glutaminase and not glutamine synthetase was induced in various tissues during starvation indicating again the utilization of amino acids through glutamate by oxidative deamination reaction of GDH. The oxidative deamination reaction of GDH has been proposed as an ATP regenerating system in organisms (Atkinson, 1968; Bidigare et al., 1982; Campbell et al., 1983; Matsushima & Kado, 1983; Teller, 1987).

There was recovery of the effects of starvation studied with relation to the alteration in the free amino acid pool, GDH (NADH and NAD^+ dependent) activity and protein level in various tissues when the fish was provided with food after 7 days of starvation (Tables 21,23&24; Figs.14,15,17-20). The external food being available, the fish must have stopped utilization of protein and amino acids from the tissues and started restoring its normal metabolic state.

Significant increase in total FAA was observed in various tissues studied during exposure of H. fossilis to hyper-ammonia ambient medium. Significant accumulation of ammonia in vivo has been reported during hyper-ammonia stress (Saha, 1986; Saha & Ratha, 1986). Induction of o-u cycle to convert accumulated ammonia to urea for its detoxification was suggested. There was also enhanced accumulation and excretion of urea indicating a transition to ureotelism under hyper-ammonia stress (Saha & Ratha 1986,1990). Accumulated ammonia also induced the activity of GS and inhibited glutaminase activity (Chakravorty, J. personal communication). There was significant accumulation of glutamate in various tissues besides significant increase in total FAA pool indicating new amino acid synthesis. In addition to the conversion of ammonia to urea, accumulated ammonia might have also converted to amino acids. Accumulation of FAA was observed during hyper-ammonia stress in carp (Dabrowska & Wlasow, 1986; Ogata & Murai, 1987), goldfish (Levi et al., 1974) and mudskipper (Iwata, 1988). Urea synthesis is restricted to only liver and kidney tissues requiring the accumulated ammonia from other tissues to be transported to them. Amino acids such as glutamate and glutamine are excellent carriers of ammonia in vivo (Campbell, 1973;

Lehninger, 1987). Besides, amino acids also serve as good osmoregulators in marine and euryhaline invertebrates (Schoffeniels & Gilles, 1970; Gilles & Schoffeniels, 1972; Schoffeniels, 1976) and in several euryhaline teleosts (Lange & Fugelli, 1965; Huggins & Colley, 1971; Lasserre & Gilles, 1971; Colley et al., 1974; Venkatachari, 1974; Ahokas & Sorg, 1977; Vislie, 1980; Jürss et al., 1984).

Present findings on the induction of GDH (NADH and NAD^+ dependent) activity (Tables 25-29; Figs.17,18,21&22) along with increased FAA level (Table 22, Figs.14&16) in various tisues of H. fossilis exposed to hyper-ammonia ambient medium support the view that amino acid synthesis via GDH→GS pathway operated in various tissues for immediate detoxification of accumulated ammonia. Studies on transaminases shall further clarify this point. Elevated ammonia level might have induced the enzyme (GDH) in various tissues of H. fossilis under hyper-ammonia stress to keep the ammonia concentration below the toxic level and also to transport it to liver and kidney for further use in ureogenesis. Ten fold increase in GDH activity in addition to the induction of urea cycle enzymes has been reported in the liver of amphibia (Xenopus laevis) exposed to higher ambient ammonia (5mM NH_4Cl) (Janssens, 1972). Elevated ammonia levels have been shown to stimulate GDH activity in several strains of algae such as Chlamydomonas (Paul & Cooksey, 1981; Cullimore & Sims, 1981b; Munoz-Blanco & Cárdenas, 1989), Chlorella (Talley et al., 1972; Isreal et al., 1977, 1978; Yeung et al., 1981; Bascomb et al., 1986; Bascomb & Schmidt, 1987; Schmidt et al., 1982; Everest & Syrett, 1983; Prunkard et al., 1986a,b) and in some plants (Shepard & Thurman, 1973; Barash et al., 1975; Skokut et al., 1978) while inhibiting the main GS→GOGAT system for glutamate production. Presence of GOGAT

has not yet been known in any animal system. Significant induction of GDH (NADH dependent) activity in liver, kidney and brain within 3 days of exposure suggests its role in immediate neutralization of ammonia toxicity at cellular level. The induction of o-u cycle enzymes in liver and kidney was reported after 7 days of exposure to ammonium chloride (Saha & Ratha, 1986).

The fishes were not taking food probably due to the hyper-ammonia stress and were starved. The ADP level, therefore, could be higher than ATP level. Reductive amination reaction was induced 8-10 fold in presence of ADP in the reaction mixture. Hence, at low energy level GDH could neutralize ammonia toxicity immediately rather than o-u cycle, which is tissue specific and a energy requiring system.

NAD^+ dependent activity of GDH showed greater induction than NADH dependent activity in liver and kidney of *H. fossilis* after 7 days of exposure. This might be the requirement of those tissue to supply ammonia for ureogenesis which was induced by 7th day (Saha & Ratha, 1990). Muscle and gill tissues were apparently indifferent to hyper-ammonia stress with relation to GDH activity even though the ammonia level and FAA level significantly increased. High GS activity has been reported in the muscle and gill of *H. fossilis* (Chakravorty, J. personal communication). Ammonia utilization in these tissues might be by GS and not by GDH activity.

The ratio of $NADH/NAD^+$ dependent activity did not show any significant variation in different tissues of *H. fossilis* during hyper-ammonia stress. It again suggests that both the GDH reactions were catalyzed by the same protein molecule.

Purification of GDH from liver of H. fossilis: (Table 30; Figs. 23&24a,b)

GDH was purified to homogeneity from the liver of H. fossilis during Summer (May) and Winter (December). The degree of purification using two anion-exchange columns and an affinity (Blue Sepharose CL-6B) column chromatography was 500 fold with 48% recovery and 420 fold with 57% recovery during Summer and Winter respectively. The fold of purification was found to be similar to those reported for hepatic GDH in other freshwater teleosts such as in tuna (102-fold and 21%) (Veronese et al., 1976); eel (76-fold and 33%) (Hayashi & Ooshiro, 1977a) and ureo-osmotic marine elasmobranch such as dogfish (150-fold and 13%) (Corman et al., 1967) and (444-fold and 33%) (Corman & Inamdar, 1970). However, the recovery rate obtained in H. fossilis was very high. The enzyme was eluted as a single peak from all the columns (Fig.23) and showed a single enzyme specific band when stained for GDH after PAGE (Figs. 24a,b). The hepatic GDH in H. fossilis seems to be a single species of protein as reported from other animal sources such as bovine (Olson & Anfinsen, 1952; Fahien et al., 1969), rat (King & Frieden, 1970; Prabakaram & Singh, 1988), Pig (Kubo et al., 1959), rabbit (Kazaryan et al., 1985), human (Julliard & Smith, 1979), chicken (Snoke, 1956), tadpole (Wiggert & Cohen, 1966), frog (Fahien et al., 1965a; King & Cohen, 1975), dogfish (Corman et al., 1967; Corman & Inamder, 1970), lungfish (Janssens & Cohen, 1968), tuna (Veronese et al., 1976) and eel (Hayashi & Ooshiro, 1977a; Hayashi et al., 1982). Multiple forms of GDH has been reported only in plant tissues (Thurman et al., 1965; Yue, 1969; Errel et al., 1973; Lee, 1973; Ratajezak et al., 1977). The purified enzyme showed also a single band when stained for protein after PAGE (Figs.24a,b).

Kinetics: (Table 31; Figs.25-28)

The apparent Km of GDH for NADH (0.07mM) was one fourth of the Km for NAD^+ (0.28mM) (Table-31)indicating four times stronger affinity for NADH than NAD^+ to favour the oxidative deamination activity in the liver of H. fossilis. However, the Km for NH_4^+ (25mM) was five times higher than that for glutamate (4.76mM). The Km for the other substrate of reductive amination, α-ketoglutarate, was 0.38mM. The physiological level of ammonia (14.5 moles/g wet wt.) (Saha, 1986) was 15 times higher than glutamate (0.99 moles/g wet wt.) (Chakravorty, J. personal communication) in this fish. The tolerance for ammonia concentration (75mM) in vivo was very high (Saha & Ratha, 1986). Therefore, it could be reasonable to expect high saturation requirement of GDH for ammonia. In nitrogen fixing micro-organisms, GDH has a higher Km for glutamate and very low Km for ammonia. (Goldin & Frieden, 1971; Smith et al., 1975). In the animals where some nitrogen is excreted the Km for ammonia has been usually higher than the Km for glutamate. However, there are tissues specific variations such as in brain glutamate serves as a neurotransmitter besides being an ammonia carrier out of the tissue. The relative Km for ammonia in brain is lower than in liver, kidney or heart. A table of the Km values for the substrates of GDH reverse reaction in different groups of animals is given below for comparison (Table B).

GDH is considered to play a role for oxidative deamination rather than the reductive amination under normal circumstances in ammoniotelic freshwater teleosts (Pequin & Serfaty, 1963,1968; Janssens & Cohen, 1968; Forster & Goldstein, 1969; Wilson, 1973a; Vellas & Serfaty, 1974 Campbell

TABLE B: Comparison of the kinetic constants of glutamate dehydrogenase from H. fosilis with those from other sources.

Source/Tissue	Km in (mM)							References
	NADH	NADPH	NAD^+	$NADP^+$	α-KG	L-glu	NH_4^+	
Bovine liver	0.096		0.10	0.057	0.70	1.10	56.0	Olson and Anfinsen (1953)
Rat liver	0.03	0.02	0.39	0.23	0.18	0.60	20.0	Chee et al.(1978)
Rat brain	0.029	0.027	0.90	0.67	0.20	2.50	10.0	Chee et al.(1979)
Pig heart	0.065	0.067	0.12	0.60	0.075	0.75	67.0	Younes et al.(1973)
Toad liver	0.20		0.022		5.0	1.8	0.5	Fahien and Cohen (1970)
Frog liver		0.20		0.50	5.0	1.8	0.5	Fahien et al. (1965a)
Xeropus liver	0.015				1.0		21.0	Lee and Balinsky (1974)
laevis kidney	0.022		0.032		2.3	0.4	20.0	"
Dogfish liver		0.40		0.08	4.5	84.0	80.0	Corman et al. (1967)
Trout liver	0.018		0.098	7.7	0.082	3.7	12.3	Walton and Cowey (1977)
Tuna liver			0.033	0.7		1.4		Veronese et al. (1976)
Mudskipper Periophthalmus cantonensis Liver	0.018 *0.032				0.65 *1.16		86.2 *66.08	Iwata and Kakuta (1983)
Muscle	0.021 *0.031				0.56 *1.05		59.73 *40.05	" "
Tridentiger obscurus liver	0.017 *0.046				0.63 *1.25		116.04 *85.80	" "
obscurus muscle	0.015 *0.038				0.85 *1.10		100.00 *83.72	" "
H. fossilis liver	**0.07** ***0.07**		***0.28**		**0.38** ***0.35**	***4.76**	**25.0** ***23.8**	**Present work**
Proteus inconstans		0.003		0.023	2.30	3.7	0.37	Shimizu et al.(1979)
Neurospora crassa		0.125		0.05	5.3	45.0	10.0	Sanwall and Lata (1961)
Thiobacillus novellus		0.077		0.061	7.4	36.0	7.5	Lejohn et al. (1968)
Bacteroides fragilis	0.20	0.013	3.0	0.019	0.14	7.3	1.7&5.1	Yamamoto et al. (1987a)
Methylotroph methanolovorus		0.07		0.05	0.25	7.5	25.0	Sokolov and Trotsenko (1988)

*Km in presence of ADP

et al., 1983; Casey *et al.*, 1983). *H. fossilis* is primarily ammoniotelic in aquatic medium (Saha *et al.*, 1988). Hence, lower Km of GDH for glutamate than ammonia suggested its role in ammonia production for excretion in liver during its aquatic life. The comparative table for Km values of GDH in various species shows that *H. fossilis* hepatic GDH has better capacity for ammonia utilization than the ureotelic bovine (Olson & Anfinsen, 1953), ureo-osmotic dogfish (Corman *et al.*, 1967) and ammoniotelic mudskipper (Iwata & Kakuta, 1983) and trout (Walton & Cowey, 1977). The liver of *H. fossilis* also had higher GDH activity compared to other freshwater teleosts (Wilson, 1973a, Walton & Cowey, 1977; van Waarde, 1981). Amphibia and *H. fossilis* showed close similarity with respect to their Km value for ammonia. May be this enzyme has evolved in *H. fossilis* in the same way as in amphibians to facilitate periods of water deprivation.

The double reciprocal Lineweaver-Burk plot with respect to NAD^+ for hepatic GDH in vertebrates has been reported to be strongly non-linear (Olson & Anfinsen, 1953; Frieden, 1959a,b; Fahien *et al.*, 1965a; Wiggert & Cohen, 1966; Corman & Kaplan, 1967; Engel & Dalziel, 1969; Dalziel & Egan, 1972; Engel & Fordinand, 1973; Smith *et al.*, 1975; Bell *et al.*, 1985). However, it was not so for the hepatic GDH of *H. fossilis*. The oxidative deamination reaction was carried out in phosphate buffer at pH-8.5 and in presence of ADP. It has been reported that phosphate buffer at pH-8.0 or above (Engel & Dalziel, 1969; Chen & Engel, 1974) and ADP removed the negative co-operativity of NAD^+ in the oxidative deamination activity (Koberstein & Sund, 1973; Koberstein *et al.*, 1973; Lee & Balinsky, 1974; Bailey *et al.*, 1982). As ADP was essential for GDH-NAD^+ dependent assay, studies could not be done in absence of ADP.

Substrate inhibition: (Figs. 25-28)

All the substrates (α-Ketoglutarate, NH_4^+, L-glutamate and NADH) except NAD^+ at higher concentrations inhibited GDH activity. The inhibition of purified hepatic GDH at higher concentration of substrates is probably due to the formation of the "dead-end" complex. They could be of several types such as E-NAD^+-α-Ketoglutarate, E-NAD^+-NH_4^+ and E-NADH-glutamate (Frieden, 1959c; Lejohn et al., 1969; Cross et al., 1972; Pantaloni & Lecuyer, 1973; Bradley et al., 1979). However, the inhibition of GDH activity by excess of NADH has been earlier suggested due to the presence of a second regulatory non-catalytic site on the enzyme (Iwatsubo & Pantaloni, 1967; Pantaloni & Desser, 1969; Engel & Dalzier, 1970; Goldin & Frieden, 1972; Hunng & Frieden, 1972; Anderson&Contestabile, 1977; McCarthy & Tipton, 1984; Agadzhanyan & Karabashyan, 1986a,b; Chalabi et al., 1987; Ozturk et al., 1990). It is not possible to definitely conclude this mechanism of NADH inhibition of hepatic GDH activity in H. fossilis with the available results.

Absorption spectra: (Figs. 29&30)

The absorption spectra of purified hepatic GDH of H. fossilis showed another peak at 228nm besides the protein peak at 280nm. Olson and Anfinsen (1952) have reported that pure hepatic bovine GDH absorbed light maximally at 279nm (protein peak). Addition of nucleotide coenzyme NAD^+ and NADH showed specific peaks at 260nm and 340nm respectively. In the presence of substrate, L-glutamate, the absorption peak at 280nm for the enzyme shifted towards 260nm indicating a conformation change which was predominant in presence of ADP and NAD^+. A broad peak between 260-280nm shown by the complete reaction mixture might be due to the

presence of the ternary complex (enzyme-coenzyme-substrate). The peak at 340nm was specific for the presence of NADH which increased with time in oxidative deamination reaction due to the formation of NADH and decreased with time in reductive amination reaction due to the utilization of NADH. It is apparent that conformational changes in the enzyme molecule did take place with the binding of various substrates and coenzymes.

Temperature optima and thermal stability: (Table 31; Figs.31&32)

The optimum temperature for the activity of purified hepatic GDH of H. fossilis was 30°C for reductive amination reaction and 45°C for oxidative deamination reaction respectively (Table 31; Fig. 31). Similar results have been reported for GDH from different animal sources (Goldin & Frieden, 1971; Hillar, 1974; Smith et al., 1975; Ruano et al., 1985). The rapid inactivation of the enzyme above 50°C in H. fossilis might be due to the thermal denaturation of the enzyme molecule (Fig.31). Different temperature optima for the reverse reactions might suggest a temperature dependent conformational change in the enzyme molecule to favour a particular reaction. At physiological temperature, which is usually low in freshwater teleosts, reductive amination will be favoured to utilize ammonia for the formation of glutamate. However, at higher temperature the enzyme could drive the reaction towards oxidative deamination to utilize glutamate for more energy production. This regulation was apparently different between the temperature range of 20-45°C for hepatic GDH of H. fossilis (Fig.31).

Presence of substrate (α-ketoglutarate or L-glutamate) and ADP in the incubation medium provided some protection to the purified GDH from thermal denaturation (Fig.32). ADP in addition to the substrate showed better

results. Increased thermal stability by binding of ADP at specific site for maintaining the conformational state of GDH has been reported (Batra & Colman, 1984,1986; Batra et al., 1989; Cioni & Strambini, 1989). Binding of substrates or some other reactants usually creates a more stable conformation thus providing some protection from thermal denaturation.

pH optima: (Table 31; Fig.33)

The optimum pH (in phosphate buffer) for GDH activity was between 7.6 to 8.0 for reductive amination reaction and 8.5 to 9.5 for oxidative deamination reaction. This phenomenon of different pH for reverse reactions has been common for most of the GDH purified from other sources (Bond & Sang, 1968; Goldin & Frieden, 1971; Smith et al., 1975). Similar results have been reported in other teleosts, osteoglossid (Aruana and Arapaima) (Storey et al., 1978a) and eel (Anguilla japonica) (Hayashi et al., 1982) using imidazole buffer. However, the optimum pH was 8.0 with phosphate buffer in goldfish (Carassius auratus) (van Waarde, 1981) for both the reactions of GDH. The result obtained shows that at physiological pH hepatic GDH favours reductive amination in H. fossilis.

Amino acid (substrate) specificity: (Table 32)

The purified hepatic GDH showed strong specificity for L-glutamate as the substrate for oxidative deamination reaction. Twenty other amino acids studied could show no activity or about five percent activity at the same concentration as glutamate. Similar results have been reported for purified hepatic GDH from bovine (Olson & Anfinsen, 1953; Strecker, 1955), lungfish (Janssens & Cohen, 1968), carp (Wiggert & Cohen, 1965) and eel

(Hayashi & Ooshiro, 1977a), and from plants and micro-organisms (Smith et al., 1975; Shimizu et al., 1979; Misono et al., 1985; Yamamoto et al., 1987a; Opden Camp et al., 1989; Vančurová et al., 1989). Struck and Sizer (1960), however, reported that crystalline bovine liver GDH could oxidatively deaminate several amino acids. The optimum pH for mono-carboxylic amino acids were 1 to 1.5 pH units higher than that of L-glutamate and Km values were much higher (from 30 to 100mM) (Rife & Cleland, 1980a,b; Bailey et al., 1982). LiMuti and Bell (1983) suggested that both the carboxyl groups on glutamate were required to allow the subunit-subunit interactions, which are essential for catalytic activity. High specificity of GDH for glutamate indicated its major role in regulating glutamate↔ammonia metabolism in H. fossilis.

Coenzyme specificity: (Table 33)

One of the important properties of GDH studied in variety of organisms is the difference observed in its coenzyme specificity. The enzyme from vertebrate sources could utilize either NAD^+ or $NADP^+$ equally well while those from micro-organisms and plants are essentially specific for NAD^+ or $NADP^+$ (Frieden, 1965, 1971; Goldin & Frieden, 1971; Fisher, 1973, 1985; Smith et al., 1975; González et al., 1976; Scheid et al., 1980; Bonete et al., 1986, 1987). The purified hepatic GDH from H. fossilis in the reductive amination activity utilized either NADH or NADPH equally well as coenzyme and the oxidative deamination reaction showed specificity for NAD^+ like other teleosts (Veronese et al., 1976; Fields et al., 1978; Storey et al., 1978a; Iwata et al., 1981; van Waarde, 1981; Iwata & Kakuta, 1983). The coenzyme specificity has been related to the specific metabolic role of GDH in the

organisms. Reducing power of NADH is used mostly for energy production and of NADPH in biosynthetic reactions mostly for lipids. H. fossilis has very little fat content and more of amino acids and proteins. Absence of NADPH might be a physiological adaptation to maintain the non-fatty character of H. fossilis.

Effect of metabolites on GDH activity:

Purified GDH activity from various sources has been shown to be modulated by a variety of metabolites such as purine nucleotides, amino acids and biogenic amines etc. (Goldin & Frieden, 1971; Hillar, 1974; Smith et al., 1975).

Purine nucleotides: (Tables 33&34; Figs.34-39)

The purified hepatic GDH (NADH and NAD^+ dependent) activity of H. fossilis showed allosteric activation and inhibition by ADP and GTP respectively (Tables 33&34) like the GDH from other animal sources (Talal & Tomkins, 1964; Freedland et al., 1967; Goldin & Frieden, 1971; Josephs et al., 1973; Chen & Engel, 1975; Chen et al., 1976; Jallon et al., 1977; Zantema et al., 1979a,b; Dieter et al., 1981; Hornby et al., 1984). GTP was reported to enhance the binding of NADH both to the active and non-active NADH sites and stabilize the enzyme-reduced coenzyme complex to cause the inhibition of the reaction. ADP activates the reaction by destabilizing the above complex (Iwatsubo & Pantaloni, 1967; Eisenberg et al., 1976; Jacobson & Colman, 1982, 1983; Inoue et al., 1984; Agadzhanyan & Karabashyan, 1986a,b). The hepatic GDH of H. fossilis might be having similar regulation by ADP and GTP. ATP, AMP and IMP were less efficient activators and

inhibitors than ADP and GTP in H. fossilis. ADP activated both NAD^+ and NADH dependent GDH activity whereas ATP and AMP activated NADH dependent activity and inhibited NAD^+ dependent activity of GDH. GTP and IMP inhibited both the reactions of GDH. The patterns of activation and inhibition were different (Table 34). These facts suggested that the hepatic GDH of H. fossilis is more tightly regulated by the energy status of the cell. The pattern of inhibition by IMP and ATP was non-competitive indicating that they can bind to both free enzyme and enzyme-substrate complex. It was also observed that presence of ADP increased the Ki value of GTP for NADH dependent GDH activity. This might be due to the reversal by ADP of the effect of GTP, facilitating the binding of NADH to non-active site to inhibit GDH activity (Cross & Fisher, 1970; Storey et al., 1978a; Iwata & Kakuta, 1983).

Various ions: (Table 35)

All the metal ions studied inhibited GDH (NADH and NAD^+ dependent) activity. Complete inhibition by mercury, zinc, silver and ferric ions as reported earlier for GDH from other sources (Olson & Anfinsen, 1953; Fahien & Cohen, 1970; Colman & Foster, 1970; Jallon & Iwatsubo, 1971; González et al., 1976; Bell et al., 1987) was observed above 25mM concentration. Destabilization of the conformational state of GDH has been suggested as the possible cause of inhibition by metal ions. Both the activities of GDH were inhibited by all the negative ions studied except PO_4^{2-} which activated the enzyme activity. Inorganic phosphate is known to be an activator for GDH activity (Sedgwick & Frieden, 1968; Godinot & Gantheron, 1971; Hillar, 1974; diPrisco & Garofano, 1975; diMatteo, 1976; Storey et al., 1978b; Fisher, 1985) probably to help the organism during low energy state.

Acetylcholine, biogenic amines and some amino acid derivatives: (Table 36)

Acetylcholine, biogenic amines (norepinephrine, epinephrine, DOPA and serotorin) and some amino acid derivatives(γ-aminobutyric acid, urea and carbamyl phosphate) inhibited purified hepatic GDH (NADH and NAD^+ dependent) activity of H. fossilis. Stimulation by epinephrine and DOPA of bovine liver GDH (Kaur & Kanungo, 1970a) and by epinephrine of rat brain GDH has been reported (Kaur & Kanungo, 1970b). Both bovine and rat GDH were inhibited by acetylcholine and norepinephrine (Kaur & Kanungo, 1970a,b). Inhibition by serotonin of bovine liver GDH has also been reported (Kaur & Kanungo 1970a). Urea and carbamyl phosphate, in general, inhibited GDH activity (Grisolia et al., 1964; Hillar, 1974; González et al., 1976). It has been suggested that inhibitory effect of these compounds on GDH activity was due to the dissociation of the enzyme subunits or due to physical changes in the enzyme molecule brought about by their binding (Yielding & Tomkins, 1960,1964; Frieden, 1963). Though it is not possible to propose any mechanism, it is clear that the activity of the enzyme was modulated by the above metabolites.

Effect of varying substrates: (Figs.40-42)

The double reciprocal plot (Fig.40) revealed that the Michaelis constant for NH_4^+ and α-ketoglutarate were independent of each other. Since the lines intersect on the abscissa, the dissociation constant and Michaelis constant were same. However, the double reciprocal plots were parallel (Fig.41) when the ammonium ion concentration was kept constant at saturation level with varying α-ketoglutarate and NADH concentration. This indicated that they are interdependent on each other. However, when the concentrations

of NAD^+ and L-glutamate were varied a pattern of intersecting lines (Fig.42) revealed the interdependence of these substrates during enzyme activity. The striking similarities between these results and those reported earlier in bovine (Frieden, 1959c), frog (Fahien et al., 1965a,b) and dogfish (Corman et al., 1967) enzymes suggest that the mechanisms proposed earlier explaining the sequence of binding of the substrates might hold good for H. fossilis GDH. NADH has been shown to bind to the free enzyme in most of the GDH studied earlier (Goldin & Frieden, 1971, Eisenberg et al., 1976; McCarthy et al., 1981). Taking that as true for H. fossilis hepatic GDH, the sequence of substrate addition may be NADH, NH_4^+ and α-ketoglutarate for reductive amination reaction as suggested for vertebrate GDH (Frieden, 1959c, Fahien et al., 1965a,b; Corman et al., 1967).

Product inhibition: (Table 37; Figs.43-47)

The inhibition by the product L-glutamate was competitive with relation to α-ketoglutarate as the substrate for GDH reductive amination reaction. However, the inhibition by α-ketoglutarate was non-competitive with relation to L-glutamate as the substrate for the oxidative deamination reaction. The binding sites for glutamate and α-ketoglutarate might be very close or overlapping. In the later case the non-competitive inhibition could be due to the interference of NADH binding. Binding to such NADH-α-keto-glutarate complex has been suggested earlier (Godinot & Gutheron, 1971; Bell et al., 1985). Inhibition by ammonia was competitive and by NADH was un-competitive with relation to glutamate as substrate. Similar reports have been made for bovine liver (Fisher & McGregor, 1960; Bell et al., 1985). It was suggested that glutamate was limited by the rate of liberation of the reduced coenzyme from the enzyme coenzyme complex. Inhibition by NH_4^+

was also non-competitive with relation to NAD^+ probably due to the formation of "dead end" complex, $E\text{-}NAD^+\text{-}NH_4^+$. The inhibition by NADH was as expected competitive with relation to NAD^+ as both have the same binding site on the enzyme. It has been reported that four coenzymes (oxidized and reduced NAD^+ and $NADP^+$) compete for the same site on the enzyme molecule (Dalziel, 1962, Cross & Fisher, 1970; Younes _et al._, 1973). The same order of substrate addition is suggested by the pattern of product inhibition. The product inhibition is of physiological importance in controlling pathways by negative feed back. The products of oxidative deamination reaction (α-ketoglutarate, NH_4^+ and NADH) were potent inhibitors of the same reaction. Inhibition of the oxidative deamination reaction of GDH by NADH concentrations well within the physiological range has been reported in eel liver (McBean _et al._, 1966; Edington _et al._, 1973). The Ki value of NADH (0.02mM) suggests that it can limit the rate of glutamate oxidation _in vivo_ in _H. fossilis._ However, the physiological level of NADH in this fish is not yet known.

Amino acids and Keto acids: (Tables 38&39; Figs.48-53)

All the amino acids studied except L-leucine and DL-isoleucine inhibited _H. fossilis_ hepatic GDH (NADH and NAD^+ dependent) activity at higher concentrations _in vitro_. Leucine is known to be an activator for GDH in other animals (Yielding & Tomkins, 1961; Gylfe, 1976; Sener & Malaisse, 1980; Iwata & Kakuta, 1983; Knudsen _et al._, 1983; Kofod _et al._, 1986; Fahien _et al._, 1988, 1990; Erecinska & Nelson, 1990). It has been suggested that leucine helped to maintain the three dimensional structure of GDH molecule. The degree of inhibition of GDH activity by other amino acids observed were different for reductive amination and oxidative deamination reaction

(Table 38). At lower concentrations, substantial inhibition of both the reactions of GDH could be observed by L-Tyr, L-Cys, L-Try, L-Asp, L-Lys and L-Ala. Arginine, ornithine, aspartate and glutamine inhibited reductive amination more than oxidative deamination reaction. Arginine, ornithine and aspartate are all associated with ureogenesis via o-u cycle and are expected to be present in liver of H. fossilis which has been reported to possess a complete o-u cycle (Saha, 1986; Saha & Ratha, 1986, 1987). The activity of glutamine synthetase, the enzyme for glutamine synthesis, has also been reported in liver of H. fossilis (Chakravorty et al., 1989). The regulation of GDH activity by these amino acids could serve as additional regulatory mechanisms to maintain amino acid/ammonia balance in H. fossilis. The inhibition pattern of L-cystein was found to be competitive for (NADH dependent) GDH activity and uncompetitive for (NAD^+ dependent) GDH activity. The L-alanine was competitive for (NAD^+ dependent) GDH activity (Table 39). These amino acids might be functional amino acid residues for catalysis or might be present near the active site of the GDH molecule. It has been reported that the cystein residue of the bovine enzyme (Hillar, 1974) appeared to be at or near the site that was involved in binding to the allosteric activator ADP. The amino acid residues involved in binding sites of hepatic GDH of H. fossilis has not been studied.

The keto acids such as pyruvate and oxaloacetate inhibited GDH (NADH and NAD^+ dependent) activity at higher concentrations (Table 38). The effects of various amino acids and keto acids indicate the possibility of self regulatory mechanism in GDH to maintain a balance between the transamination and deamination reactions.

Immunological studies: (Figs. 54&59)

No structural differences were observed between the summer and winter purified GDH from liver of H. fossilis to elicit any antigenic variations to be apparent during immunological studies. This result suggests that GDH molecule does not change instead the alteration in the level of various physico-chemical modulators might have caused the seasonal variations in the activity of GDH reverse reactions during Summer and Winter in H. fossilis. Antisera developed against the two antigens (summer and winter purified GDH) showed similar rate of inhibition of both the enzymes (summer and winter purified) activity. However, the reverse reactions showed different rate of inhibition. The amount of antisera which caused 100% inhibition of NAD^+ dependent activity caused only 80% inhibition of NADH dependent activity. The antigenic sites could be more in the oxidative deamination domain than the reductive amination domain of the GDH molecule.

Determination of molecular weight: (Table 31; Figs.55-58)

The molecular weight and the subunit structure of purified GDH were determined by exclusion chromatography and polyacrylamide gel electrophoresis (PAGE). The purified hepatic GDH of H. fossilis during Summer and Winter showed the same molecular weight of about 3,38,000. The enzyme molecule was composed of six identical subunits with molecular weight of about 56,000 each. The molecular weight and subunit pattern of GDH observed were very close to those reported in the liver of teleost, tuna (Veronese et al., 1976) and marine ureo-osmotic elasmobranch, dogfish (Corman et et al., 1967). A comparative table of the molecular structure of GDH reported from various sources is given below (Table C). The molecular weight did not increase with increasing protein concentration indicating that H. fossilis

TABLE C: Comparison of the molecular weight of glutamate dehydrogenase from H. fossilis with those from other sources.

Source/Tissue	Native enzyme molecular weight	Subunit molecular weight	No.of subunit	References
Bovine liver	3,10,000-3,50,000 ±25,000	53,000-57,000 ±3,000	6	Olson and Anfinsen (1952) Fisher et al. (1962), Eisenberg and Tomkins (1968), Eisenberg and Reisler, (1970), Reisler et al. (1970)
Ox brain		55,000±5,000		McCarthy et al (1980)
Rat liver	3,50,000±20,000	48,000-53,000 ±5,000±5,000	6-8	Sedgwick and Frieden(1968) King and Frieden (1970)
Rat brain	>2,00,000	56,000		Chee et al. (1979)
Human liver		55,200±1,500		Julliard and Smith (1979)
Human placenta		55,155±2,204		Julliard and dePaulet (1978)
Frog liver	2,50,000			Fahien et al. (1965a,b)
Dogfish liver	3,30,000±20,000			Corman et al (1967)
Tuna liver	3,33,000±15,000	53,900±2,500	6	Veronese et al. (1976)
Eel liver	3,15,000			Hayashi et al (1982)
H. fossilis	**3,38,000**	**56,000**	**6**	**Present work**
Pisum sativum Lemna minor	2,30,000	58,500	4	Scheid et al. (1980)
Blastocladiclla emersoni	2,00,000±21,000	48,500±7,000	4	Lejohn et al. (1968,1969)
Neurospora crassa	2,88,000	48,800	6	Sanwall and Lata (1961) Blumerthal and Smith (1973)
Cnlorella sorkiniana	2,90,000-3,60,000	53,000-58,500	6	Bascomb et al (1986,1987) Bascomb and Schmidt(1987)
Methylophilus methanolovorus	3,00,000	49,000	6	Sokolov and Trotsenko (1988)
Lactooacillus fermentum	3,00,000	50,000	6	Misono et al. (1985)

enzyme had no tendency to polymerize like GDH from liver of tuna (Veronese et al., 1976), dogfish (Corman et al., 1967), rat (King & Frieden, 1970) and micro-organisms (Winnacker & Barker, 1970; Coulton & Kapoor, 1973; Veronese et al., 1974a; Smith, 1980).

The subunits dissociated by urea and separated by exclusion column chromatography did not show any enzyme activity either by spectrophotometric assay or by specific staining on PAGE even after dialysis.

Mitochondrial localization in various tissues and the molecular characters of hepatic GDH from Heteropneustes fossilis showed close similarities of the enzyme with the GDH reported from other sources. However, high reductive amination activity, induction of the GDH activity during starvation, hyper-ammonia stress and water deprivation suggest the significance of GDH in ammonia detoxification at cellular level particularly in non-ureogenic tissues and before the o-u cycle was induced. It also trapped excess ammonia to synthesize more glutamate and probably other amino acids for storage and transport of toxic ammonia. The results on substrate specificity, binding affinity and the regulation by various physico-chemical factors of the purified hepatic GDH activity favoured the above proposed function in Hetaropneustes fossilis. However, the multiple regulations by various metabolites and metal ions need more specific studies to determine their exact functions at the physiological level.

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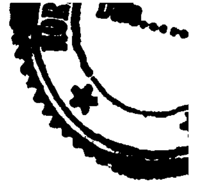

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