

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

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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
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CONTENTS

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CONTENTS

	<u>Page</u>
LIST OF ABBREVIATIONS	i-iii
INTRODUCTION	1-18
Ammoniogenesis	
Toxicity of ammonia	
Utilization and detoxification of ammonia	
Classification of GDH	
Purification, molecular characterization and kinetics of GDH	
Plan of Work	
MATERIALS AND METHODS	19-40
Animals	
<u>Experimental set up</u>	
Physiological level, circadian and seasonal variation	
Effect of temperature acclimation	
Effect of water deprivation	
Effect of starvation, refeeding and ambient hyper-ammonia stress	
<u>Tissue processing</u>	
<u>Estimations</u>	
Enzyme assays	
Glutamate dehydrogenase	
Lactate dehydrogenase	
Cytochrome oxidase	
Protein	
Total free amino acid (FAA)	
Purification of hepatic GDH and its physico-chemical properties	
Crude extract	
Ammonium sulphate fractionation	
Ion exchange chromatography on DEAE-Cellulose	
Ion exchange chromatography on DEAE-Sepharose	
Affinity chromatography on Blue Sepharose	
Polyacrylamide gel electrophoresis (PAGE)	
GDH specific staining	
Protein staining	
Absorption spectra	

Temperature optima and thermal stability
pH optima
Kinetic studies
Product inhibition of GDH

Molecular weight determination

Exclusion chromatography
PAGE
Preparation of standard protein and GDH sample for SDS-PAGE

Immunological Studies

Preparation of antiserum
Immuno-diffusion

Chemicals

Statistical analysis and presentation of data

RESULTS

41-52

Effect of buffer on stability on GDH activity
ADP and nicotinamide coenzyme requirement
Physiological level of GDH in various tissues
Sub-cellular distribution of GDH
Circadian (24 hr) cycle
Annual cycle
Effect of temperature
Effect of water deprivation
Effect of Starvation, refeeding and hyper-ammonia stress
Purification of GDH from liver of H. fossilis
Kinetics and substrate inhibition
Absorption spectra
Temperature optima and thermal stability
pH optima
Amino acid (substrate) specificity
Coenzyme specificity
Effect of various nucleotides
Effect of various ions
Effect of acetylcholine, biogenic amines and some amino acid derivatives
Effect of varying substrates
Product inhibition
Effect of amino acids and keto acids
Effect of antibody
Determination of molecular weight
Immuno diffusion

TABLES 1 - 39

FIGURES 1 - 59

DISCUSSION

53-86

- Effect of buffer on stability of GDH activity
- ADP and nicotinamide coenzyme requirement
- Tissue distribution
- Sub-cellular distribution
- Circadian (24 hr) cycle
- Annual cycle
- Effect of temperature
- Effect of water deprivation
- Effect of starvation, refeeding and hyper-ammonia stress
- Purification of GDH from liver of H. fossilis
- Kinetics
 - Substrate inhibition
 - Absorption spectra
 - Temperature optima and thermal stability
 - pH optima
 - Amino acid (substrate) specificity
 - Coenzyme specificity
 - Effect of metabolites on GDH activity
 - Purine nucleotides
 - Various ions
 - Acetylcholine, biogenic amines and some amino acid derivatives
 - Varying substrates
 - Product inhibition
 - Amino acids and keto acids
- Immunological studies
- Determination of molecular weight

REFERENCES

87-145

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
$^{\circ}\text{C}$	- 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
μ l	- Microlitre
μ M	- Micromolar
μ mole	- 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 signigance)
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(hydroxy 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

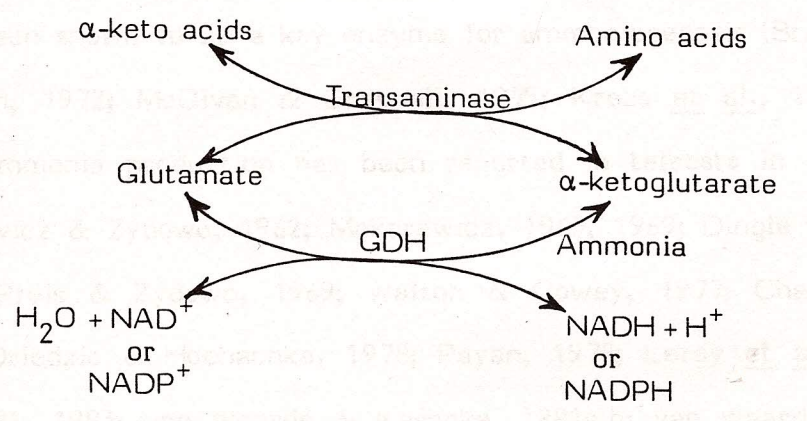
INTRODUCTION

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 form (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 electroencephalogram (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 coefficient, 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.

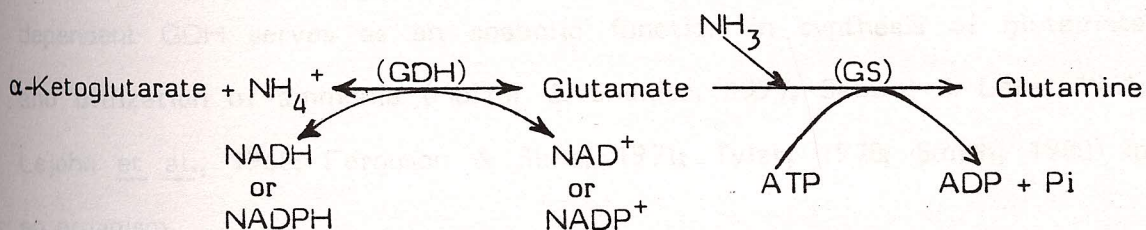
Aquatic organisms rapidly dispose off ammonia to their ambient medium by diffusion across the gills and through body surface immediately after its formation to avoid its accumulation in vivo (Smith, 1929; Wood, 1958; Fromm & Gillette, 1968; Goldstein 1972; Vellas & Serfaty, 1974; Payan & Matty, 1975; 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.

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; Ehmke 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; IdeToma & Laugidge, 1974; Peters & Sypherd, 1979; Janssen et al., 1980, Osmani & Scurtten, 1983; Kumar & Nicholas, 1984; Turner et al., 1986; Bischoff & Garraway, 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 (Mifflin & 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 & Mäntsälä, 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 Kritzman (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 GDHs:

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 $3,50,000 \pm 20,000$ and composed of six to eight polypeptide chains, each of which had a molecular weight of $48,000 \pm 5,000$ (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 (Chondrichthyes). 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 K_m values differed markedly depending on the condition of the reaction and the sources. The vertebrate GDH, in general, showed lowest K_m values for NADH and highest for NH_4^+ . On the other hand, in the micro-organisms the GDH had K_m 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.

102298



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.