GLUTAMINE METABOLISM IN REGULATING AMMONIA LEVEL *IN VIVO* IN AN AIR-BREATHING FRESHWATER TELEOST, *Heteropneustes fossilis*

ABSTRACT

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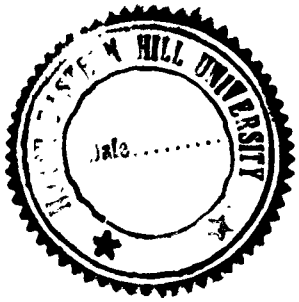

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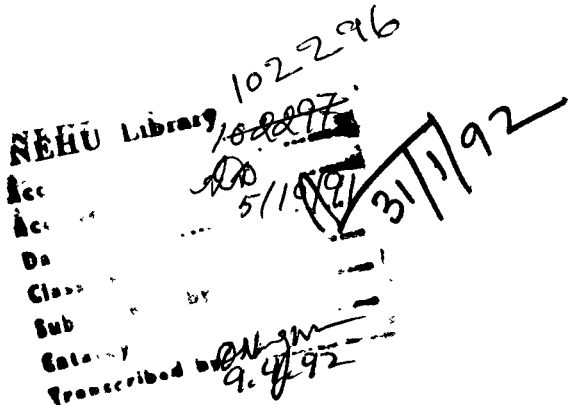

ABSTRACT

Glutamine occupies a central position in nitrogen metabolism. Besides being used in protein synthesis, it serves as a substrate for the synthesis of many biologically important molecules in the organisms such as purines and pyrimidines. It helps in storage and transport of both glutamate and ammonia. Glutamine synthesis plays a major role in detoxifying ammonia at cellular level maintaining it at a lower non-toxic level besides controlling the level of glutamate a neurotransmitter in brain. Ammonia is produced continuously during catabolism of nitrogen containing biomolecules *in vivo*. It is highly toxic for the normal physiological function of living organism even at low concentrations. Therefore, the cells try to eliminate the toxic ammonia immediately or detoxify it by conversion to some less toxic/non-toxic substances such as amino acid, urea or uric acid for temporary storage *in vivo*.

The nature of nitrogen excretory products in animals has evolved depending upon the availability of water in the surrounding medium. Animals are thus classified as ammoniotelic (aquatic), ureotelic (amphibians and terrestrial) and urecotelic (desert and aerial). The fishes are primarily ammoniotelic and excrete ammonia to the aquatic medium. However, african lungfishes, marine teleosts and elasmobranchs

synthesize and retain urea in their body with a functional ornithine-urea (o-u) cycle. African lungfishes, mudskippers and aquatic amphibians showed transition from ammoniotelism to ureotelism mainly to avoid ammonia toxicity during water restricted conditions.

Ornithine-urea cycle could not be detected in the freshwater teleosts and hence it was suggested to have been deleted in freshwater teleosts during its evolution from marine ancestors. However, presence of a complete o-u cycle with appreciable level of activity of all the enzymes were reported from this laboratory in four species of freshwater air-breathing teleosts including Heteropneustes fossilis . Further studies on ammonia metabolism with H. fossilis indicated its high tolerance for ambient ammonia (upto 75mM NH_4Cl), longer periods (60-70hrs) of water deprivation and hyper-osmolar (300mOsm) ambient medium without any apparent deleterious effect. During such exposures, the fish suppressed excretion of ammonia and accumulated excess ammonia in vivo. The induction of o-u cycle was followed by enhanced rate of conversion of accumulated ammonia to urea in liver and kidney. The high ammonia tolerance limit in this fish was suggested to be due to the existence of better ammonia management mechanisms including induction of ureogenesis in H. fossilis.

A direct correlation between the urea production and glutamine synthesis has been reported in marine fishes

where CPS III utilized glutamine as the amino donating substrate for urea cycle. The synthesis of glutamine and its degradation has been suggested to play a major role in the regulation of ammonia besides serving as a amino donating substrate for different metabolic pathways. Ammonia produced in different tissues is fixed in the form of glutamine by the enzyme glutamine synthetase (GS) and transported to the target organs for its direct entry to o-u cycle through CPS III or deaminated to release ammonia by glutaminase (Glnase) either for its excretion or utilization by o-u cycle through CPS I. Therefore, the role of glutamine metabolism was evaluated in ammonia management in the ammoniotelic but ureogenic fresh water teleost, Heteropneustes fossilis.

The present study conducted in H. fossilis included the following:

1. Normal distribution of glutamine synthetase (GS) and glutaminase (Glnase) activity in various tissues such as brain, liver, kidney, muscle and gill.
2. The sub-cellular localization of GS and Glnase in brain, liver and kidney tissues.
3. The diurnal variation of GS and Glnase activity in brain, liver and kidney tissues.
4. The alteration in the levels of ammonia and glutamate and the activity of GS and Glnase in different

tissues during starvation, exposure to hyperambient ammonia and various periods of dehydration.

5. GS was purified from the brain tissue and its molecular characters, kinetics and regulation were studied in vitro.

GS and Glnase activity were assayed in five tissues of H. fossilis with brain showing the highest level of enzyme activity followed by liver kidney and gill. Only GS but not Glnase activity could be detected in muscle. High GS and Glnase activity in brain might be helping in prompt detoxification of ammonia and maintenance of glutamate-glutamine pool. The GS activity in liver and kidney was 2-3 times higher than those reported in ammoniotelic freshwater fishes but lower compared to ureo-osmotic marine fishes. Thus, H. fossilis occupies an intermediate position between the ammoniotelic freshwater fishes and ureo-osmotic marine fishes with respect to level of GS activity. The ratio of the activity of GS/Glnase in various tissues of H. fossilis, in general, favoured detoxification of ammonia for the production of metabolically important glutamine.

The sub-cellular distribution of GS and Glnase were studied in brain, liver and kidney by separating the sub-cellular fractions by differential centrifugation and identifying them using marker enzymes such as GDH, LDH, G-6-Pase for mitochondrial, cytosolic and microsomal fractions

respectively. The cytosolic GS in brain and mitochondrial Glnase in all the tissues were similar to the earlier reports in other animals. However, the mitochondrial GS in the liver and kidney of H. fossilis was a unique observation for any ammoniotelic animal showing close similarity with ureo-osmotic marine elasmobranchs. It suggested that active glutamine utilization might exist in the mitochondria of liver and kidney of this fish.

Diurnal variation of GS and Glnase activity in brain, liver and kidney of H. fossilis was studied at 3 hrs interval during 24 hrs cycle. There was no significant variation in any of the tissues studied indicating that glutamine metabolism operated at the same rate throughout the 24 hr cycle in this freshwater teleost.

The high GS activity besides its unique pattern of sub-cellular localization in the liver and kidney suggested the effective involvement of glutamine metabolism in ammonia management in H. fossilis. A direct experiment was conducted to study the effect of higher ambient ammonia on GS and Glnase activity and levels of ammonia and glutamate in various tissues. The fish was exposed to 50, 75 and 100 mM concentration of NH_4Cl for 14 days. The effect of treatment with 100 mM NH_4Cl was restricted to 4 days due to complete mortality of fishes after 4 days. Ths fish did not take any food and continued to starve during their exposure to NH_4Cl. As starvation does influence amino acid metabolism,

the effect of starvation was also monitored simultaneously with the treatment with NH_4Cl.

The metabolic ammonia and the activity of GS did not change significantly in any of the tissues during starvation. However, tissue specific accumulation of glutamate and the induction of Glnase activity was observed in different tissues. Non-accumulation of ammonia might have been the cause of unchanged GS activity during starvation. The induction of Glnase activity in brain and kidney during starvation might have produced sufficient glutamate in liver and kidney. The induction of GDH activity for ammonia detoxification by glutamate formation might have contributed to the accumulation of glutamate. Brain glutamate level remained unchanged during starvation period. GS/Glnase activity decreased significantly favouring also glutamate formation which might have been needed for energy production.

Exposure of H. fossilis to various concentrations of NH_4Cl resulted in significant accumulation of ammonia and glutamate in different tissues accompanied by high induction of GS activity. Brain showed early induction of GS activity followed by liver and kidney. Brain being most sensitive to ammonia toxicity and also the o-u cycle being absent, the early induction of GS activity might have controlled the ammonia level in the brain by converting it to glutamine. In liver and kidney, inspite of the presence

of functional o-u cycle, induction of GS activity in both the tissues might have helped in accelerating the ammonia detoxification process under hyper-ammonia stress. The glutamate accumulation in these tissues might have been caused due to induction of GDH activity during hyper-ammonia stress. Glutaminase activity was significantly inhibited in brain and kidney exposed to 75 and 100 mM. NH_4Cl There was no change at 50 mM ambient NH_4Cl Glnase activity in liver significantly increased only at early stages of exposure to NH_4Cl. The inhibition of Glnase activity might have been an adaptation to decrease addition of metabolic ammonia and hence, to tolerate the stress of higher ambient ammonia. The increase in the ratio of GS/Glnase activity in all the tissues at various concentrations of NH_4Cl indicated more synthesis of glutamine rather than its hydrolysis to glutamate and ammonia.

The effect of dehydration was studied in H. fossilis kept in glass jars covered with bilayer cheese cloth for 36 hrs. There was significant accumulation of glutamate accompanied by induction of GS activity and inhibition of Glnase activity in the three tissues studied. The results were similar to those obtained for NH_4Cl experiment. The increase in accumulation of ammonia in vivo during emersion has been shown earlier in this fish. This increase might have induced GS activity to detoxify the excess ammonia.

Brain being highly sensitive to ammonia, and in absence of o-u cycle there was early induction of GS activity within 3 hr of dehydration. Induction of GS was comparatively late in liver and kidney (with 9-12 hrs). These two organs are the primary ammonia metabolizing organs and hence, might have high ammonia tolerance limit. Significant accumulation of glutamate along with the synthesis of glutamine by GS might be due to higher induction of GDH activity for ammonia detoxification. The inhibition of Glnase in all the tissues might be an adaptation to avoid undue ammonia formation by hydrolysis of glutamine. The ratio of GS/Glnase increased with the increase in duration of dehydration indicating increase in ammonia detoxification through glutamine synthesis.

Glutamine synthetase was purified from the brain of H. fossilis acclimatised to laboratory conditions. The protocol followed for purification such as $(NH_4)_2SO_4$ precipitation, DEAE Sephacel and Sephadex G-200 column chromatography resulted in 62% recovery of activity with 58 fold of purification. It showed a single band on protein staining and specific staining on PAGE. The enzyme was probably a single species of protein like the GS reported from other sources. The molecular weight was found out to be 3.91×10^5 by gel filtration. It was similar to the molecular weight of GS from animal origin. The purified GS showed instability

in the purification buffer (Tris-HCl pH 7.4). Presence of 10% glycerol and 0.25 N NaCl separately or in combination could give better stability to the pure enzyme for more than a month. A broader pH maxima of 7.2-11 was observed for H. fossilis brain GS compared to the pH optima of GS known from other sources. The broader pH range indirectly indicate better adaptation of the brain enzyme of H. fossilis to fluctuations in pH in derelict water bodies. The temperature maxima was found to be 45°C. However, the native enzyme showed decline in activity after 15 minutes at 45°C. The prevention of thermal denaturation of the pure enzyme was observed in presence of the reaction mixture. Among the substrates/cofactors studied Mg^{2+} alone and in combination with ATP were found to stabilize the pure enzyme. Mg^{2+} in presence or in absence of ATP could bind to the enzyme to protect the enzyme from thermal denaturation. The substrate and cofactors of GS reaction could also protect the enzyme from 2-mercaptoethanol denaturation. The Km values of the enzyme were determined for ATP (2.3 mM), Mg^{2+} (6.25 mM), Glutamate (50 mM) and hydroxylamine (0.5 mM). Higher Km values for glutamate and hydroxylamine showed lower affinity of the enzyme towards these substrates. This might have been compensated by the presence of higher (2-60 times higher) GS specific activity compared to specific activity reported for ureotelic animals. The purified brain GS

activity has been shown to be modulated by variety of metabolites. The purified GS showed higher affinity towards Mg^{2+} for its optimum activity. In addition to Mg^{2+}, Co^{2+} and Mn^{2+} could activate the enzyme to a lesser extent in absence of Mg^{2+}. However, Co^{2+} and Mn^{2+} inhibited the enzyme at higher concentrations. Cu^{2+}, Zn^{2+}, Po_4^{-2}, Co^{2+}, Ca^{2+} showed inhibitory effect on GS. Thus, divalent cations showed both activatory or inhibitory effects. The purified enzyme was inhibited by several amino acids, such as Ala, Gly, CP, Asp, Asn, Orn, Arg and Cit. The inhibition by amino acids might be due to the feedback or product inhibition mechanisms suggested in other animals and micro-organisms. In addition to metal ions and amino acids some nucleotides such as ADP, AMP and IMP inhibited GS activity. The effect of inhibition was ADP > AMP > IMP. ADP inhibited the enzyme significantly with non-competitive type of inhibition with Ki value of 3.15 mM. The inhibition of GS by ADP is of physiological significance as it could aggravate taxaemic condition at lower energy level.

Higher tissue level of GS compared to Glnase suggested capability of H. fossilis to effectively detoxify ammonia by converting it to glutamine. The high activity of GS and Glnase in the brain where o-u cycle was absent, indicated the major role of this reaction in ammonia management besides maintaining the level of glutamate a neurotransmitter.

The mitochondrial localization of GS in liver and kidney tissues unlike the ammoniotelic species was a unique finding resembling with uricotelic birds and reptiles and ureo-osmotic marine elasmobranchs. The induction of GS activity and general inhibition of Glnase activity and the accumulation of glutamate during dehydration and hyper-ambient ammonia treatment strongly suggested the synthesis of glutamate and glutamine as an important pathway for ammonia detoxification besides o-u cycle in the ureogenic teleost. Localization and induction of mitochondrial glutamine synthetase in liver and kidney suggested the possibility of utilization of glutamine for urea synthesis through the CPS III isoenzyme like ureo-osmotic marine fishes. The inhibition of glutaminase during ammonia accumulation might be a physiological adaptation to avoid the undue formation of ammonia in addition to already high accumulated ammonia level *in vivo*. Thus metabolism of glutamine plays an important role in ammonia management in *H. fossilis* besides o-u cycle and thereby providing higher tolerance limit for ammonia. Some of the characters of GS also indicate evolutionary closeness of this species with marine ancestors.

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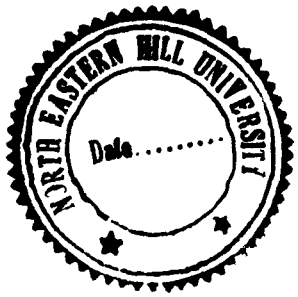

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*Certified that the thesis entitled "**Glutamine Metabolism In Regulating Ammonia Level in vivo, In An Air-breathing Fresh Water Teleost Heteropneustes Fossilis**", submitted by Ms Jharna Chakravorty for the degree of Doctor of Philosophy in Zoology of the North-Eastern Hill University, Shillong embodies the record of original investigation under my supervision. She has been duly registered and completed required tenure. This work has not been submitted for any other degree in any university.*

SHILLONG
JUNE 30, 1990

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Jharna Chakravorty.
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CONTENTS

LIST OF ABBREVIATIONS

°A	Anstrom
ADP	Adenosine di-phosphate
Ala	Alanine
AMP	Adenosine mono-phosphate
Arg	Arginine
Asn	Aspargine
Asp	Aspartic acid
ATP	Adenosine tri-phosphate
°C	Degree centigrade
Cm	Centimetre
CP	Carbamyl phosphate
Cit	Citrulline
CPS	Carbamyl phosphate synthetase
DEAE	Diethyl aminoethyl
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetra acetic acid
Fig.	Figure
g	Gram
GDH	Glutamate dehydrogenase
GHA	γ-glutamyl hydroxamate
Gln	Glutamine
Glnase	Glutaminase
Gly	Glycine
GOGAT	Glutamate Oxoglutarate amino transferase
G-6-Pase	Glucose-6-Phosphatase
GS	Glutamine synthetase
H. fossilis	Heteropneustes fossilis
HEPES	Hydroxyethyl piperazine ethane sulphonic acid
Hrs.	Hour

IMP	Inosine mono-phosphate
Kav	Partition coefficient
Kg	Kilogram
Ki	Inhibitor constant
Km	Michaelis constant
l	Litre
λ	Lamda (wave length)
LDH	Lactate dehydrogenase
M	Molar
µl	Microlitre
µM	Micro molar
µ mole	Micro mole
mg	Miligram
min	Minute
ml	Millilitre
mM	Milli molar
M.W	Molecular weight
N	Normal concentration
nm	Nenometer
NAD	Nicotinamide adenine dinucleotide oxidized
NADH	Nicotinamide adenine dinucleotide reduced
N.S.	Not significant
O.D.	Optical density
Orn	Ornithine
o-u	ornithine urea
p	Probability (Level of significance)
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
PPi	Inorganic pyrophosphate
PRPP	Phosphorybosyl pyrophosphate
r.p.m.	Revolution per minute
S.D.	Standered deviation
Sec.	Second

TCA	Trichloro acetic acid
Tris	Tris(hydroxy methyl) amino methane (a buffer component)
UV	Ultraviolet
V	Volume
V_e	Elution volume
V_{max}	Maximal velocity
V_o	Void volume
V_t	Total volume
Wt/w	Weight

INTRODUCTION

Nitrogen metabolism is one of the major metabolic path ways in all living organisms. Microorganisms and plants are capable of using nitrogen gas and simple inorganic nitrogen compounds such as nitrites, nitrates and ammonia to synthesize variety of nitrogenous biomolecules such as amino acids, nucleotides, amino sugars, coenzymes etc. Animals are not capable of using inorganic nitrogen compounds to synthesize nitrogenous biomolecules. They receive organic nitrogenous compounds in the diet which are taken in largely as amino acids, proteins and nucleic acids along with other biomolecules such as carbohydrates and fat. Most animals use carbohydrates as the primary source for metabolic energy production releasing CO_2 and H_2O as the end product. Other biomolecules are used as energy source after depletion of carbohydrates (Phillips, 1969). However, in teleostean fish the main source of energy is the catabolism of proteins and amino acids releasing ammonia as one of the major end products (Goldstein & Forster, 1970; Watts & Watts, 1974; French et al., 1981; Walton & Cowey, 1982).

Normal dietary intake of protein provides excess of amino acids than the amount required to sustain protein turnover. Elevated level of amino acids in plasma has been reported in fishes with high protein diet (Cowey et al, 1977). Excess

amino acids can not be stored (as stored proteins) as can be carbohydrates (as glycogen) or lipids (as fat) in animals. Vitellogenin is the only special storage protein in the egg of oviparous animals (Driedzic & Hochachka, 1978). The excess of amino acids are deaminated and the carbon residues utilized for energy production or used in gluconeogenesis or lipogenesis. In carnivorous fishes, the natural diet is rich in protein and low in carbohydrate. Amino acids serve as the major source of energy in these fishes (Cowey et al, 1972). Cells utilize amino acids as energy source during starvation either directly by the oxidation of carbon skeleton or indirectly by conversion of carbon skeleton to glucose (Bever et al, 1981).

Irrespective of the pathways for utilization of the carbon skeleton, the first step in amino acid catabolism is the removal of α-amino group as ammonia. Ammonia produced from the amino acids becomes highly toxic to animals even at a very low concentration in vivo (Campbell, 1973; Cooper & Plum, 1987). It is either eliminated out immediately or detoxified by conversion to some less toxic products for temporary storage in vivo. The three major end products of nitrogen catabolism in animals are - ammonia, urea and uric acid. The predominance of a particular end product in the nitrogen excretion has evolved depending upon the availability of water to the animal groups. Animals are thus classified into three categories on the basis of major

nitrogenous waste product as ammoniotelic - predominently excreting ammonia mostly by diffusion to the aquatic environment, ureotelic - primarily excreting urea in urine by terrestrial and amphibious animals and uricotelic - excreting primarily insoluble uric acid by animals with little availability of water. However, each animal species is neither strictly ammoniotelic nor ureotelic nor uricotelic. They mostly have more than one type or mixed type of ammonia detoxification path ways to meet their physiological needs. Amphibians can live both in land as well as in water and they show both ammoniotelic and ureotelic conditions. They are generally ammoniotelic in water and ureotelic on land. The tadpole is ammoniotelic during early stages and ureotelic during later stages of development. Purines such as guanine is excreted predominantly by some groups of animals which are called purinotelic or guanotelic. It has geen suggested that nitrogen excretory pattern is one of the most sensitive physiological processes to respond effectively to environmental variations (Gordon, 1970).

Source of ammonia and ammonia toxicity <u>in vivo</u>

Protein ingested in the diet are hydrolysed in the digestive tract by proteolytic enzymes and absorbed into the body as amino acids. Protein aminoacids are the major source of ammonia in fish (Walton & Cowey, 1977; 1982). These amino acids are deaminated to keto acids with the

removal of α-amino group as ammonia (Watts & Watts, 1974) which is about 90% of the total nitrogen liberated by catabolism of biomolecules (Baldwin, 1970). Campbell et al (1983) showed that alanine, aspartate, aspargine, glutamate, glutamine and serine are the main amino acids for ammonia production in channel catfish with glutamate playing the major role. In addition to direct deamination of amino acids ammonia is produced by transdeamination of amino acids and deamination of nucleosides and nucleotides.

In transdeamination process the amino group from most amino acids are transfered to the keto acid, α-ketoglutarate forming the amino acid, glutamate by transamination reaction. Glutamate, so formed, then oxidatively deaminated in presence of the enzyme glutamate dehydrogenase (GDH) and coenzyme NAD(P) yielding ammonia, α-ketoglutarate and NAD(P)H. The activity of GDH has been reported in a variety of fish species and in most tissues with liver showing the highest level of activity (Forster & Goldstein, 1969; Watts & Watts, 1974). GDH has been considered as the N-storage enzyme playing important role in ammonia metabolism in fish (Forster & Goldstein, 1969; Walton & Cowey, 1977, 1982). Another source of ammonia in fish is deamination of different nucleosides and nucleotides (Cohen & Brown, 1960). Hydrolysis of particularly adenosine monophosphate (AMP) by AMP deaminase has been shown to play an important role in ammonia production in some fishes (Makarewicz & Zydowo, 1962; Makareweicz, 1963).

However, precise and specific mechanisms of ammonia formation are still not completely understood in all the fishes. Available reports suggest that the major pathways of ammoniogenesis in fish are by deamination and transdeamination of amino acids (Vanslyke et al, 1943; Janssens, 1964; Wilson, 1973a,b; Watts & Watts, 1974; Walton & Cowey, 1982; Campbell et al, 1983; Casey et al, 1983). Deamination of nucleotides has been suggested to be an additional pathway for metabolic ammonia production in fishes (Waarde, 1981).

Ammonia from external aquatic environment enters into the fish due to its easy diffusion in favour of concentration gradient causing toxicity in vivo. The level of ammonia in external environment is first increasing from domestic sewage, agricultural run off and industrial effluents besides the excretory ammonia. This increasing level of ammonia in ambient environment has been shown to cause serious damage to the aquatic fauna.

Ammonia exists in two forms i.e. ionic form (NH_4^+) and non-ionic form (NH_3) in aqueous medium. The relative proportion of these two forms of ammonia is dependent on the pH, temperature and ionic strength of the medium. Unionized form of ammonia (NH_3) is more toxic to fish than the ionized form (Wuhrmann & Woker, 1948; Downing & Merkens, 1955; Evans & Cameron, 1986). Although the unionized form of ammonia determines the toxicity level in water, Hillaby and Randall (1979) suggested that in blood either total

or even the ionized form could produce the toxic effect. Ammonium ion influences the normal physiological functions in the living organisms in various ways (Prosser, 1973). Acute toxicity studies with ammonia has been done on cutthroat trout, Salmo clarki (Thurston et al, 1978) and channel catfish Ictalurus punctatus (Knepp & Arkin, 1973; Colt & Tchobanoglous, 1976; Robinette, 1976). Extensive proliferation and consolidation of gill lamellae and generative tissue damage in kidney were observed in salmonids exposed to ammonia (Burrows, 1964; Reichenbach-Klinke, 1967; Olson & Fromm, 1971; Smart, 1976; Thurston et al, 1978). Sublethal exposure to ammonia have shown to reduce the oxygen carrying capacity of hemoglobin (Sousa & Meade, 1977), increased oxygen consumption, respiratory rate and rate of heart beat (Smart, 1978), increased urine output (Lloyd & Orr, 1969), and hyperexcitability (Wuhrman & Woker, 1948; McCay & Vars, 1950; Fromm & Gillette, 1968; Olson & Fromm, 1971) in fish. Sousa and Meade (1977) proposed that the mechanism of ammonia toxicity involves stimulation of glycolysis by the ammonium ion (NH_4^+) and the simultaneous suppression of the Krebs cycle due to the depletion of α-ketoglutarate which removes ammonia by amination to form glutamate and then glutamine. These two concurrent actions would result in an increase in acidic metabolites from glycolysis and early Krebs cycle which would lower blood pH. The resulting acidemia would shift to right the oxygen dissociation curve (Bohr effect) and reduce maximal oxygen saturation of hemoglobin causing hypoxia. Tomasso et al (1980)

suggested that toxic action of ammonia might also involve in osmoregulatory disturbances in channel catfish as it has been reported to increase the permeability of biomembranes to water (Dennis, 1966; Lloyed & Orr, 1969). Another site of action suggested was inhibition of ATP production due to the uncoupling of oxidative phosphorylation by ammonium ion (Smart, 1978). The observable symptoms noticed in fish are hyper-ventilation, hyper excitability followed by coma and ultimately death (Alabaster & Lloyd, 1980). All these toxic effects besides any unknown ones might be acting on the fish individually or simultaneously and the actual contribution of each to the total stress would depend on the concentration and form (ionized or unionized) of ammonia present (Tomasso et al, 1980).

Excretion and detoxification of ammonia

Ammonia can be easily eliminated from the body of aquatic animals by diffusion into the surrounding water due to its small molecular size, high solubility in water as free base and higher partition co-efficient (Forster & Goldstein, 1969; Maetz & Gracia, 1964; Evans & Cameron, 1986). Fishes are primarily ammoniotelic excreting ammonia as the major nitrogenous waste. Ammonia diffuse out of animal body through the excretory surfaces to the ambient aquatic medium without utilization of metabolic energy (Hochachka & Somero, 1973). Teleosts mostly excrete

ammonia by diffusion through the extra-renal sources such as gills or body surface immediately after its formation to avoid its accumulation in vivo (Smith, 1929; Forster & Goldstein, 1969; Campbell, 1973; Watts & Watts, 1974). More than 90% of ammonia is excreted by diffusion through the gills in freshwater teleosts (Smith, 1929; Fromm & Gillette, 1968; Vellas & Serfaty, 1974; Payan & Matty, 1975; Saha et al, 1988) and atleast 50% of the waste nitrogen through the gills in marine fish such as Leptocottus armatus (Sculpin), Platichthys stellatus (Starry flounder) and Taeniotoca lateralis (blue sea perch) (Wood, 1958).

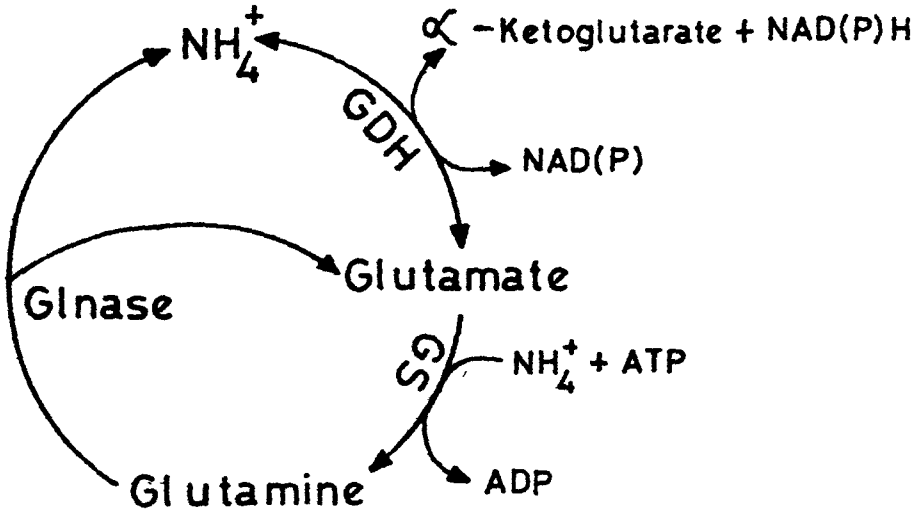
In terrestrial animals such as mammals and amphibians during their stay outside water, excretion of ammonia by diffusion is not possible due to non-availability of water. In these animals ammonia is mostly detoxified to urea via ornithine-urea cycle (Krebs & Henseleit, 1932; Cohen, 1976) as urea is lesser toxic than ammonia. Existence of a functional urea cycle has also been demonstrated in elasmobranchs (Baldwin, 1958, 1960; Campbell, 1961; Schooler, 1964; Watts & Watts, 1966) which synthesize and retain urea mainly for osmoregulation in marine environment (Smith, 1929). Aestivating lungfishes also convert excess of ammonia to urea during the period of aestivation for detoxification (Forster & Goldstein, 1966; Janssens , 1964; Brown, 1965; Janssens & Cohen, 1966). The urea cycle was thought to have been deleted in fresh water teleosts during their evolution (Brown & Cohen, 1960;

Huggins et al, 1969; Wilson, 1973b). However, this cycle was reported to be functional in some marine teleosts such as Opsanus tau (Read, 1971), O. betta (Mommsen & Walsh, 1989) and in a species of Tilapia, Oreochromis alcalicus grahami living in an alkaline lake (Randall et al, 1989). Reports from our laboratory have shown functional urea cycle in atleast four species of fresh water air-breathing teleosts (Saha, 1986; Saha & Ratha, 1987, 1989a).

Animals living in acute water restricted conditions such as birds and reptiles convert their metabolic ammonia to uric acid through glutamine path ways instead of being converted to urea. The amide nitrogen of glutamine contribute N-3 and N-9 of the purine ring and the insoluble uric acid is excreted as solid pellets. Production of ammonia takes place in all the tissues but its elimination or conversion to urea or uric acid takes place in some specific tissues.

Gills, skin and kidney tubules are the sites for ammonia elimination from body in fishes. Conversion of ammonia to urea or uric acid takes place in liver and kidney in ureotelic and uricotelic animals. Therefore, in all the animals ammonia formed in other tissues such as brain, muscle etc. is immediately transported through the blood to avoid its toxicity due to accumulation and to make it reach the site of its excretion or conversion. Transporting excess ammonia in blood is dangerous due to its toxicity. Therefore,

most of the tissues have the mechanism to convert ammonia to glutamate and glutamine for its immediate detoxification and safe transport. Ammonia is first trapped by α-ketoglutarate to form the amino acid glutamate by the enzyme glutamate dehydrogenase (GDH), which also catalyses the reverse reaction. Glutamate traps another molecule of ammonia to form a diamino aminoacid glutamine in presence of the enzyme glutamine synthetase (GS). The release of glutamate and ammonia from glutamine by the reverse reaction is catalysed by the enzyme glutaminase (Glnase). The possible interaction of GDH, GS and Glnase in the cellular regulation of ammonia can be shown as follows.



Duda and Handler (1958) suggested that rapid incorporation of exogenous ammonia into glutamine act as a general cellular mechanism for ammonia detoxification. Glutamine is a major amino acid in blood plasma in mammals which accounts for

about 20% of the total amino acid content (Meister, 1984). Many tissues such as brain, liver, intestine and muscle, have shown to have very high concentration of glutamine. Glutamine can be effectively transported across the blood-brain barrier and across cell membrane than glutamate (Haussinger & Sies, 1984). It has free access to mitochondria inspite of osmotic changes (Salganicoff & DeRobertis, 1965). The glutamate $\rightleftharpoons$ glutamine reaction is of particular importance in the central nervous system for removal of ammonia and maintenance of the level of glutamate, a neuro-transmitter (Hamberger et al, 1978, 1979a,b; Ward et al, 1983; Ottersen & Storm-Methisen, 1984; Hoop et al, 1988; Farrant & Roy, 1989; Paulsen & Fonnum, 1989). Glutamine help in energy production through deamination or transamination and the subsequent metabolism of the keto acid through TCA cycle. It is a major energy source in kidney (Pitts, 1975), small intestine (Windmueller & Spaeth, 1980), bone (Biltz et al, 1982) and in certain cells grown in vitro (Zielke et al, 1978; Reitzer et al, 1979). Glutamine is the amide form of glutamate and thus serve as a storage and transport form of both ammonia and glutamate.

Production of renal ammonia from glutamine has been attributed to the glutaminase reaction (Katunuma et al 1970). This reaction is also required for the release of ammonia from glutamine in liver and kidney for urea synthesis. Besides,

in some species glutamine also substitutes for ammonia as substrate with CO_2 for carbamyl phosphate synthesis through CPS II or III.

$$\text{L-glutamine} + 2\text{ATP} + HCO_3 + H_2O \xrightarrow{\text{CPS(II or III)}} \text{Carbamyl phosphate} + 2\text{ADP} + Pi + \text{L-glutamate}$$

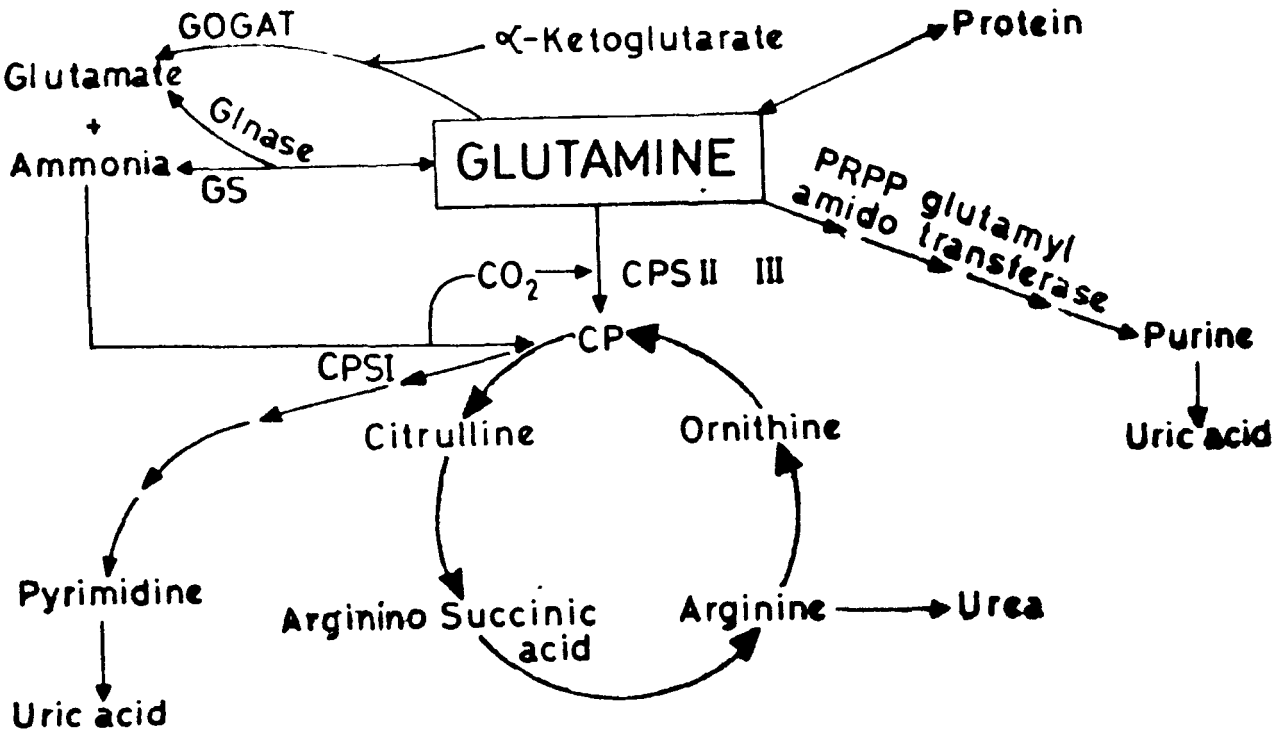
Thus, glutamine serves as the nitrogen donor in the synthesis of carbamyl phosphate for either the formation of arginine and urea or in pyrimidine biosynthesis. Glutamine is also used in the synthesis of purines. This pathway starts with the synthesis of 5-phosphorybosyl amine by the enzyme phosphorybosyl pyrophosphate (PRPP) glutamyl amido transferase.

$$\text{PRPP} + \text{Glutamine} \xrightarrow{\text{PRPP glutamyl amido transferase}} 5\text{ phosphorybosylamine} + \text{glutamate} + PP_i$$

In microorganisms and plants, glutamine has been shown to transfer its amide group to α-ketoglutarate and produce two glutamate molecules without releasing any free ammonia (Tempest et al, 1970; Meister, 1985). This reaction is catalyzed by the enzyme glutamate synthetase or glutamate oxoglutarate transamidase (GOGAT).

$$\text{Glutamine} + \alpha\text{-ketoglutarate} \xrightarrow[\text{GOGAT}]{\text{NADPH} + H^+ \quad \text{NADP}} 2\text{ Glutamate}$$

However the existence of this enzyme catalysed reaction is yet to be demonstrated in any animal.

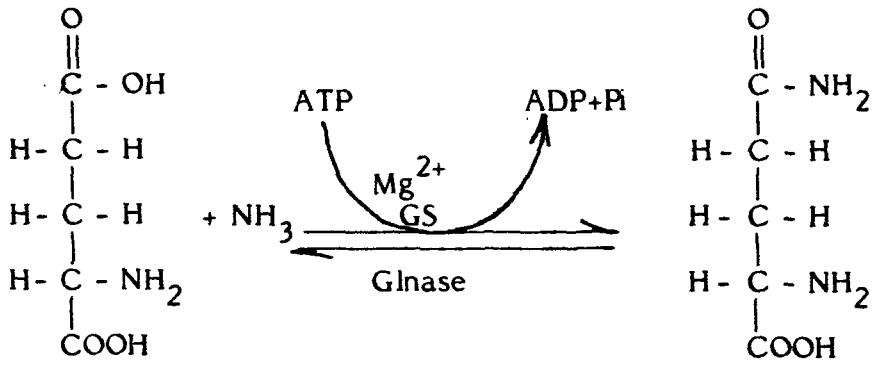


Major metabolic pathways of glutamine metabolism

It could be seen from the above that glutamine plays several important metabolic functions. However, the present study has been restricted to glutamine synthesis and hydrolysis.

Enzymes of glutamine synthesis and hydrolysis

Glutamine synthesis from ammonia and glutamate and the reverse reaction of its hydrolysis are catalysed by the two enzymes glutamine synthetase (GS) and glutaminase (Glnase) respectively.



Glutamine Synthesis and glutamine synthetase

Synthesis of glutamine from glutamate and ammonia by the enzyme GS in presence of ATP and Mg^{2+} is the only known route for glutamine in most of the living organism. Coulson and Hernandez (1983) reported in caiman and lizard (Chameleon) that glutamine is derived from variety of precursors such as arginine, ornithine, glutamate, pyruvate and γ-methyl γ-hydroxyl α-ketoglutarate.

Glutamine synthetase activity has been reported in various tissues from wide range of animal groups including fish, both marine (Webb & Brown, 1976, 1980; Anderson, 1980, 1981; Casey & Anderson, 1982, 1985; Smith et al, 1987) and fresh water fishes (Pequin et al, 1969; Wilson & Fowlkes, 1976; Webb & Brown, 1976; Walton & Cowey, 1977; Waarde & Kesbeke, 1982). Liver and kidney of fresh water fishes showed lower activity of GS compared to marine fishes. However, brain has been shown to have very high GS activity which

has been associated with the detoxification of ammonia (Wu, 1963a,b; Levi et al, 1974; Wilson & Fowlkes, 1976; Walton & Cowey, 1977; Waarde & Kesbeke, 1982) and maintenance of the level of the neurotransmitters-glutamate and γ-amino-butyrate (GABA) (Shank & Aprison, 1981). Glutamine synthetase in the liver and kidney of elasmobranchs provide glutamine for urea production via CPS-III for osmoregulation (Webb & Brown, 1976; Casey & Anderson, 1982). In addition to the detoxification of metabolically generated ammonia, glutamine synthetase has been shown to play significant role in the detoxification of exogenous ammonia in different animal groups (Krebs, 1935; Örström et al, 1939; Brown et al, 1957; Kamin & Handler, 1957; Duda & Handler, 1958; Wu, 1963a; Addei & Lotspeich, 1968; Wilson et al, 1966; Vorhaben & Campbell, 1972, 1977; Campbell et al, 1984).

The sub-cellular distribution of glutamine synthetase vary in different tissues within the same animal and also among different groups of animals. The hepatic and renal GS activity have been shown to be cytosolic in ureotelic mammals and amphibians (Wu, 1963b; Katunuma et al, 1970; Campbell et al, 1984) and in catfish, Ictalurus punctatus (Casey et al, 1983) and mitochondrial in ureo-osmotic marine elasmobranchs (Webb & Brown, 1976, 1980; Anderson, 1980; Casey & Anderson, 1982; Smith et al, 1987), uricotelic birds and reptiles (Vorhaben & Campbell, 1972; Smith & Campbell, 1983) and predominantly ammoniotelic american alligator

(Smith & Campbell, 1987). However, in a uricotelic tree frog it was reported to be cytoplasmic (Campbell et al, 1984). GS activity in brain has been generally cytosolic in all animals.

Isoenzymic forms of GS has been reported in plant tissues (Winter et al, 1982; McNally et al, 1983; Hirel et al, 1984; Alekhina & Klyuikova, 1988). However, in animals except in dogfish, Squalus acanthias, stringray, Dasyatis sabina and holocephalan, Hydrologus colliei (Shankar & Anderson, 1985; Smith et al, 1987) isoenzymic forms of GS has not yet been reported.

Glutamine synthetase has been extensively studied in bacteria (Alef et al, 1981; Prusiner & Stadtman, 1973; Streicher & Tyler, 1980), cyanobacteria (Sampaio et al, 1979; Stacye et al, 1979; Streicher & Tyler, 1980; Murray & Zinder, 1984), marine elasmobranchs (Shankar & Anderson, 1985; Ritter et al, 1987; Smith et al, 1987) birds (Seyama et al, 1972; Vorhaben et al, 1982; Maurizi et al, 1986; Smith & Campbell, 1987) and mammals (Pamiljans et al, 1962; Rao & Kanungo, 1972; Seyama et al, 1972; Denman & Wedler, 1984; Wedler & Denman, 1984; Meister, 1985; Rowe, 1985; Maurizi et al, 1986). The enzyme was first purified to homogeneity from sheep brain among vertebrates (Pamiljans et al, 1962). The enzyme has also been purified from human brain (Yamamoto et al, 1987), rat liver (Rowe, 1985), pig

brain (Meister, 1974; Tate & Meister, 1973), chicken liver (Seyama et al, 1972; Vorhaben & Campbell, 1972) and elasmobranch (Squalus acanthias) liver (Shankar & Anderson, 1985). The bacterial GS showed higher molecular weight and had more number of subunits. The GS from Bacillus brevis and Bacillus subtilis, Bacillus polymya had molecular weight of $6x10^5$ and 12 subunits each having molecular weight of 50,000 (Deuel et al, 1970; Tiwari et al, 1989; Ojha & Kantengwa, 1989). Krishnan et al (1986) reported the molecular weight of GS from Clostridium pasteurianum to be $1x10^6$ having 20 subunits of 50,000 molecular weight each. The animal GS have been reported to consist of generally eight apparently identical subunits each having molecular weight of 44,000-49,000. The molecular weight of GS was found to be $3.24x10^5$ in fleshfly flight muscle (Dowton & Kennedy, 1985), $3.64x10^5$ in chicken brain (Tholey et al, 1987), $3.6x10^5$ in rat liver (Rowe, 1985) and $4.3x10^5$ in sheep brain (Pamiljans et al, 1962). The enzyme purified from liver mitochondria of dog fish (Squalus acanthias), a representative of elasmobranch, had the molecular weight of $4x10^5$ (Shankar & Anderson, 1985).

MgATP has been shown to be essential for GS activity and to protect from denaturation of the activity of GS isolated from sheep brain (Pamiljans et al, 1962), Clostridium pasteurianum (Krishnan et al, 1986) and dogfish liver (Shankar & Anderson, 1985). The pH optima of GS isolated from different sources was reported to range from 7.0-7.4 (Seyama et al,

1972; Shankar & Anderson, 1985; Krishnan et al, 1986). The presence of divalent metal ions influenced the pH optima of the enzyme. In plant GS, pH optima was about 8 when Mg^{2+} was used in the reaction mixture (Bray, 1983). The apparent Km values of GS for MgATP, glutamate and ammonia were reported to be 0.7, 11.0, 0.015 mM respectively in dogfish (Shankar & Anderson, 1985); 0.9, 6 and 0.5 mM in chicken liver (Vorhaben et al, 1982) and 0.24, 12.8, 0.13 mM in fleshfly flight muscle (Dowton & Kennedy, 1985).

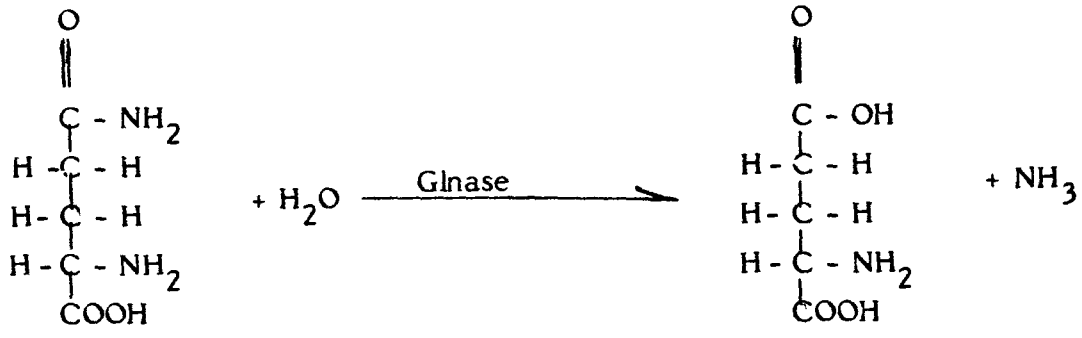
GS isolated from various sources bound to divalent metal ions for the catalytic activity. Mn^{2+} and Mg^{2+} have been most commonly used metal ions for in vitro catalysis. Mg^{2+} ion has been shown to be required for the maximal activity of GS from Squalus liver (Shankar & Anderson, 1985), chick brain (Tholey et al, 1987) and human brain (Yamamoto et al, 1987). Although Mg^{2+} and Mn^{2+} both supported the enzyme activity, Mg^{2+} rather than Mn^{2+} appeared to remain bound to the brain GS (Maurizi et al, 1986). Several metal ions such as Ca^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cr^{2+} and Li^{2+} could not replace Mg^{2+} for GS activity in either chick brain (Tholey et al, 1987) or C. pasteurianum (Krishnan et al, 1986). Mn^{2+} and Ca^{2+} inhibited the GS activity in sheep brain and in flesh fly flight muscle (Elliot, 1951; Tate et al, 1972; Dowton & Kenney, 1985) and PO_4^{--} inhibited in rat liver (Tate et al, 1972). Dogfish liver GS has been shown to be activated by Cl^-, Br^-, I^- (Shankar & Anderson, 1985). α-ketoglutarate

was also found to activate rat liver GS activity (Tate et al, 1972; Rowe, 1985).

Woolfolk and Stadtman (1967) reported inhibition of E. coli GS by nine end products of glutamine metabolism such as CTP, AMP, glucosamine-6-P, histidine, tryptophan, carbamyl phosphate, alanine, glycine and serine, ADP and GDP showed strong inhibition in rat muscle, human brain and pig heart but poor inhibition in sheep brain (Rowe, 1985; Yamamoto et al, 1987). UDP, CDP, TDP, AMP, GMP, cAMP and guanine did not inhibit significantly the mammalian GS (Rowe, 1985). The GS from rat muscle was inhibited by phosphate, pyrophosphate and carbamyl phosphate, and from rat liver by L-glycine, L-alanine, L-serine and carbamyl phosphate (Rowe, 1985). GS from C. pasteurianum was inhibited by L-serine, L-glycine, L-alanine and L-aspartic acid, (Krishnan et al, 1986). However, L-alanine, L-arginine, L-aspargine, L-aspartic acid, γ-amino butyric acid, L-glutamine, L-glycine, L-histidine, L-lysine, and L-serine had no significant effect on fleshfly flight muscle GS (Dowton & Kennedy, 1985).

Glutamine degradation and glutaminase

The formation of glutamine is also associated with its degradation by the enzyme glutaminase (glnase) with the formation of glutamate and ammonia. This reaction is independent of metabolic energy requirement and any co-factor or coenzyme . It also plays a key role in glutamine metabolism (Kovacevic & McGiven, 1983).



Glutamine **Glutamate**

The enzyme has been studied extensively in mammalian systems. However, the studies in fish, amphibians and avian systems are very limited. Krebs (1935) reported first the presence of Glnase activity in mammalian brain, liver, kidney and neural retina. Glnase activity could also be detected later in various tissues of amphibians, birds and mammals (Makarewicz & Zydowo, 1962). Mammals showed the highest glutaminase activity (Makarewica & Zydowo 1962) with birds showing Glnase activity of less than one tenth of that in mammals (Katunuma et al, 1970). Significant Glnase activity in fish have been reported in liver, kidney and gill of Walley, Stizostidion vitreum (Stieber & Covancara, 1977) and gold fish (Waarde & Kesbeke, 1982), skeletal muscle of cod (Siebert et al, 1965) and in liver, muscle and gill of mud skipper (Chew & Ip, 1987).

Glnase activity has been shown to be mitochondrial (Vorhaben & Campbell, 1977 ; Chew & Ip, 1987). Some reports indicate its localization in mitochondrial matrix in mammals (Errera & Greenstein, 1949; Kalra & Brosnan, 1973, 1974) where as others suggested the enzyme to be associated with the inner mitochondrial membrane (Curthoys & Weiss, 1974; Kovacevic, 1976). Errera and Greenstein (1949) reported the association of Glnase activity with the outer surface as well as inner surface of inner mitochandrial membrane.

Glnase has been reported to play a significant role in glutamine deamination and ammonia production in the Kidney (Davies & Yudkin, 1952; Curthoys & Lowry, 1973; Goldstein, 1967,1976), in releasing ammonia from glutamine for ureogenesis in liver (Welbourne & Joshi, 1986) and to maintain a high level of glutamic acid in brain (Weil-Melherbe, 1969). Several modulators have been shown to regulate Glnase activity. It required the presence of a polyvalent anion for activity (Curthoys et al, 1976a,b). Phosphate has been most commonly used activator along with phosphate containing compounds such as ATP, CTP, GTP, ITP, and phospho-enol-pyruvate which at equimolar concentration were as effective as phosphate. (Sayre & Roberts, 1958; Svenneby, 1972; Curthoys et al, 1976b; Pinkus & Windmueller, 1977). Acetyl CoA, sulphate and leucine have been shown to be the activators of glutaminase activity (Kvamme & Torgner, 1974a,b, 1975; Ardawi & Newsholme,1984).

The glutaminase-glutamine synthetase reverse reaction has been postulated to be a major mechanism for the regulation of ammonia level and ammonia excretion rate in fish (Waarde & Kesbeke, 1982). Ammonia arising from amino acid catabolism in various tissues are fixed in the form of glutamine and transported to fish liver, gill and kidney for further metabolism. In rat kidney, glutaminase and glutamine synthetase operate as a reversible system. Under normal conditions the reverse reactions operate with approximately equal rate. During alkalosis, there is net amide synthesis while during acidosis large amount of ammonia are produced and excreted through urine (Dannian & Pitts, 1970).

Ammonia detoxification via glutamine formation is much more advantageous in most animal tissues than the five step energy dependent urea cycle. It involves only a single step for conversion of ammonia to glutamine. Urea synthesis via urea cycle is also tissue specific. It, therefore, requires the transportation of ammonia from different tissues to those specific tissues where urea cycle is functional for its conversion to urea.

The urea retaining marine elasmobranchs (Sharks, skates and rays) possess a complete o-u cycle for the production of urea for osmoregulation and ammonia detoxification. These fishes have shown very high activity of glutamine synthetase

compared to fresh water teleosts. Webb and Brown (1980) suggested that glutamine synthetase activity and production of urea are directly correlated in these urea retaining species. Ammonia assimilation for citrulline synthesis (and therefore urea synthesis) in marine elasmobranchs involves intermediate formation of glutamine (Anderson & Casey, 1984) with the presence of high levels of both GS and a unique glutamine and N-acetyl-L-glutamate dependent CPS III (Anderson, 1980, 1981; Casey & Anderson, 1983). However, most of the fresh water teleosts studied so far by various workers did not show a complete ornithine-urea cycle (Brown & Cohen, 1960) except for some fresh water teleosts which are primarily aquatic but breathe predominantly air through their secondary respiratory organs (Saha & Ratha, 1987,1989a). They tolerate varying periods of dehydration (60-70 hrs), high concentrations of ambient ammonium chloride (75mM) and high osmolar ambient medium (300 mosm manitol) (Saha, 1986; Saha & Ratha, 1989a,b). The urea cycle was found to be induced both in liver and kidney of H. fossilis under these extreme environmental conditions resulting in enhanced synthesis, retention and excretion of urea (Saha, 1986; Saha & Ratha 1986, 1989b, 1990). Thus, H. fossilis represents an intermediate group (fresh water air-brething teleost) between purely fresh water ammoniotelic teleosts and ureosmotic marine forms and ureotelic amphibians. It has been further suggested that air-breathing fresh water teleosts might possess some

other adaptations in their nitrogen metabolism processes.

Glutamine metabolism which has been reported as an important nitrogen metabolic pathway for production of nucleotides, urea, citrulline and maintaining glutamate-glutamine balance particularly in neural tissue was studied with relation to ammonia management in H. fossilis as a model animal for freshwater air-breathing teleosts.

The plan of work proposed was as follows:

1. Normal distribution of glutamine synthetase (GS) and glutaminase (Glnase) activity was studied in various tissues such as brain, liver kidney, muscle and gill.

2. Sub-cellular distribution of GS and Glnase activity was studied in brain, liver and kidney tissues.

3. Diurnal variation of GS and Glnase activity was studied in brain liver and kidney tissues.

4. The alterations in the levels of ammonia and glutamate and the activity of GS and Glnase in different tissues were studied during exposure to hyper-ambient ammonia and various periods of dehydration.

5. Purification and studies on the molecular characters kinetics and regulation of purified GS activity from brain tissue were undertaken.

MATERIALS AND METHODS

Animal :

Freshwater air-breathing teleost, Heteropneustes fossilis (Bloch) were purchased from commercial sources. The fishes were maintained in plastic aquaria containing bacteria free filtered tap water at 20±2°C temperature with 12 hr: 12 hr light : dark period. Minced pork liver (100g/Kg body wt.) and rice bran (50g/Kg body wt.) were provided as food on alternate days. Water was changed regularly on the day after feeding. The fishes were used after their acclimatization to the laboratory conditions for atleast four weeks when their death rate became almost zero and food consumption was normal. They were used for experiments 24 hrs after their last feeding and no food was given during the experimental period. The experiments were conducted under the same environmental conditions as they were acclimatized unless otherwise mentioned.

Chemicals:

All the enzymes, coenzymes and substrates were obtained from Sigma Chemical Co., St. Louis, U.S.A.The chromatography media and molecular weight determination kit (Proteins) were from Pharmacia Fine Chemicals, Sweden. Other chemicals used were of analytical grade and obtained indegenously.

Deionised and double glass distilled water was used in all preparations.

Experimental set up:

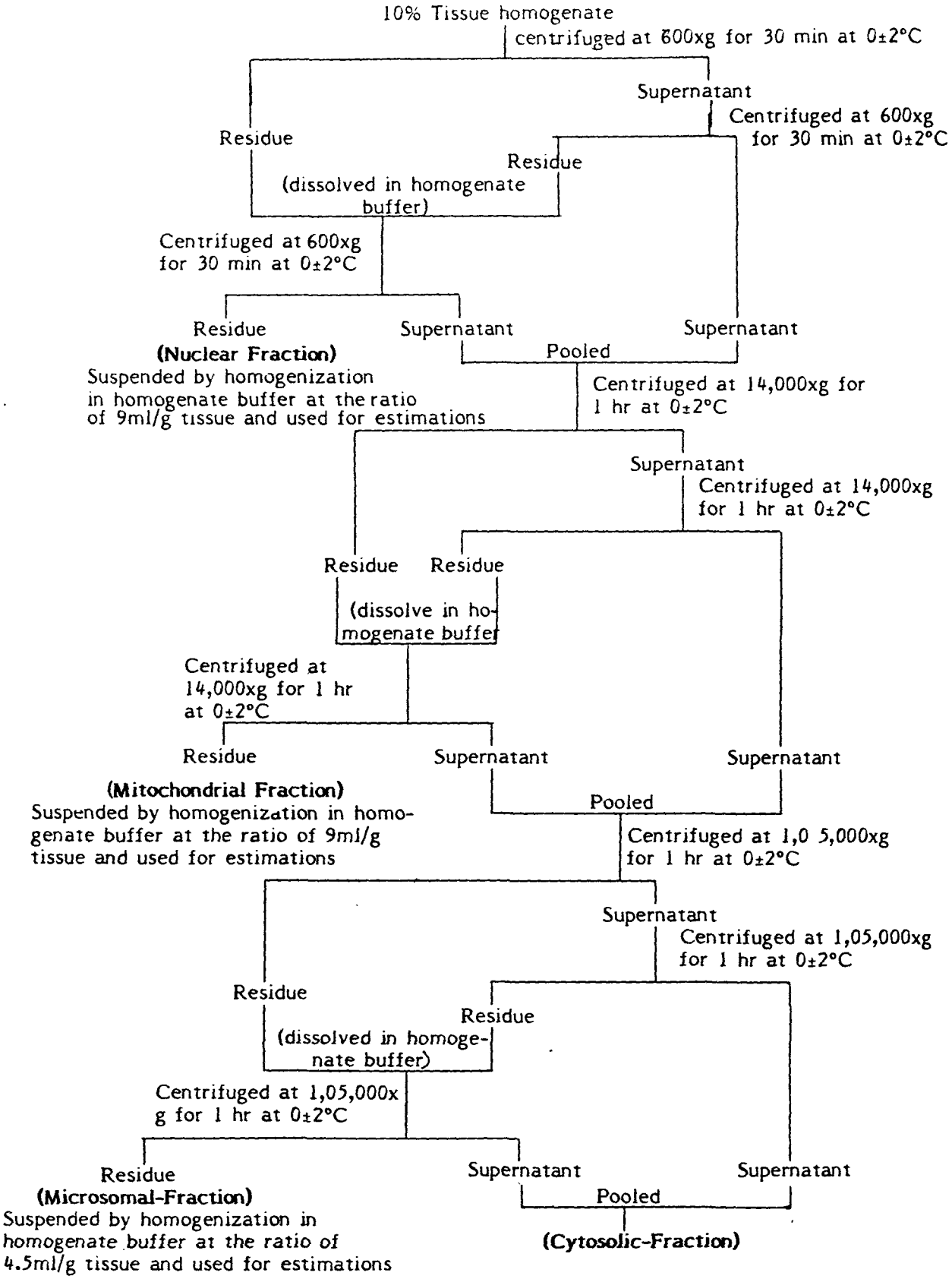
Only female H. fossilis weighing 20-25g were used in the experiments.

(i) **Tissue distribution:** The distribution pattern of glutamine synthetase (GS) and glutaminase (Glnase) activity was studied in various tissues of H. fossilis. Five fishes were killed at 10 A.M. and the activity of both the enzymes were estimated in brain, liver, kidney, muscle and gills. Due to the absence or low activity of both the enzymes in gill and muscle, further studies were carried out only in brain, liver and kidney of H. fossilis.

(ii) **Sub-cellular distribution:** Sub-cellular distribution of GS and Glnase were studied in brain, liver and kidney tissues of H. fossilis. The sub-cellular fractions were separated by differential centrifugation of the tissue homogenates. The schematic representation for the separation procedure is given in the following chart. The fractions were identified using marker enzymes such as glutamate dehydrogenase(GDH), lactate dehydrogenase(LDH) and glucose-6-phosphatase (G-6-Pase) for mitochondrial, cytosolic and microsomal fractions respectively.

(iii) **Diurnal variation:** Fishes were kept in groups of five

The schematic representation of sub-cellular fractionation



each in plastic buckets containing two litres of filtered tap water. Five fishes were sacrificed by decapitation at 3 hr interval starting at 6 A.M. for 24 hrs. Glutamine synthetase and glutaminase activity (total and specific) were determined in their brain, liver and kidney tissues to find out the diurnal variation.

(iv) **Effect of NH_4Cl treatment:** Fishes were exposed in groups of five each in plastic buckets containing two litres of different concentrations of NH_4Cl (50, 75 and 100mM). Ammonium chloride solutions were prepared in bacteria free filtered tap water containing streptopenicillin (20mg/l) to stop microbial growth in the medium. The pH of the ammonium chloride solution did not alter significantly from that of the filtered tap water. The treatment was continued for 14 days in 50mM and 75mM NH_4Cl respectively. In case of 100mM concentration, the experiment was limited to 4 days due to complete mortality of the fish after 4 days. Groups of five fishes each as parallel controls were maintained in plastic buckets containing two litres of bacteria free filtered tap water with streptopenicillin (20mg/l). The media were replaced at 24 hrs interval in both experimental and control buckets. A group of five fishes from each concentration and control were sacrificed after 4,7,10 and 14 days of treatment. Brain, liver and kidney from each fish were removed, processed and activities of GS and Glnase (both total and specific) and concentrations of ammonia and glutamate were estimated.

(v) **Effect of dehydration:** Fishes were kept separately in groups of five each in glass jars without water under the laboratory condition. Each jar was covered with bilayers of cheese cloth. The humidity around the fish was 70-80% as determined by a hygrometer. The fishes were sacrificed after 3,6,9,12,15,18,21,24,27,30 and 36 hrs of water deprivation and the activity of GS and Glnase (total and specific) and concentration of glutamate were estimated in brain, liver and kidney tissues.

Tissue Processing:

The fishes were killed by decapitation. Tissues such as liver, kidney, brain, muscle and gill were quickly removed, blotted dry and frozen immediately at -20°C until used for estimations. All the estimations were completed within three days of sampling during which enzyme activities did not alter.

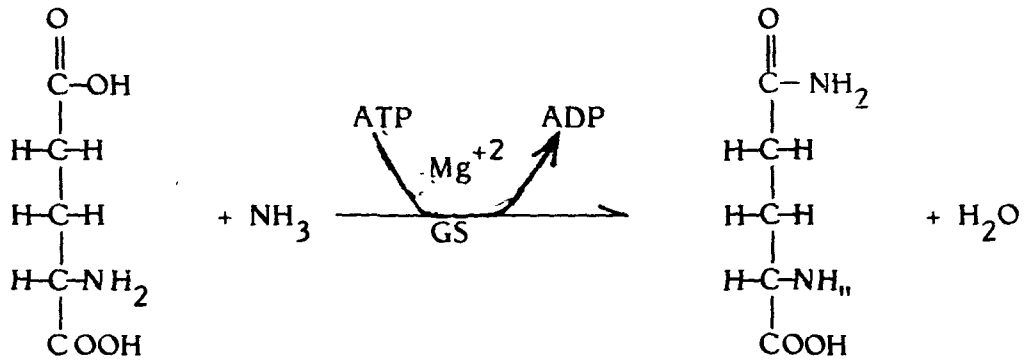
Each frozen tissue was thawed on ice and 10% homogenate (w/v) was prepared following Haser et al (1985) in buffered sucrose solution (0.33 M Sucrose/20mM Tris-HCl/1mM EDTA) pH 7.4 for the experimental tissues (except for subcellular distribution) with Potter-Elvehjem type motor driven glass homogenizer with a teflon pestle. The tissue homogenates and different sub-cellular fractions were prepared in 0.25 M Sucrose containing 5mM HEPES and 1mM EDTA (pH 7.4) for sub-cellular localization studies following Smith and Campbell (1983). Tris buffer was replaced due to its inactivation of GDH activity.

The homogenates were centrifuged at 600xg at 0±2°C for removal of nuclei and cell debris and the supernatant was used for enzyme assay. Half of the supernatant was sonicated using the microprobe of MSE ultrasonic disintegrator operated at an amplitude of 6 microns for two seconds at 0-4°C before glutaminase assay and the other half was treated separately with Triton X-100 (0.5% v/v final concentration) for 20 min before GS assay. These treatments were standardised and found to be optimum.

Estimations:

(i) Glutamine synthetase (GS) (E.C.6.3.1.2):

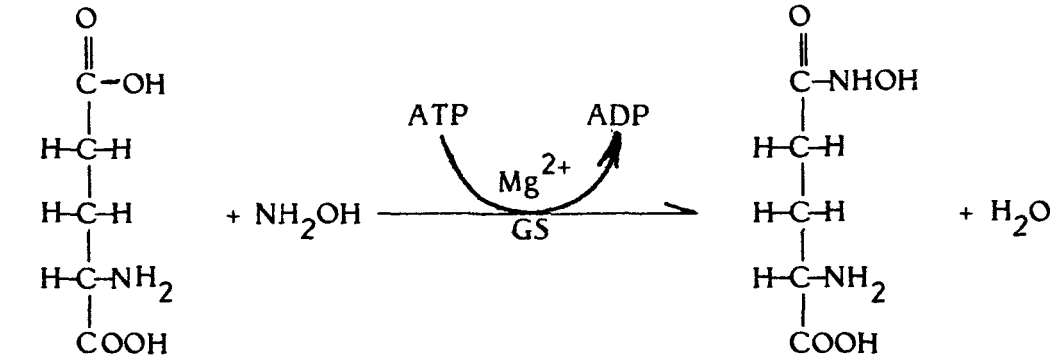
Glutamine synthetase(GS) catalyses the following reaction:



Glutamate Ammonia **Glutamine** **Water**

The enzyme activity was assayed following Wilson and Fowlkes (1976) where ammonia was replaced by hydroxylamine

and amount of γ-glutamyl hydroxamate formed was estimated colorimetrically.



Glutamate **Hydroxylamine** **γ-glutamyl hydroxamate** **Water**

The assay mixture in a final volume of 1 ml contained the following:

Imadazole - HCl-buffer pH 7.5	50 μ mole
$MgCl_2$	20 μ mole
2-Mercaptoethanol	25 μ mole
Hydroxylamine hydrochloride	25 μ mole
ATP (di-sodium salt)	10 μ mole
L-Glutamate (sodium salt)	50 μ mole
Phosphoenol pyruvate (tri-sodium hydrate)	10 μ mole
Creatine phosphokinase (Sigma Type-I from rabbit muscle)	0.4 Unit
Tissue extract as enzyme source	0.1-0.2 ml

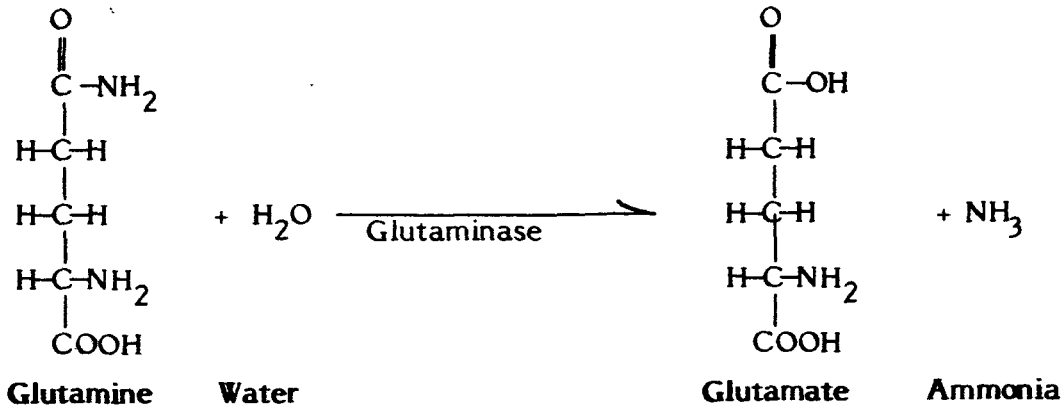
The incubation was carried out at 30°C in glass centrifuge tube for 15 min and the reaction was stopped by adding 0.5 ml of 20% TCA. In blank, TCA was added to

the reaction mixture prior to the addition of enzyme. The precipitated protein was separated by centrifugation at 5000 r.p.m. for 10 min. The amount of γ-glutamyl hydroxamate (GHA) formed in the reaction was determined in the supernatant by adding 1.5 ml of ferric chloride reagent (containing 0.37 M $FeCl_3$ and 0.67 N HCl) and measuring the absorbance at 600 nm λ in a Beckman model-26 spectrophotometer within 30 min. The amount of γ-glutamyl hydroxamate produced was calculated from a linear standard graph prepared using different concentrations (0.1-1.0 μ mole) of γ-glutamyl hydroxamate).

One unit of GS activity was defined as that amount which catalysed the formation of 1 μ mole of GHA/hr at 30°C. The enzyme activity was expressed as total activity (units/g wet wt. of tissue) and the specific activity (units/mg protein).

(ii) Glutaminase (Glnase) (E.C. 3.5.1.2):

Glutaminase catalyses the following reaction:

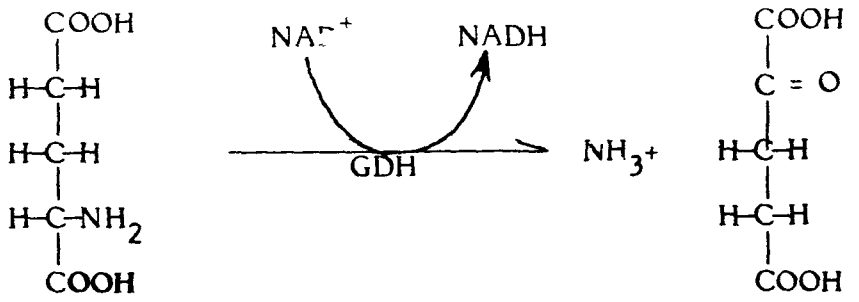


Glutaminase was assayed following the method of Curthoys and Lowry (1973). The amount of glutamate produced was measured quantitatively by oxidising glutamate by glutamate dehydrogenase in presence of NAD to yield equivalent amount of NADH and α-Ketoglutarate at pH 8.6. The amount of NADH formed was calculated from the increase in the O.D. at 340 nm λ in a Backman model-26 UV-visible spectrophotometer.

The assay mixture in a final volume of 1 ml contained the following:-

Tris-HCl buffer pH 8.6	10 μ mole
L-Glutamine	30 μ mole
Phosphate buffer (sodium salt)pH 8.6	40 μ mole
Tissue extract as enzyme source	0.2 ml

The reaction was started with the addition of tissue extract. After 15 min of incubation at 30°C the reaction was stopped by addition of 0.2 ml of 10%(v/v) PCA. A tissue blank was prepared for each assay with addition of PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The glutamate formed was estimated in the supernatant using its oxidative deamination by GDH and NAD^+.



Glutamate **Ammonia α-ketoglutarate**

The reaction mixture in a final volume of 2.5 ml contained the following:

Supernatant	0.05 ml
Tris-HCl buffer (pH 8.6)	50 µ mole
ADP (di-sodium salt)	0.3 µ mole
H_2O_2	0.004 %
NAD^+(free acid)	1.0 µ mole
GDH (Sigma Type-II)	5 units

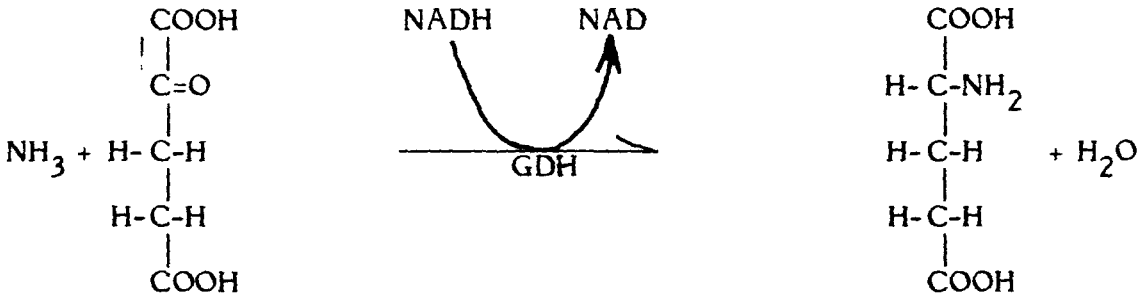
The reaction mixture was mixed well and allowed to stand for 60 min at room temperature for complete conversion of glutamate. The reading for the NADH formed was taken at 340 nm λ in a uv-visible spectrophotometer (Beckman model-26) against zero time blank (without GDH). The concentration of glutamate was determined from the calibration curve prepared with oxidation of different concentrations of glutamate (0.1-1 µ mole of glutamate) following the above method which was linear.

One unit of glutaminase activity was defined as that amount which catalysed the formation of 1 µ mole of

glutamate/hr at 30°C. The enzyme activity was expressed as total activity (units/hr/g wet wt. of tissue) and the specific activity (units/mg protein).

(iii) Glutamate dehydrogense (GDH) (E.C. 1.4.1.3):

GDH activity was assayed by NADH oxidation following the method of Olson and Anfinsen (1952).



Ammonia α-Ketoglutarate **Glutamate Water**

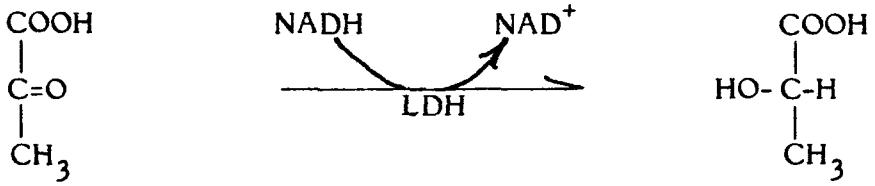
The assay mixture in a final volume of 3 ml contained the following:

Potassium phosphate buffer pH 7.6	117.56	µmole
NH_4Cl	450	µmole
α-Ketoglutarate (sodium salt)	7	µmole
NADH	0.3	µmole
Tissue extract as enzyme source	0.02	ml

The decrease in O.D. at 340 nm λ was recorded at 30 sec interval. The calculation of enzyme activity was done as per the method described below under LDH assay.

(iv) Lactate dehydrogenase (LDH) (E.C. 1.1.2.7):

LDH activity was assayed by NADH oxidation following the method of Vorhaben and Campbell (1972).



Pyruvate **Lactate**

The assay mixture in a final volume of 3 ml contained the following:

Potassium phosphate buffer pH 7.4	150 µmole
Pyruvate (sodium salt)	2 µmole
NADH	0.4 µmole
Tissue extract as enzyme source	0.1 ml

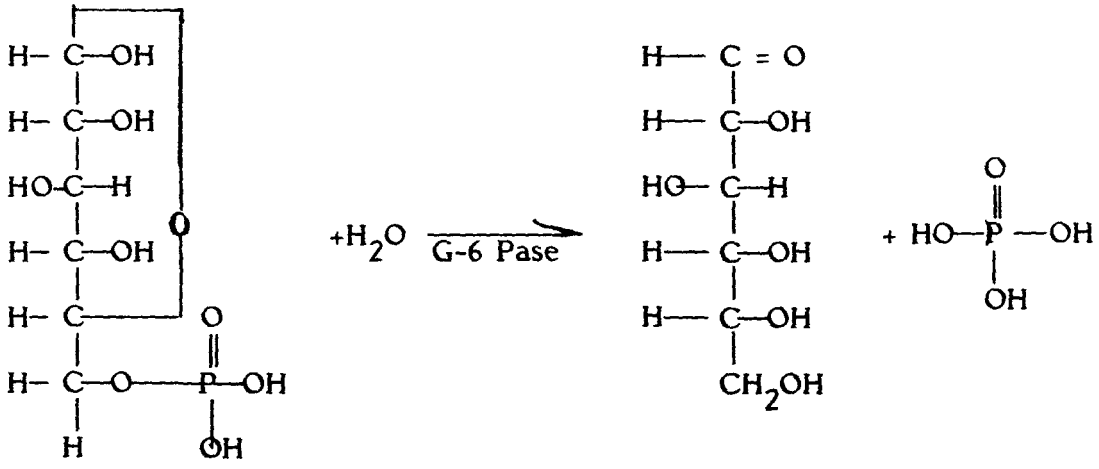
The reaction for both GDH and LDH activity was started with the addition of NADH in a rectangular quartz cuvette having 1 cm light path at 30°C using a variable temperature control accessory in a spectrophotometer (Beckman model 26). Decrease in O.D. at 340 nm λ was recorded at every 30 sec for GDH and 15 sec for LDH. The reaction was allowed to continue till the decrease in O.D. was linear (atleast for 5 min). The amount of NADH oxidised per hour was calculated taking the molar extinction coefficient for NADH/hr at 30°C as 6.37×10^{-3}.

One unit of GDH/LDH activity was defined as that

amount which oxidised 1 μ mole of NADH/hr at 30°C. The enzyme activity was expressed as total activity (units/g wet wt. of tissue).

(v) Glucose-6 phosphatase (G-6 Pase) (E.C. 3.1.3.9):

Glucose-6-phosphatase was assayed by estimating the free phosphate released following the method of Nordlie and Arion (1966).



Glutose-6-P **Water** **Glucose** **Phosphate**

The assay mixture in a final volume of 0.75 ml contained the following:

Na-acetate buffer pH 6.5	100 μmole
Glucose-6-phosphate (di-sodium salt)	30 μmole
Tissue extract as enzyme source	0.25 ml

The assay mixture was preincubated for 5 min at 30°C before addition of the tissue extract to initiate the reaction. The reaction was terminated after 30 min

by addition of 0.5 ml of 10% trichloro acetic acid (TCA) to the reaction mixture. The tissue blank was prepared for each assay with the addition of TCA prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The phosphate formed was estimated in the supernatant following the method of Fiske & Subba Row (1957). To 0.5 ml of the supernatant was added 0.5 ml H_2O, 0.5 ml H_2SO_4(5N), 0.5 ml ammonium molybdate (2.5% w/v) and 0.25 ml freshly prepared Fiske-Subba Row reducer (2.5% w/v solution in water of the mixture of 6 part sodium sulphate: 6 part sodium metabisulfite : 1 part 1-amino, 2-napthol, 4-sulphonic acid). The reaction mixture was incubated for 10 min and the reading was taken at 700 nm λ at room temperature against zero time tissue blank where TCA was added prior to the addition of enzyme. The amount of phosphate present was calculated from a standard graph prepared using different concentrations of a monophosphate (0.1-0.5 µ mole of NaH_2PO_4) which was linear.

One unit of glucose-6-phosphatase activity was defined as that amount which catalysed the release of 1 µ mole of phosphate/hr at 30°C from glucose-6-phosphate. The enzyme activity was expressed as total activity (units/g wet wt. of tissue).

(vi) Glutamate:

The tissue homogenates (10%) were treated with 10% PCA and the precipitated protein separated out by centrifugation

The supernatant were used for glutamate estimation following the method as described under glutaminase assay using the oxidative deamination reaction. Glutamate content was expressed as μ mole glutamate/g wet wt. of tissue.

(vii) Ammonia:

Ammonia was estimated following the method of Kawakubo et al (1983) with modification by Saha (1986).

A suitable aliquot of deproteinized tissue extract was taken and the volume was made upto 0.6 ml with distilled water. To it 0.6 ml of 0.1N sodium hydroxide (NaOH) followed by 0.2 ml of ethanol (to facilitate the mixing) were added. The solution was diluted with 1.0 ml water and 0.2 ml of 0.5% sodium hypochlorite (to form monochloramine). Then 1 ml of 0.2 N sodium hydroxide and 0.4 ml of 1-napthol (5% w/v in acetone) were added in the above order. The solution was mixed thoroughly and O.D. taken immediately between 5-15 min in a spectrophotometer at 740 nm λ against reagent blank. The concentration of ammonia was calculated from a linear standard graph prepared with different concentrations of NH_4Cl (0.1-1.0 μM) following the same method.

(viii) Protein:

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

Purification of brain glutamine synthetase and its physico-chemical properties

Glutamine synthetase was purified from the brain of H. fossilis acclimatized to laboratory conditions. All the steps of purification were carried out between 0-4°C unless and otherwise mentioned. The GS assays with the purified enzyme were done as described earlier but without the ATP regenerating system (Phospho-enol-pyruvate and Creatine phosphokinase) as that did not alter the enzyme activity.

(i) Purification

Step I - Preparation of crude extract: The fishes were killed by decapitation and the brain was immediately removed, cleaned and blotted dry. 5g of brain tissue was homogenised (20% w/v) in 20mM Tris-HCl buffer pH 7.4 using a Potter-Elvehjem type glass homogenizer fitted with a mortor driven teflon pestle. The homogenate was treated with 1% Triton X-100 in the ratio of 1:1 (final concentration of 0.5% Triton X-100) for 30 min and then centrifuged at 10,000xg in a refrigerated centrifuge for 30 min. The supernatant was used as the crude extract for further purification steps.

Step II - Ammonium sulfate fractionation: Solid ammonium sulfate was added without pH adjustment to the crude extract gradually with stirring to a saturation of 10% and kept for

30 min. The precipitate was removed by centrifugation at 14,000xg for 30 min. The supernatant was collected and adjusted to 30% saturation by further addition of ammonium sulfate with stirring for 30 min. The precipitate was then collected by centrifugation at 14,000xg for 30 min and dissolved in 15ml of 20mM Tris-HCl buffer (pH 7.4) at the ratio of 3ml/g tissue.

Step III - Dialysis: Above solution was dialysed for 12-16 hrs with three changes of the same buffer to completely remove the ammonium ions.

Step IV - Ion exchange chromatography: DEAE Sephacel column (20x1.6cm) was equilibrated with 20mM Tris-HCl buffer (pH 7.4). The ammonia free dialysed fraction was loaded on the column and the column was washed with the homogenizing buffer till no protein appeared in the eluent. 5 ml fractions were collected using a fraction collector and protein peaks recorded using a Pharmacia chromatography system. The flow rate was maintained at 15 ml/Hr using a peristaltic pump. The elution of enzyme was carried out with a linear gradient of NaCl prepared by taking 200ml of 20mM Tris-HCl (pH 7.4) and 200ml of 0.6 N NaCl prepared in the same buffer in the two chambers of a Pharmacia gradient mixer. The protein peaks obtained were assayed for GS activity. The enzyme activity was eluted as a single major peak with NaCl. Three fractions (15ml) having very high enzyme activity were

pooled and used for further purification.

Step V - Gel filtration with Sephadex G-200: Sephadex G-200 was preswollen at room temperature for 72 hr, equilibrated with 20mM Tris-HCl buffer (pH 7.4) before being loaded in a column (90x1.6cm) and equilibrated with the same buffer containing 0.25 N NaCl. The NaCl was added to the gel to protect the enzyme from denaturation. The pooled enzyme fraction was loaded and the elution was carried out with 20mM Tris-HCl buffer (pH 7.4) containing 0.25 N NaCl in 5 ml fractions. The flow rate was maintained at 5ml/hr using a peristaltic pump. The fractions having the protein peaks were assayed for GS activity. The enzyme was eluted as a single sharp peak and 2-3 fractions containing the highest specific activity were pooled and used for studying the molecular characters, kinetics and regulation of enzyme activity.

(ii) Purity study of GS by Polyacrylamide gel electrophoresis:

Electrophoretic separation of the enzyme fractions from each purification step was carried out simultaneously on 7.5% polyacrylamide gel in Tris-glycine buffer (5mM Tris and 38mM glycine) at pH 8.3.100-200 µl of sample containing 20-30 units of GS activity in 50% sucrose was loaded on each gel after 30 min of pre-run. Bromophenol blue was used as tracking dye. Duplicate gels were run for each sample and electrophoresis was carried out at 0-4°C using

a constant current of 3mA/tube for 2-3 hr at 2-4°C. The electrophoresis was stopped when the dye was about to migrate out of the gels. The gels were quickly removed from their tubes by ringing them with narrow water jets. One set of gels was stained for glutamine synthetase activity following the method of Vorhaben and Campbell (1977). The gels were incubated at 30°C in the reaction mixture as used for glutamine synthetase assay. After incubation for 1 hr the gels were washed with distilled water and treated with a colour reagent made up of 20% TCA and ferric chloride reagent (0.37 N $FeCl_3$ and 0.67 N HCl) in the ratio 1:3. A sharp brown band appeared at the site of GS due to reaction of γ-glutamylhydroxamate (formed due to enzyme activity) with ferric chloride in acidic medium. The band started diffusing after 15 min. Therefore, the photograph was taken within 15 min. The set of duplicate gels was fixed in 20% TCA for 1 hr and stained for protein with 0.25% Coomassie brilliant blue R-250 in 20% TCA for 4 hrs. The gels were then destained by diffusion in 7% glacial acetic acid to get sharp blue protein bands removing the back ground colour. Photographs were taken for the gels stained for protein and compared with those for GS activity to determine the purity.

(iii) **Molecular weight determination:**

The molecular weight of purified glutamine synthetase was determined by gel filtration chromatography on a Sephadex

G-200 column (90x1.6cm) equilibrated with 20mM Tris-HCl buffer (ph 7.4) containing 0.25 N NaCl. A constant flow rate was maintained at 5 ml/hr using a peristaltic pump. The column was calibrated with reference proteins (5 mg in 1 ml) such as thryroglobulin (M.W. 6,69,000) ferritin (M.W. 4,40,000), catalase (M.W. 2,10,000), aldolase (M.W. 1,58,000) and albumin (M.W. 67,000). 5mg of enzyme protein also was eluted in the same manner. The void volume (V_o) of the column was determined with blue dextran. The elution volume of each of the reference and the purified enzyme was determined. The Kav value for each protein was calculated using the following equation.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$ where Ve = Elution volume for the protein

Vo = Column void volume = elution volume for Blue Dextron2000

Vt = Total bed volume.

The molecular weight of glutamine synthetase was determined by superimposing its Kav value on a semi-log calibration curve prepared by plotting the Kav values against the log values of the molecular weights of the reference proteins.

(iv) Stability of purified enzyme on storage:

GS activity was assayed after different time intervals while preserving it in 20mM Tris-HCl (pH 7.4), 20mM Tris-HCl (pH 7.4) containing 10% glycerol, 20mM Tris-HCl (pH 7.4) containing 0.25 N NaCl and 20mM Tris-HCl (pH 7.4) containing 0.25 N NaCl and 10% glycerol to determine the stability of the enzyme activity in these storage media.

(v) **Determination of optimum pH for enzyme activity:**

GS activity was assayed at pH - 4, 5, 6, 7, 7.2, 7.5, 8, 8.5, 9, 10, 11 and 12 separately using imidazole buffer to determine the optimum pH for enzyme activity.

(vi) **Effect of temperature:**

(a) Optimum temperature for enzyme activity: The enzyme (GS) activity was assayed by incubating the reaction mixture at 5, 10, 20, 30, 40, 45, 50, 60 and 70°C separately for 30 min to find out the optimum incubation temperature for enzyme activity.

(b) Thermal stability: The thermal stability of GS in Tris-HCl buffer was studied by preincubating the purified enzyme in 20mM Tris-HCl-buffer (pH 7.4) at 0, 10, 20, 30, 40, 45 and 60°C separately for 30 min and then assaying the enzyme activity with usual assay procedure.

(c) Half life at 45°C: The purified GS was incubated in 20mM Tris-HCl buffer (pH 7.4) at 45°C for 0, 5, 10, 15, 20, 25, 30, 35, 40 and 50 min and then the enzyme activity was assayed following usual assay procedure. The time taken to loose half of the zero time enzyme activity was taken as half life of GS at 45°C.

(d) Factors protecting the thermal denaturation at 45°C: GS activity was assayed after pre-incubating the enzyme in 20mM Tris-HCl buffer (7.4) at different temperature

with the following components for 30 min.

1. Enzymne in buffer (20mM Tris-HCl pH 7.4)
2. Enzyme + 50 µM glutamate in buffer
3. Enzyme + 40 µM $MgCl_2$ in buffer
4. Enzyme + 0·5 µM ATP in buffer
5. Enzyme + 25 µM 2-mercaptoethanol in buffer
6. Enzyme + 25 µM hydroxylamine in buffer
7. Enzyme + 4·0 µM $MgCl_2$ +0·5 µM ATP in buffer.

The enzyme activity was assayed after different time of incubation in each of the above seven combinations to find out the factor(s) which can protect GS from thermal denaturation.

(vii) **Factors protecting the enzyme from 2-mercaptoethanol denaturation:**

GS activity was assayed after preincubating the enzyme in the following different combinations of substrate and cofactors along with 2-mercaptoethanol at 30°C to find out the possible factor(s) which could protect the enzyme activity against 2-mercaptoethanol denaturation.

1. Enzyme in buffer (20mM Tris-HCl pH 7.4).
2. Enzyme + 25 µM 2-mercaptoethanol
3. Enzyme + 4·0 µM $MgCl_2$ + 25 µM 2-mercaptoethanol
4. Enzyme + 0·5 µM ATP + 25 µM 2-mercaptoethanol
5. Enzyme + 0·5 µM ATP + 40 µM $MgCl_2$+25 µM 2-mercaptoethanol

6. Enzyme + 2·5 μ M 2-mercaptoethanol + 4·0 μ M $MgCl_2$+ 0·5 μM ATP

7. Enzyme + 5·0 μ M glutamate + 2·5 μ M 2-mercaptoethanol

(viii) **Kinetic studies:**

(a) Determination of Km and V_{max}: The activity of purified glutamine synthetase was assayed at different concentrations of the substrates and cofactors such as glutamate, hydroxylamine, ATP and Mg^{+2} separately keeping other components of the assay mixture at saturation level as mentioned in the GS assay method. The concentrations used were 5,10,20,40, 60,80 and 100mM for glutamate, 0.4, 0.5, 1, 2.5, 5, 10 and 20mM for hydroxylamine, 0.5, 1, 2, 4, 5, 7, 10, 20, 25 and 30mM for Mg^{2+} and 1,2,3,5,10,20 and 30mM for ATP respectively. Km and V_{max} values were determined by plotting both Michaelis-Menten and Lineweaver-Burk plot of the data.

(b) Effect of metal ions: The purified enzyme was dialysed exhaustively against 20mM Tris-HCl buffer (pH 7.4) to remove all the ions from the enzyme source. The effect of various metal ions such as Na^+, K^+, Li^+, Co^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} and Po_4^{3-} were studied at various concentrations on the GS activity both in presence of and in absence of Mg^{2+}

(c) Effect of modulators on GS activity: The effect of various concentrations of amino acids such as glutamine(Gln), aspartic acid (Asp), aspargine(Asn), ornithene (Orn), citrulline

(Cit), alanine (Ala), arginine (Arg) glycine (Gly) and carbamylphosphate (CP) and nucleotides such as IMP, AMP and ADP were studied on purified glutamine synthetase activity.

(d) Inhibition of GS activity by ADP: The nature of inhibition of ADP was determined by assaying the enzyme activity at varying concentrations of glutamate (5,10,20,40, 60,80 and 100mM) with 0, 2.5, 5.0 and 10mM ADP separately. The data were plotted on a Lineweaver-Burk plot to determine the nature of inhibition. The Ki for ADP was a determined by assaying the enzyme activity at 0, 0.5, 1, 2, 3, 4 and 5mM ADP with 10, 25 and 50mM glutamate separately and by plotting the data on a Dixon plot.

Statistical Analysis:

Several repeatations were done for each experiment and estimation. The data for each point has been presented as the Mean ± Standard deviation (S.D.) fo at least five different observations. Comparison of paired mean values were made using Student's "t" test (Croxton et al, 1982) and p values more than 5% (> 0.05 were taken as nonsignificant (N.S.)

RESULTS

The tissue distribution of glutamine synthetase and glutaminase: (Table 1)

The total and specific activities of GS and Glnase in different tissues such as brain, liver, kidney, muscle and gill of H. fossilis have been presented in Table 1.

Glutamine synthetase: GS activity could be detected in all the five tissues studied. The total activity of GS was found to be maximum in brain followed by kidney, liver, muscle and gill. The total activity in brain was higher by about 8 times than in liver and kidney, 15 and 35 times than in muscle and gill respectively. The specific activity was also maximum in brain, but followed by gill, kidney, liver and muscle. The specific activity in brain was higher by about 7, 11, 12 and 16 times more than in gill, kidney, liver and muscle respectively.

Glutaminase: Glutaminase activity could be detected in the brain, liver, kidney and gill of H. fossilis. However, in muscle the enzyme activity could not be detected under assay conditions used. The total activity of Glnase was found to be maximum in brain followed by kidney, liverand gill in decreasing order. The total activity was 2.5 times

higher than in liver and kidney and about 10 times higher than in gill. The specific activity was also found maximum in brain but followed by gill, kidney and liver. The specific activity in brain was twice that of gill but about 3.5 times those of kidney and liver.

GS/Glnase ratio: The ratio of GS and Glnase activity was highest in brain followed by kidney, liver and gill. The ratio was almost same in liver, kidney and gill (2.12-2.22) but in the brain the ratio was about 7.1 in favour of GS.

Sub-cellular distribution of glutamine synthetase and glutaminase: (Table 2-9)

The sub-cellular distribution of GS and Glnase was studied in brain, liver and kidney tissues of H. fossilis. Different marker enzymes were used for characterization of the fractions obtained by differential centrifugation. The activity of different enzymes and protein content of various sub-cellular fractions of brain, liver and kidney have been presented in Tables 2-4. The marker enzyme used were GDH (NADH dependent) for mitochondrial, LDH for cytosolic and G-6-Pase for microsomal fractions.

GS was found to be localized primarily in the mitochondrial fraction of liver and kidney and in the cytosolic fraction of the brain of H. fossilis (Table 2-4).

Sonication and treatment with Triton X-100 was found

to be effective in the release of the GS activity in tissue homogenates. Treatment with Triton X-100 released the enzyme activity by more than 90% in kidney and liver and 26% in brain homogenate (Table 5&6). However, sonication even for 60 sec at 6μ did not release more than 75% of the enzyme activity. The enzyme was found to be stable upto 48 hrs both after sonication and with Triton X-100 treatment (Table 7).

Glnase activity was predominantly mitochondrial in liver, kidney and brain. It was about 70% for brain, 68% for liver and 72% for kidney mitochondrial fractions respectively (Table 2-4). Glnase activity was about 15-20% in nuclear fractions, 7-12% in cytosolic fractions and 3-5% in microsomal fractions of brain, liver and kidney.

Both Triton X-100 and sonication could solubilize the Glnase activity from mitochondrial fractions of brain, liver and kidney (Table 8). The release of Glnase activity was 65-78% by both sonication and Triton X-100 treatment from brain, liver and kidney homogenates. However, the released enzyme activity was found to be more stable after sonication than with Triton X-100 treatment. (Table 9). Brain and kidney glutaminase maintained its activity upto 12-24 hrs after Triton X-100 treatment and 36-48 hrs after sonication. Liver glutaminase was found to be more sensitive both after sonication and Triton X-100 treatment. However, after sonication it maintained its activity only upto 4-8 hrs

compared to Triton X-100 treatment, where it lost its activity about 18% by 4 hrs.

Diurnal variation of glutamine synthetase and glutaminase activity: (Table 10, 11; Fig.1).

GS and Glnase activity during the 24 hr cycle in brain, liver and kidney of H. fossilis have been presented in Table 10 & 11 and Fig.1. Both total and specific activity of GS and Glnase did not show any diurnal variation in any of the tissues studied.

Effect of starvation and NH_4Cl treatment: (Table 12-21; Fig. 2-8).

The alteration in the concentrations of glutamate and ammonia and the activities (total and specific) of GS and Glnase in brain, liver and kidney tissues of H. fossilis during starvation and exposure to different concentrations of NH_4Cl for different periods of time have been presented in Table 12-21 and Fig.2-8.

Concentration of ammonia: (Table 12; Fig.2&3).

The concentration of ammonia in vivo did not show significant change in any of the tissues during starvation. However, ammonia accumulated significantly in all the tissues when the fishes were exposed to different concentrations of

NH_4Cl. The accumulation of ammonia in the tissues continued all through the treatment of NH_4Cl reaching the highest level between 10-14 days of the treatment. The accumulation was concentration dependent showing greater accumulation at higher concentration of NH_4Cl. Maximum tissue ammonia accumulation in treated fish was in kidney (164%) followed by liver (~120%) and brain (~103%) compared to their starved controls.

Concentration of glutamate: (Table 13; Fig. 2&4)

There was significant accumulation of glutamate in kidney after 4 days and in liver after 7 days of starvation reaching the highest level by 14th day. The maximum accumulation was about 40% in liver and 30% in kidney compared to normal control. However, there was no significant change in glutamate level in brain during 14 days of starvation.

Exposure to various concentrations of NH_4Cl resulted into significant accumulation of glutamate in all the three tissues. Maximum accumulation of glutamate was found in kidney (58%) and brain (56%) followed by liver (40%). The accumulation continued in brain and liver whereas in kidney the accumulation was highest on the 7th day of exposure to NH_4Cl after which it decreased to a relatively lower level. However, in all the cases the accumulation level was significantly higher than the starved control. The rates of

accumulation were, in general, directly related to the ambient concentrations of NH_4Cl.

Glutamine synthetase activity: (Table 14-16; Fig.5-8).

GS activity (both total and specific) did not show significant change throughout the starvation period of 14 days in any of the tissues studied.

The exposure to various concentrations of NH_4Cl showed significant induction in GS activity in all the three tissues. The maximum induction of enzyme activity during exposure to NH_4Cl was found in brain (57-58%) followed by liver (50%) and in kidney (48%). The brain enzyme showed an early induction of activity (4th day) followed by liver (7th day) and kidney (10th day) when exposed to 50mM concentration of NH_4Cl. The induction of the enzyme activity reached almost to plateau level at higher ambient NH_4Cl concentration and with increase in the time of exposure. Exposure to 100mM NH_4Cl resulted in sudden induction of GS activity within 4 days to the highest level obtained for other concentrations of NH_4Cl and the fishes did not survive beyond 4 days, at that concentration of NH_4Cl.

Glutaminase activity: (Table 17-19; Fig.5-8).

Alteration in glutaminase activity (both total and specific) showed tissue specificity with brain and kidney showing a common pattern and liver showing exactly the

opposite pattern. During starvation Glnase activity was induced significantly in brain and kidney. The maximum induction was found in kidney (58%) which was almost twice that found in brain (30%). However, liver did not show any significant change in glutaminase activity during starvation period.

Exposure to different concentrations of NH_4Cl inhibited Glnase activity in brain and kidney tissues of H. fossilis. The inhibition was generally not significant at 50mM NH_4Cl exposure for 10-14 days. However, at 75mM and 100mM concentration of NH_4Cl, Glnase activity was significantly inhibited by about 20% in brain and 43% in kidney tissues compared to the starved control fishes. Glnase activity in liver increased significantly within 4 days of exposure to higher concentrations (75 and 100mM) of NH_4Cl which decreased with increasing exposure time. It was significantly inhibited in liver on the 14th day of exposure at 75mM NH_4Cl. Lower concentration (50mM) of NH_4Cl resulted in gradual increase in Glnase activity in liver till 10 days showing maximum of about 40% induction after which it started declining to about 17% by 14th day of exposure.

The ratio of GS/Glnase activity: (Table 20 & 21).

The ratio of GS/Glnase activity during starvation declined in the brain and kidney, with kidney showing greater reduction by about 30%. However, liver did not show very conspicuous change.

Exposure to various concentrations of NH_4Cl increased the ratio in all the three tissues. The maximum increase was observed at 75mM NH_4Cl treatment for 14 days with the highest rate in kidney (135%) followed by brain (97%) and liver (78%).

Effect of dehydration: (Table 22-27; Fig.9-11)

Concentration of glutamate: (Table 22; Fig.9)

Alteration in glutamate concentration in brain, liver and kidney tissues of H. fossilis during dehydration have been presented in Table 22 and Fig.9. There was accumulation of glutamate in all the three tissues. However, the pattern of accumulation was different in different tissues with increase in time of dehydration. The glutamate level in brain increased significantly by 12 hrs reaching the highest level by 18 hrs of dehydration. Then the level gradually decreased with increasing time of dehydration although it remained significantly higher than the control till 36 hrs. There was no significant alteration in glutamate level in liver during the first 12 hrs of dehydration. However, glutamate level increased significantly from 15 hrs onwards with increasing time of dehydration having maximum accumulation between 30 to 36 hrs of dehydration. Kidney also did not show any significant change in glutamate level during the first 12 hrs of dehydration. Significant increase in glutamate level was observed in kidney at 15 hrs of

dehydration. It increased further to reach the highest level (51%) by 24 hrs. Then the level declined gradually but remained significantly above the control level by 36 hrs of dehydration. Glutamate accumulation occured immediately in brain and in all the tissues within 24 hrs of dehydration. The accumulation decreased in brain and kidney after 24 hrs where as in liver the accumulation further enhanced to a still higher level between 30-36 hrs.

Glutamine synthetase activity: (Table 23 & 24; Fig. 10 & 11).

Effect of dehydration on glutamine synthetase activity (total and specific) in brain, liver and kidney of H. fossilis have been presented in Table 23 and 24 and Fig. 10 & 11. GS activity (both total and specific) which was highest in the brain among the three tissues increased significantly reaching a platue level from 12 hrs to 36 hrs of dehydration. The liver showed two phase increase - first one during first 12 hrs and the level was maintained till 24 hrs of dehydration. Then the second increase was between 24 to 36 hrs of dehydration. However, the GS activity in kidney continued to increase slowly but steadily till 36 hrs being significant after 9 hrs of dehydration. The highest induction of GS activity was 70% in liver followed by 48% in brain and 32% in kidney at 36 hrs of dehydration.

Glutaminase activity: (Table 25 & 26; Fig. 10 & 11).

Effect of dehydration on glutaminase activity (both

total and specific) in brain, liver and kidney of H. fossilis have been presented in Table 25 & 26 and Fig. 10 & 11. The enzyme activity in all the three tissues decreased gradually with increasing time of dehydration. The percent decrese by 36 hrs of dehydration was maximum in liver followed by kidney and brain. However, the decrease during the early period of dehydration was relatively more in brain and kidney than in liver.

The ratio of GS/Glnase activity (Table 27; Fig.11) in all the three tissues increased with increasing time of dehydration upto 36 hrs. The increase was about 170% in liver followed by 112-120% in brain and 90-100% in kidney by 36 hrs of dehydration.

Purification of brain glutamine synthetase and its physico-chemical properties: (Table 28; Fig.12).

Glutamine synthetase (GS) was purified from the brain of H. fossilis. The protocol of purification has been presented in Table 28. The degree of purification obtained was about 58 fold with 62% of recovery of the activity. The enzyme activity was eluted as a single peak coinciding with one of the protein peaks from the ion exchange DEAE sephacel and on Sephadex G-200 columns (Fig.12).

Polyacrylamide gel electrophoresis: (Fig.13)

The enzyme samples from different stages of purification

were separated by polyacrylamide gel electrophoresis. The gels were stained specifically for GS activity and parallel gels were stained with Coomassie blue for protein. The enzyme showed a single band during all the stages of purification. The purified GS showed also a single protein band on PAGE (Fig.13).

Molecular weight determination: (Table 29; Fig.14)

Molecular weight of the purified glutamine synthetase was determined by gel filtration on a Sephadex G-200 column (90x1.6cm). The void volume (V_o) was determined by Blue Dextran followed by the calibration of the column with marker proteins such as thyroglobulin (M.W. 6,69,000), ferritin (M.W. 4,40,000), catalase (M.W. 2,33,000), aldolase (M.W. 1,58,000) and albumin (M.W. 67,000). Purified GS was eluted as a single peak and its molecular weight was found out from the Kav values superimposed on the calibration graph for known proteins to be 3,91,300 (Table 29; Fig.14).

Preservation of purified enzyme: (Table 30)

Purified glutamine synthetase was not stable beyond 12 hrs in purifying buffer (20mM Tris-HCl pH 7.4). Storage at 0-4°C in the same buffer resulted in a total loss of enzyme activity within 96 hrs. However, preservation in the presence of 10% glycerol or 0.25 n NaCl in buffer at 0-4°C temperature could retain 90-95% of the enzyme activity for

more than a month. Albumin (0.25%) could also protect the enzyme from denaturation to some extent (95%). However, the presence of both 0.25N NaCl and 10% glycerol in purifying buffer gave better stability for longer time and easy handling of the enzyme than other methods of preservation.

pH optima: (Table 31; Fig.15A)

The GS activity at different assay pH has been shown in Table 31 and Fig.15A. The enzyme activity increased with increasing pH from 4-7.2 after which the activity was maintained at that high level upto pH 11. At pH 12 there was a decrease in the enzyme activity. Thus GS purified from brain of H. fossilis showed a broad pH optima ranging from 7.2-11.

Temperature optima: (Table 31; Fig.15B)

The activity of GS assayed at different temperatures keeping other assay conditions normal showed a gradual increase from 4-40°C. The activity was maintained from 40 to 45°C after which there occured a sharp decrease. The temperature optima for purified GS from brain of H. fossilis was found to be between 40-45°C.

Half life and thermal stability: (Table 32; Fig.16 & 17).

Purified GS was incubated at 45°C in a water bath. Aliquots were withdrawn at different time intervals, immediately

cooled in ice-bath and assayed for enzyme activity. Maximum activity was continued upto 15 min and then there was a gradual decrease losing total activity by 50 min of incubation at 45°C. The half life of GS activity (50% of enzyme activity) at 45°C was found to be 25 min. (Fig.16).

Thermal stability of purified GS was studied by incubating the purified enzyme in the purification buffer containing 0.25N NaCl for 30 min at different temperature and then immediately cooling the enzyme on ice-bath and assaying the activity under normal assay condition. The enzyme activity showed a sharp decline after 30°C reaching almost 50% at 40°C and below 10% at 50°C. Thus GS purified from brain of H. fossilis showed thermal stability for 30 min at 30°C.

Factors influencing thermal stability: (Table 33; Fig.18)

The half life as well as thermal stability studies showed the enzyme being unstable at 45°C in the purification buffer containing 0.25N NaCl. Addition of various substrates and cofactors in the incubating medium individually or in combinations did influence the thermal stability of the purified enzyme. 2-mercaptoethanol made the enzyme highly heat sensitive loosing all the activity even at 30°C. However, addition of glutamate protected the enzyme activity completely against thermal denaturation even upto 45°C. ATP and Mg^{2+} individually protected 63 and 70% of

the enzyme activity respectively but together they protected the enzyme activity completely even at 45°C. Hydroxylamine had rather no effect on temperature sensitivity of the purified enzyme.

Factors protecting the enzyme from 2-mercaptoethanol denaturation: (Table 34 & 35)

Absence of any of the components except 2-mercaptoethanol from the reaction mixture resulted into complete loss of GS activity (Table 34). However, the enzyme preincubated with 2-mercaptoethanol completely inhibited GS activity. To find out the factors, which could protect the enzyme activity, purified GS was preincubated at 30°C in presence of substrates and cofactors along with 2-mercaptoethanol. Mg^{2+} protected the enzyme activity completely from 2-mercaptoethanol denaturation followed by ATP and glutamate.

K_m and V_{max}: (Table 36; Fig. 19-22)

The apparent Michaelis constant (Km) of pure brain glutamine synthetase for Mg^{2+}, glutamate, hydroxylamine and ATP were found to be 6.25, 5.0, 0.5, 2.5 mM respectively as determined by both Michaelis-Menten and Lineweaver-Burk plot of the data. The saturation curve (Michaelis-Menten plot) for Mg^{2+} was sigmoidal where as the same for glutamate, hydroxylamine and ATP were hyperbolic.

Effect of Modulators on GS activity:

Metal ions: (Table 37; Fig.23).

Mg^{2+} in the reaction mixture showed highest GS activity above 10mM concentration. Replacing Mg^{2+} in the assay mixture with divalent cations such as Mn^{2+} or Co^{2+} showed very low enzyme activity in indicating the specificity of GS for Mg^{2+} (Fig.23).

In presence of Mg^{2+} in the reaction mixture the monovalent cations such as Na^{+}, K^{+} and Li^{+} had no effect on the GS activity. Divalent cations such as Co^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} Ca^{2+} and Zn^{2+} and divalent Po_4^{2-} inhibited GS activity in presence of Mg^{2+}. At 5mM concentration of Zn^{2+}, Mn^{2+} were more effective than Cu^{2+}, Cd^{2+} and Ca^{2+} in inhibiting GS activity. Co^{2+} and Po_4^{2-} inhibited GS activity only by about 25% at 5mM concentration. However, at 10mM concentration all the divalent cations and anions except Co^{2+} were extremely effective in inhibiting GS activity in presence of Mg^{2+} (20mM); in the assay mixture.

Amino Acids: (Table 38)

Purified Gs activity was inhibited by Asp., Asn., Orn, Cit, Ala, Arg. Gly and CP at 5, 50 and 125 mM concentration. Gly and CP inhibited GS activity completely at 125mM concentration. The inhibitory effect of other amino acids were Orn > Arg > Ala> Asp > Cit> Asn. Glutamine did not show any inhibitory effect.

Nucleotides: (Table 39)

IMP, AMP and ADP inhibited the purified glutamine synthetase activity. ADP inhibited the enzyme activity more effectively even at low concentrations showing 55% inhibition at 0.5mM ADP. AMP inhibition became similar to ADP only at higher concentrations. However, IMP inhibition was very poor in comparison to ADP or AMP both at lower and higher concentrations.

Nature of inhibition and Ki of ADP: (Fig. 24 & 25)

A strong inhibitor of GS activity, ADP was found to be a noncompetitive inhibitor (Fig.24) with respect to the substrate glutamate. The inhibition constant (Ki) of ADP was found to be 3.2mM as determined by Dixon Plot (Fig.25).

Table 1. Physiological level of total (units/g wet wt.) and specific (units/mg protein) activity of glutamine synthetase and glutaminase and their ratios in different tissues of Heteropneustes fossilis. (Mean±S.D.)

Tissues	GS		Glnase		Ratios of GS / Glnase	
	Total	Specific	Total	Specific	Total	Specific
Brain	495.5 ±49.5	3.50 ±0.85	69.52 ±5.28	0.494 ±0.04	7.13	7.09
Liver	60.90 ±9.7	0.289 ±0.02	28.72 ±6.30	0.136 ±0.008	2.12	2.13
Kidney	64.80 ±6.4	0.326 ±0.03	29.19 ±2.9	0.147 ±0.008	2.22	2.22
Muscle	32.2 ±0.44	0.22 ±0.04	BLD	BLD	-	-
Gill	14.36 ±2.42	0.51 ±0.09	6.73 ±1.32	0.24 ±0.02	2.13	2.12

Table 2. Sub-cellular distribution of glutamine synthetase and glutaminase activity (units/g wet wt.) and protein (mg/g wet wt.) in the brain of Heteropneustes fossilis (Mean ± S.D.)

Enzymes	Crude homogenate	Sub-cellular fractions				Recovered activity
		Nuclear	Mitochondrial	Microsomal	Cytosolic	
GS	495.02±49.5	32.2±5.3 (5.4)	64.2±12.4 (10.7)	86.1±7.9 (14.4)	416.7±35.5 (69.5)	599.2 (121.1)
Glnase	69.52±5.28	16.20±3.6 (20.18)	56.47±3.68 (70.35)	2.2±0.8 (2.74)	5.4 ± 0.52 (6.73)	80.27 (115.46)
GDH	261.6±25.3	58.1±9.3 (21.9)	171.7±10.3 (64.8)	20.6±2.4 (7.8)	14.5±3.6 (5.5)	264.8 (101.3)
LDH	5806.4±150.7	648.1±40.5 (11.0)	1011.9±58.4 (17.2)	239.2±25.3 (4.1)	3976.9±129.3 (67.7)	5876.1 (101.2)
G 6-Pase	33.6±4.3	3.5±0.3 (12.2)	5.5 ± 0.3 (19.3)	15.1 ± 3.9 (52.8)	4.5 ± 0.2 (15.7)	28.6 (85.1)
Protein	140.7±18.8	20.3 ± 3.3 (15.1)	38.1 ± 5.3 (28.4)	5.7 ± 1.3 (4.2)	70.0 ±5.27 (52.2)	134.0 (95.2)

% of crude homogenate activity are given in the parentheses.

Table 3. Sub-cellular, distribution of glutamine synthetase and glutaminase activity (units/g wet wt.) and protein (mg/g wet wt.) in the liver of Heteropneustes fossilis (Mean ± S.D.)

Enzymes	Crude homogenate	Sub-cellular fractions				Recovered activity
		Nuclear	Mitochondrial	Microsomal	Cytosolic	
GS	60.9±9.7	6.7±0.6 (9.4)	53.2±10.3 (74.2)	3.2±0.2 (4.4)	8.6±1.3 (12.0)	71.7 (117.8)
Glnase	28.72±6.3	4.4±0.28 (15.3)	19.22±2.5 (67.8)	1.5±0.05 (5.3)	3.2±1.6 (11.30)	28.32 (98.60)
GDH	868.0±66.2	86.8±15.6 (8.4)	799.8±55.3 (77.0)	57.8±5.4 (5.6)	94.7±10.5 (9.1)	1039.0 (119.7)
LDH	12599.3±790.0	630.6±80.9 (4.5)	484.9±58.2 (3.5)	920.7±70.8 (6.6)	11977.6±805.5 (85.5)	14013.8 (111.2)
G 6-Pase	235.4±10.6	20.9±2.3 (9.7)	80.9±11.4 (37.5)	94.0±5.5 (43.6)	20.0±3.4 (9.3)	215.8 (91.7)
Protein	210.6±15.3	24.3±2.3 (12.4)	41.4±7.8 (21.1)	24.8±6.7 (12.7)	105.4±15.3 (53.8)	195.9 (97.2)

% of crude homogenate activity are given in the parentheses.

Table 4. Sub-cellular distribution of glutamine synthetase and glutaminase activity (units/g wet wt.) and protein (mg/g wet wt.) in the kidney of Heteropneustes fossilis (Mean ± S.D.)

Enzymes	Crude homogenate	Sub-cellular fractions				Recovered activity
		Nuclear	Mitochondrial	Microsomal	Cytosolic	
GS	64.8±6.4	6.5±0.3 (9.3)	51.5±8.9 (73.6)	3.0±0.2 (4.2)	9.0±2.1 (12.9)	69.9 (108.0)
Glnase	29.19±2.9	3.2±0.5 (11.89)	19.12±1.4 (71.02)	1.4±0.08 (5.2)	3.2±0.89 (11.89)	26.92 (92.22)
GDH	551.6±58.8	46.3±8.3 (7.8)	439.2±29.7 (74.5)	50.1±5.3 (8.5)	54.4±10.0 (9.2)	589.9 (106.9)
LDH	2888.2±195.2	99.5±10.9 (3.2)	325.1±30.4 (10.5)	254.3±28.3 (8.2)	2429.4±150.4 (78.2)	3108.3 (107.6)
G 6-Pase	69.4±7.1	6.0±2.0 (8.1)	11.5±2.4 (15.5)	49.0±10.2 (65.9)	7.9±1.3 (10.6)	74.3 (107.1)
Protein	198.5±27.6	30.8±12.0 (15.2)	31.2±10.0 (15.4)	20.2±5.7 (10.0)	120.3±12.7 (59.4)	202.5 (102.2)

% of crude homogenate activity are given in the parentheses.

Table 5. Release of glutamine synthetase activity (units/g wet wt.) by treatment with Triton X-100 and sonication of the brain, liver and kidney homogenates of Heteropneustes fossilis. (Mean ± S.D.)

Triton X-100 Treatment (20 min)				Sonication (6 micron)			
Concentration of Triton X-100 (%)	Brain	Liver	Kidney	Period of sonication	Brain	Liver	Kidney
0 (Control)	394.85±30.25	31.03±2.25	33.81±3.39	0 (Control)	394.85±30.25	31.03±2.25	33.81±3.39
0.1	440.02±35.39 (+11.43)	55.25±3.10 (+78.05)	58.75±5.5 (+73.77)	2 Sec x 1	428.25±20.3 (+8.45)	45.25±5.34 (+45.82)	40.25±7.3 (+19.04)
0.2	496.75±45.25 (+25.80)*	60.30±7.75 (+94.32)	64.25±7.35 (+90.30)	5 Sec x 1	465.35±30.35 (+17.85)*	54.35±8.5 (+75.15)	45.75±8.5 (+35.31)
0.5	495.02±30.45 (+25.36)	60.75±9.72 (+95.77)*	64.75±8.25 (+91.61)*	20 Sec x 1	463.35±45.02 (+17.34)	55.10±7.3 (+77.57)*	59.25±4.25 (+75.24)*
				60 Sec x 1	465.32±40.39 (+17.84)	54.10±5.6 (+74.34)	59.25±8.25 (+75.24)

% increase from control activity are given in parentheses.

* Maximum activity obtained.

Table 6. Effect of Triton X-100(0.5%)on the activity (units/g wet wt.) of glutamine synthetase in brain, liver and kidney homogenates of Heteropneustes fossilis. (Mean ± S.D.)

Time of Treatment	Brain	Liver	Kidney
0 (Control)	394.85±30.25	31.03±2.25	33.81±3.39
5	495.02±49.5 (+25.37)	41.18±5.25 (+32.71)	57.10±5.70 (+68.88)
10	494.02±47.03 (+25.12)	60.89±9.72 (+96.23)*	58.37±8.97 (+72.63)
20	497.85±30.89 (+26.09)*	59.87±7.83 (+92.94)	64.75±6.35 (+91.52)*
30	495.02±35.39 (+25.37)	55.63±3.03 (+79.28)	60.60±5.25 (+79.25)

% increase from control activity are given in parentheses.

* Maximum activity obtained.

Table 7. Stability of glutamine synthetase activity (units/g wet wt.) after Triton X-100 treatment and sonication of brain, liver and kidney homogenates of Heteropneustes fossilis. (Mean ± S.D.)

Time in hrs / Tissue homogenate	After Triton X-100 treatment (0.5%)							After Sonication (6 micron)						
	0 (Control)	4	8	12	24	36	48	0 (Control)	4	8	12	24	36	48
Brain	495.02 ±30.45 (100)	490.25 ±20.25 (99.03)	495.12 ±30.35 (100)	490.35 ±25.75 (99.0)	500.25 ±40.85 (101.05)	498.75 ±37.37 (100.7)	490.85 ±35.45 (99.15)	445.32 ±40.3 (100)	447.22 ±30.35 (100.4)	450.25 ±25.10 (101.1)	446.75 ±30.75 (100)	451.25 ±40.40 (101.33)	448.25 ±15.37 (100.6)	449.75 ±25.75 (100.4)
Liver	60.75 ±9.72 (100)	57.25 ±7.5 (94.2)	55.95 ±7.8 (92.09)	57.10 ±5.2 (93.99)	56.95 ±6.40 (93.74)	56.25 ±4.5 (92.59)	56.85 ±3.5 (93.58)	55.10 ±7.0 (100)	55.25 ±7.70 (100.2)	55.10 ±6.33 (100)	53.85 ±5.25 (97.73)	55.85 ±5.25 (101.36)	58.75 ±4.08 (106.62)	59.05 ±7.5 (107.17)
Kidney	64.75 ±8.25 (100)	61.64 ±7.2 (95.0)	62.25 ±5.6 (95.0)	62.75 ±4.5 (96.9)	61.25 ±7.8 (94.5)	62.05 ±7.5 (95.8)	61.45 ±4.25 (94.90)	59.25 ±8.25 (100)	60.25 ±3.85 (101.68)	60.65 ±5.2 (102.36)	59.85 ±4.5 (101.01)	61.06 ±5.4 (103.05)	62.06 ±3.5 (104.74)	61.85 ±4.5 (104.38)

% activity are given in parentheses.

Table 8. Release of glutaminase activity (units/g wet wt.) by treatment with Triton X-100 and by sonication of the brain, liver and kidney homogenates of Heteropneustes fossilis. (Mean ± S.D.)

Triton X-Treatment (20 min)				Sonication (6 micron)			
Concentration of Triton X-100 (%)	Brain	Liver	Kidney	Period of sonication	Brain	Liver	Kidney
0 (Control)	40.7±5.7	17.5±3.2	16.35±1.5	0 (Control)	40.7±5.6	17.5±3.2	16.35±1.5
0.1	64.8±6.2 (+59.2)	28.7±2.9 (+61.23)	27.85±3.2 (+70.33)	2 Sec x 1	70.5±6.5 (+73.2)*	28.85±4.3 (+64.50)*	28.95±2.8 (+77.6)*
0.2	69.5±6.5 (+70.8)*	28.8±3.8 (+64.57)*	28.10±3.1 (+71.86)	5 Sec x 1	69.9±7.6 (+71.7)	27.6±2.9 (+57.7)	28.95±3.4 (+77.6)
0.5	69.23±8.2 (+70.0)	28.70±2.5 (+61.23)	28.20±2.5 (+72.42)*	20 Sec x 1	60.2±5.2 (+47.9)	26.4±3.2 (+50.86)	25.25±3.5 (+54.43)
				60 Sec x 1	50.3±2.5 (+23.58)	0.0	24.3±2.5 (+48.62)

% increase from control activity are given in parentheses.

* Maximum activity obtained.

Table 9. Stability of glutaminase activity (units/g wet wt.) after Triton X-100 treatment and sonication of the tissue homogenates of brain, liver and kidney of Heteropneustes fossilis. (Mean ± S.D.)

Time in hr / Tissue homogenate	After Triton X-100 treatment (0.5%)							After Sonication (6 micron)						
	0 (Control)	4	8	12	24	36	48	0 (Control)	4	8	12	24	36	48
Brain	69.65 ±5.60 (100)	70.72 ±5.70 (101.5)	68.95 ±4.70 (98.9)	69.75 ±7.34 (100)	69.52 ±5.70 (99.8)	65.70 ±6.20 (94.3)	62.25 ±7.25 (89.37)	69.52 ±4.39 (100)	69.52 ±2.63 (100)	70.12 ±7.37 (100.8)	68.85 ±8.65 (99.03)	70.12 ±7.73 (100.86)	69.27 ±5.35 (99.64)	65.25 ±8.50 (93.85)
Liver	28.72 ±2.85 (100)	23.63 ±2.50 (82.27)	22.25 ±1.25 (77.47)	22.02 ±2.30 (76.67)	15.85 ±3.34 (55.19)	8.75 ±1.25 (30.46)	2.30 ±0.5 (8.0)	28.65 ±2.25 (100)	28.65 ±3.60 (100)	28.25 ±3.90 (98.60)	25.30 ±2.30 (88.31)	20.85 ±3.35 (72.77)	15.75 ±1.39 (54.97)	8.39 ±2.25 (29.28)
Kidney	29.20 ±3.25 (100)	28.95 ±3.70 (99.14)	29.28 ±5.70 (100.3)	30.05 ±4.27 (102.9)	27.03 ±2.35 (92.56)	24.35 ±3.25 (83.39)	20.32 ±2.25 (69.58)	29.19 ±2.39 (100)	29.30 ±1.50 (100.4)	30.39 ±3.25 (104.11)	29.19 ±3.29 (100)	30.25 ±3.25 (103.63)	28.85 ±4.80 (98.8)	29.30 ±4.75 (100.4)

% activity are given in parentheses.

Table 10. Total activity (units/g wet wt.) of glutamine synthetase and glutaminase in brain, liver and kidney of Heteropneustes fossilis during 24 hrs cycle. (Mean ± S.D.)

Tissue	Enzyme	Time of the 24 hrs cycle							
		6 A.M.	9 A.M.	12 A.M.	3 P.M.	6 P.M.	9 P.M.	12 P.M.	3 A.M.
Brain	GS	450.50 ±45.63	445.25 ±40.75	455.75 ±30.37	460.07 ±35.27	450.25 ±30.39	452.05 ±30.09	450.27 ±45.60	449.50 ±42.60
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	72.63 ±10.69	70.25 ±8.75	69.34 ±7.50	72.75 ±8.50	70.75 ±7.50	73.04 ±10.34	72.07 ±9.34	74.25 ±10.24
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Liver	GS	55.27 ±6.20	54.85 ±5.34	56.25 ±4.30	57.35 ±6.60	54.07 ±4.45	55.96 ±6.05	54.97 ±6.35	55.27 ±6.28
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	28.26 ±3.30	28.35 ±3.25	26.34 ±4.45	28.26 ±3.96	25.07 ±4.45	27.47 ±5.57	28.37 ±5.05	30.35 ±6.76
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Kidney	GS	60.75 ±5.28	62.28 ±6.65	60.05 ±5.24	63.29 ±7.76	60.25 ±6.30	61.05 ±5.25	59.09 ±6.07	62.96 ±6.75
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	29.07 ±4.70	29.26 ±5.50	28.25 ±3.76	26.25 ±4.25	30.35 ±4.45	31.35 ±5.56	30.05 ±5.56	32.65 ±6.15
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

p - level of significance compared to 6 A.M. value.

Table 11. Specific activity (units/mg protein) x 10 of glutamine synthetase and glutaminase in brain, liver and kidney of Heteropneustes fossilis during 24 hrs cycle. (Mean ± S.D.)

Tissue	Enzyme	Time of the 24 hrs cycle							
		6 A.M.	9 A.M.	12 A.M.	3 P.M.	6 P.M.	9 P.M.	12 P.M.	3 A.M.
Brain	GS	30.2±2.50	30.2±3.3	30.3±2.5	30.2±5.0	30.4±4.0	30.4±3.0	30.2±4.0	30.3±2.50
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	5.5±0.40	5.5±0.50	5.4±0.40	5.5±0.50	5.6±0.70	5.6±0.40	5.5±0.40	5.6±1.0
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Liver	GS	2.9±0.50	2.9±0.40	3.0±0.70	2.9±0.40	3.1±0.30	3.1±0.20	2.9±0.40	3.0±0.50
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	1.40±0.50	1.45±0.20	1.44±0.10	1.39±0.20	1.48±0.05	1.45±0.10	1.38±0.10	1.39±0.20
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Kidney	GS	3.30±0.50	3.25±0.50	3.34±0.50	3.40±0.20	3.30±0.10	3.40±0.40	3.35±0.60	3.30±0.20
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Glnase	1.50±0.10	1.50±0.15	1.45±0.15	1.50±0.15	1.48±0.20	1.55±0.10	1.52±0.10	1.48±0.10
	P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

p - level of significance compared to 6 A.M. value.

Table 12. Alteration in the concentration (μmoles/g wet wt.) of ammonia in the brain, liver and kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride. (Mean±S.D.)

Tissue	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Brain	0 (Normal/Starved control)	7.15±0.63	6.95±0.42(- 2.80)[c]	6.35±0.41(-11.19)	6.45±0.5 (- 9.79)	6.65±0.46(- 6.99)
			a N.S.	N.S.	N.S.	N.S.
	50		9.75±0.30(+40.29)[d]	10.36±0.54(+63.15)	10.95±1.0 (+69.77)	11.69±0.74(+75.79)
			b < 0.001	< 0.001	< 0.001	< 0.001
	75		10.56±0.45(+51.94)[d]	12.25±1.25(+92.91)	13.09±1.5(+102.95)	12.75±0.40(+91.72)
			b < 0.001	< 0.001	< 0.001	< 0.001
	100		11.30±0.35(+62.59)[d]			
			b < 0.001	did not survive		
Liver	0 (Normal/Starved control)	15.30±2.75	14.50±1.75(- 5.23)[c]	14.85±2.07(- 2.94)	15.25±1.85(-0.33)	14.67±1.25(- 4.12)
			a N.S.	N.S.	N.S.	N.S.
	50		21.75±1.05(+47.96)[d]	29.70±3.50(+100.00)	33.35±3.70(+118.69)	28.95±2.50(+97.34)
			b < 0.001	< 0.001	<0.001	<0.001
	75		27.80±3.25(+89.12)[d]	32.90±4.5(+121.55)	32.25±2.25(+111.48)	30.20±1.25(+105.81)
			b < 0.001	< 0.001	<0.001	< 0.001
	100		32.35±2.25(+119.32)[d]			
			b < 0.001	did not survive		
Kidney	0 (Normal/Starved control)	11.27±0.84	12.40±1.25(+10.03)[c]	13.25±1.85(+17.57)	12.75±1.32(+13.13)	12.50±1.42(+10.91)
			a N.S.	N.S.	N.S.	N.S.
	50		22.25±2.25(+79.43)[d]	30.35±1.75(+129.06)	28.90±2.02(+126.67)	31.75±2.75(+154.00)
			b < 0.001	< 0.001	< 0.001	< 0.001
	75		25.37±1.25(+104.60)[d]	31.05±2.25(+134.34)	32.37±1.85(+153.88)	33.01±2.30(+164.08)
			b < 0.001	< 0.001	<0.001	<0.001
	100		32.75±2.50(+164.11)[d]			
			b < 0.001	did not survive		

a-p values compared to normal control; b-p values compared to starved control;

c-% change compared to normal control; d-% change compared to starved control.

Table 13. Alteration in the concentration (μmoles/g wet wt.) of glutamate in the brain, liver and kidney of *Heteropneustes fossilis* during starvation and exposure to various concentrations of ammonium chloride. (Mean±S.D.)

Tissue	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Brain	0 (Normal/Stadard control)	1.57±0.36	1.70±0.25(+ 8.28)[c]	1.44±0.20(- 8.28)	1.60±0.25(+ 1.91)	1.80±0.25(+14.65)
			a N.S.	N.S.	N.S.	N.S.
	50		1.65±0.22(- 2.94)[d]	1.66±0.26(+15.27)	2.04±0.22(+27.50)	2.28±0.23(+26.67)
			b N.S.	N.S.	<0.02	<0.02
	75		2.10±0.23(+23.53)[d]	2.24±0.51(+55.55)	2.40±0.2 (+50.00)	2.56±0.43(+42.22)
			b <0.05	<0.02	<0.005	<0.05
	100		2.32±0.36(+36.47)[d]			
			b <0.02	did not survive		
Liver	0 (Normal/Starved control)	0.996±0.14	0.986±0.07(- 1.00)[c]	0.990±0.08(- 0.60)	1.24±0.2 (+24.50)	1.40±0.2 (+40.56)
			a N.S.	N.S.	<0.05	<0.01
	50		1.02±0.05 (+ 3.44)[d]	1.17±0.20(+18.18)	1.31±0.22(+ 5.65)	1.32±0.22(- 5.7)
			b N.S.	N.S.	N.S.	N.S.
	75		1.20±0.20(+21.70)[d]	1.33±0.22(+34.34)	1.53±0.20(+23.39)	1.83±0.25(+30.71)
			b <0.05	<0.02	<0.05	<0.02
	100		1.38±0.36(+39.96)[d]			
			b <0.02	did not survive		
Kidney	0 (Normal/Starved control)	1.10±0.12	1.21±0.33(+10.0)[c]	1.53±0.18(+39.10)	1.41±0.17(+28.18)	1.43±0.14(+30.00)
			a N.S.	<0.005	<0.01	<0.02
	50		1.51±0.33(+24.79)[d]	2.32±0.16(+51.63)	2.19±0.29(+55.32)	2.08±0.23(+45.45)
			b N.S.	<0.025	<0.001	<0.001
	75		1.43±0.22(+18.18)[d]	2.42±0.28(+58.17)	2.02±0.22(+43.26)	2.02±0.28(+41.26)
			b N.S.	<0.001	<0.005	<0.005
	100		1.69±0.15(+39.67)[d]			
			b <0.02	did not survive		

a-p values compared to normal control; b-p values compared to starved control;

c-% change compared to normal control; d-% change compared to starved control.

Table 14. Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein) X10 of glutamine synthetase in the brain of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal/Starved control)	475.25±30.34	486.99±20.2(+ 2.47)[c]	502.00±35.3(+ 5.63)	507.70±20.2(+ 6.83)	478.35±35.3(+10.65)
			a N.S.	N.S.	N.S.	N.S.
	50		530.38±17.4(+ 8.91)[d]	598.28±24.3(+19.18)	684.73±30.3(+34.87)	686.86±30.3(+43.59)
			b <0.01	<0.001	<0.001	<0.001
	75		666.93±30.3(+36.95)[d]	707.16±40.5(+40.37)	714.3 ±40.4(+40.63)	749.00±35.4(+56.58)
			b <0.001	<0.001	<0.001	<0.001
	100		733.55±45.3(+50.62)[d]			
			b <0.001	did not survive		
Specific	0 (Normal/Starved control)	30.96± 5.2	40.70±6.0 (+ 2.78)[c]	43.30±5.5 (+ 9.34)	44.20±4.5 (+11.62)	41.1 ± 6.5(+ 3.79)
			a N.S.	N.S.	N.S.	N.S.
	50		46.3±4.30(+13.76)[d]	52.40±3.5 (+21.02)	62.50±8.5 (+41.40)	61.30± 9.0(+49.15)
			b N.S.	<0.02	<0.005	<0.005
	75		58.3±5.5 (+43.24)[d]	60.9±4.5 (+40.65)	61.1±10.2 (+38.24)	64.8±2.5 (+57.56)
			b <0.001	<0.001	<0.01	<0.005
	100		60.8±8.4 (+49.39)[d]			
			b <0.005	did not survive		

a-p values compared to normal control b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 15. Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein)X10 of glutamine synthetase in the liver of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal/Starved control)	55.34±7.5	55.95±7.5 (+ 1.10)[c]	59.51±7.50(+ 7.54)	55.85±8.25(+ 0.92)	58.25±9.70(+ 5.26)
		a	N.S.	N.S.	N.S.	N.S.
	50		56.05±7.9 (+ 0.17)[d]	73.13±5.60(+22.89)	80.67±7.70(+44.44)	87.37±8.27(+49.99)
		b	N.S.	< 0.02	< 0.005	< 0.001
	75		63.69±3.20(+13.83)[d]	71.56±5.70(+20.25)	79.82±8.6(+42.92)	85.95±7.35(+47.56)
		b	N.S.	< 0.025	< 0.005	< 0.001
	100		80.03±8.90(+43.04)[d]			
		b	< 0.005	did not survive		
Specific	0 (Normal/Starved control)	2.90±0.5	2.97±0.3(+ 2.41)[c]	3.15±0.5(+ 8.62)	3.30±0.7(+13.79)	3.10±0.3(+ 6.90)
		a	N.S.	N.S.	N.S.	N.S.
	50		2.93±0.4(- 1.03)[d]	3.75±0.3(+19.05)	4.97±0.5(+50.61)	4.51±0.7(+45.48)
		b	N.S.	< 0.05	< 0.005	< 0.005
	75		3.29±0.6(+10.77)[d]	3.88±0.4(+23.17)	4.66±0.4(+41.21)	4.58±0.6(+47.74)
		b	N.S.	<0.05	<0.005	< 0.005
	100		4.31±0.4(+45.12)[d]			
		b	< 0.001	did not survive		

a-p values compared to normal control; b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 16. Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein)X10 of glutamine synthetase in the kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal/Starved control)	62.30±3.50	65.30±8.39(+ 4.82)[c]	64.39±4.50(+ 3.35)	65.35±4.5 (+ 4.90)	62.30±5.6 (0)
			a N.S.	N.S.	N.S.	N.S.
	50		66.45±3.50(+ 1.76)[d]	65.35±5.80(+ 1.49)	85.92±10.68(+31.48)	91.26±5.4 (+46.48)
			b N.S.	N.S.	< 0.005	< 0.001
	75		66.45±3.70(+ 0.23)[d]	79.59±5.7 (+23.61)	88.25±3.70(+35.04)	92.04±5.7(+47.74)
			b N.S.	<0.005	< 0.001	< 0.001
	100		87.32±8.02(+33.72)[d]	did not survive		
			b < 0.005			
Specific	0 (Normal/Starved control)	3.41±0.5	3.58±0.8(+ 4.99)[c]	3.44±0.5(+ 0.87)	3.50±0.4(+ 2.64)	3.51±0.8(+ 2.90)
			a N.S.	N.S.	N.S.	N.S.
	50		3.59±0.5(+ 0.28)[d]	3.46±0.4(+ 0.58)	4.57±0.8(+30.57)	4.97±0.4(+41.59)
			b N.S.	N.S.	< 0.025	< 0.01
	75		3.56±0.4 (- 0.56)[d]	4.21±0.4 (+22.38)	4.85±0.6(+38.57)	5.02±0.3(+43.02)
			b N.S.	<0.05	< 0.005	< 0.005
	100		5.00±0.4(+39.66)[d]	did not survive		
			b < 0.001			

a-p values compared to normal control; b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 17. Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein)X10 of glutaminase in the bra[illegible] of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride. (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal/Starved control)	69.81±10.51	79.54±8.25(+13.94)[c]	91.02±8.52(+30.38)	85.87±8.52(+23.00)	86.51±9.67(+23.92)
			a N.S.	< 0.001	< 0.025	< 0.025
	50		75.37±5.40(- 5.24)[d]	97.21±11.22(+ 6.80)	85.22±6.20(- 0.76)	69.69±3.20(-19.44)
			b N.S.	N.S.	N.S.	< 0.005
	75		72.25±5.2 (- 9.16)[d]	69.69±7.90(-23.43)	68.81±6.20(-19.87)	68.81±6.2 (-20.46)
			b N.S.	<0.001	< 0.005	< 0.005
	100		67.33±3.03(-15.35)[d]			
			b < 0.01	did not survive		
Specific	0 (Normal/Starved control)	5.80±0.5	6.25±0.5 (+ 7.75)[c]	7.85±0.7 (+35.34)	7.48±0.5 (+28.97)	7.44±0.9 (+28.28)
			a N.S.	< 0.001	< 0.001	< 0.01
	50		6.14±0.5 (- 1.76)[d]	8.52±0.6 (+ 8.53)	7.78±0.7 (+ 4.02)	6.22±0.5 (-16.40)
			b N.S.	N.S.	N.S.	< 0.05
	75		5.73±0.8 (- 8.32)[d]	6.09±0.5 (-22.42)	6.23±0.4(-16.71)	6.02±0.4 (-19.08)
			b N.S.	< 0.001	<0.005	< 0.01
	100		5.24±0.5 (-16.16)[d]			
			b < 0.01	did not survive		

a-p values compared to normal control; b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 18. Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein)X10 of glutaminase in the liver of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride. (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal Starved control)	28.36±5.90	29.01±4.60(+ 2.29)[c]	30.54±7.5 (+ 7.69)	26.26±4.60 (- 7.40)	30.84±2.6 (+ 8.74)
			a N.S.	N.S.	N.S.	N.S.
	50		30.39±3.5 (+ 4.67)[d]	37.99±4.15(+24.39)	36.52±5.4 (+39.07)	35.98±2.6 (+16.67)
			b N.S.	< 0.025	< 0.02	<0.025
	75		36.54±4.8 (+25.96)[d]	36.54±3.50(+19.65)	29.27±2.6 (+11.46)	25.54±2.05(-17.19)
			b < 0.05	< 0.05	N.S.	< 0.01
	100		34.65±2.7 (+19.44)[d]	did not survive		
			b < 0.05			
Specific	0 (Normal/Starved control)	1.50±0.03	1.54±0.1 (+ 2.67)[c]	1.62±0.2 (+ 8.0)	1.57±0.1 (+ 4.67)	1.63±0.2 (+ 8.67)
			a N.S.	N.S.	N.S.	N.S.
	50		1.59±0.08(+ 8.25)[d]	1.95±0.1 (+20.37)	2.01±0.2 (+28.02)	1.90±0.09(+16.56)
			b N.S.	< 0.01	< 0.025	<0.02
	75		1.89±0.2 (+22.73)[d]	1.88±0.05(+16.05)	1.71±0.2 (+ 8.92)	1.42±0.01(-12.88)
			b < 0.01	<0.02	N.S.	< 0.05
	100		1.78±0.2 (+15.58)[d]	did not survive		
			b < 0.025			

a-p values compared to normal control; b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 19 Alteration in the total activity (units/g wet wt.) and specific activity (units/mg protein)X10 of glutaminase in the kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride. (Mean±S.D.)

Activity	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Total	0 (Normal/Starved control)	29.24±2.90	29.54±2.80(+ 1.03)[c]	46.11±4.70(+57.69)	45.96±3.80(+57.18)	40.63±2.8 (+38.95)
			b N.S.	< 0.001	< 0.001	<0.001
	50		29.25±2.50(- 0.98)[d]	40.77±5.0 (-11.58)	38.96±3.4 (-15.23)	33.04±3.4 (-18.68)
			b N.S.	N.S.	< 0.02	< 0.005
	75		29.25±5.6 (- 0.98)[d]	29.51±1.52(-36.00)	26.39±6.8 (-42.58)	25.68±3.5 (-36.80)
			b N.S.	<0.001	< 0.001	< 0.001
	100		19.35±4.04(-34.83)[d]			
			b < 0.005	did not survive		
Specific	0 (Normal/Starved control)	1.60±0.08	1.62±0.03(+ 1.25)[c]	2.46±0.02(+53.75)	2.46±0.02(+53.75)	2.29±0.03(+43.13)
			a N.S.	< 0.001	< 0.001	<0.001
	50		1.58±0.04(- 2.47)[d]	2.16±0.02(-12.20)	2.07±0.04(-15.85)	1.80±0.01(-21.40)
			b N.S.	< 0.001	< 0.001	<0.001
	75		1.59±0.06(- 1.85)[d]	1.56±0.1 (-36.59)	1.45±0.06(-41.05)	1.40±0.1 (-38.86)
			b N.S.	<0.001	< 0.001	< 0.001
	100		1.02±0.2 (-37.04)[d]			
			b < 0.001	did not survive		

a-p values compared to normal control; b-p values compared to starved control; c-% change compared to normal control; d-% change compared to starved control.

Table 20. The ratio of the total activity of glutamine synthetase/glutaminase in various tissues of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride.

Tissue	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Brain	0 (Normal/Starved control)	6.80	6.12 (-10.01)	5.52 (-18.82)	5.91 (-13.09)	5.53 (-18.68)
	50		7.04 (+15.03)	6.15 (+11.41)	8.03 (+35.87)	9.86 (+78.30)
	75		9.23 (+50.82)	10.15 (+83.88)	10.38 (+75.63)	10.89 (+96.93)
	100		10.89 (+77.94)	did not survive		
Liver	0 (Normal/Starved control)	1.95	1.93 (- 1.03)	1.95 (0)	2.13 (+ 9.23)	1.89 (- 3.08)
	50		1.84 (- 4.66)	1.92 (- 1.53)	2.21 (+ 3.76)	3.36 (+77.78)
	75		1.74 (- 9.84)	1.96 (+ 0.51)	2.73 (+28.17)	3.37 (+78.31)
	100		2.31 (+10.36)	did not survive		
Kidney	0 (Normal/Starved control)	2.13	2.21 (+ 3.76)	1.40 (-34.27)	1.42 (-33.33)	1.53 (-28.16)
	50		2.27 (+ 2.71)	1.60 (+14.29)	2.21 (+55.63)	2.76 (+80.39)
	75		2.24 (+ 1.35)	2.70 (+92.86)	3.34 (+135.20)	3.58 (+133.9)
	100		4.51 (+104.0)	did not survive		

% changes are given in parentheses.

Table 21. The ratio of the specific activity of glutamine synthetase/glutaminase in different tissues of Heteropneustes fossil during starvation and exposure to various concentrations of ammonium chloride.

Tissue	NH_4Cl (mM)	Days of starvation/NH_4Cl treatment				
		0	4	7	10	14
Brain	0 (Normal/Starved control)	5.34	6.51 (+21.91)	5.52 (+ 3.37)	5.90 (+10.49)	5.52 (+ 3.37)
	50		7.54 (+15.82)	6.15 (+11.41)	8.03 (+36.10)	9.86 (+78.62)
	75		10.17 (+56.22)	10.0 (+81.16)	9.81 (+66.27)	10.76 (+94.92)
	100		11.60 (+78.19	did not survive		
Liver	0 (Normal/Starved control)	1.93	1.93 (0)	1.94 (+ 0.52)	2.10 (+ 8.80)	1.90 (- 1.56)
	50		1.84 (- 4.66)	1.92 (- 1.03)	2.47 (+17.62)	2.37 (+24.74)
	75		1.74 (- 9.8)	2.06 (+ 6.19)	2.73 (+30.00)	3.22 (+69.47)
	100		2.42 (+25.39)	did not survive		
Kidney	0 (Normal/Starved control)	2.13	2.21 (+ 3.76)	1.40 (-34.27)	1.42 (-33.33)	1.53 (-28.17)
	50		2.27 (+ 2.71)	1.60 (+14.29)	2.21 (+55.63)	2.76 (+80.39)
	75		2.24 (+1.35)	2.70 (+92.86)	3.34 (+135.2)	3.59 (+134.6)
	100		4.90 (+22.72)	did not survive		

% changes are given in parentheses.

Table 22. Alteration in the glutamate level (μ mole/g wet wt.) in brain, liver and kidney of Heteropneustes fossilis during dehydration (Mean ± S.D.)

Tissue	Time (hr) of dehydration											
	0 (control)	3	6	9	12	15	18	21	24	27	30	36
Brain	1.57 ±0.36	1.76 ±0.25	1.94 ±0.21	2.06 ±0.20	1.98 ±0.16	2.35 ±0.32	2.42 ±0.19	2.40 ±0.20	2.12 ±0.33	2.15 ±0.32	2.06 ±0.25	1.96 ±0.15
	a	+12.10	+ 23.56	+31.21	+26.11	+49.68	+54.14	+52.86	+35.03	+36.94	+31.21	+24.84
	b	N.S.	N.S.	N.S.	< 0.05	< 0.01	< 0.005	< 0.005	< 0.05	< 0.05	<0.05	N.S.
Liver	0.996 ±0.14	0.952 ±0.15	1.04 ±0.15	1.10 ±0.20	0.901 ±0.36	1.25 ±0.20	1.49 ±0.163	1.39 ±0.18	1.40 ±0.15	1.58 ±0.35	1.95 ±0.20	1.95 ±0.4
	a	-4.41	-4.42	+10.44	-9.53	+25.50	+49.59	+39.55	+40.56	+58.63	+95.78	+95.78
	b	N.S.	N.S.	N.S.	N.S.	< 0.05	< 0.001	< 0.005	<0.005	< 0.01	< 0.001	< 0.001
Kidney	1.10 ±0.12	1.15 ±0.15	0.98 ±0.075	1.25 ±0.13	1.22 ±0.23	1.30 ±0.12	1.34 ±0.12	1.49 ±0.32	1.66 ±0.10	1.45 ±0.2	1.34 ±0.13	1.36 ±0.16
	a	+4.54	-10.90	+13.64	+10.90	+18.18	+21.82	+35.45	+50.91	+31.82	+21.82	+23.63
	b	N.S.	N.S.	N.S.	N.S.	< 0.05	< 0.02	< 0.05	< 0.001	< 0.01	< 0.02	<0.025

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

Table 23. Alteration in the total activity (units/g wet wt.) of glutamine synthetase in brain, liver and kidney of Heteropneustes fossilis during dehydration (Mean ± S.D.)

Tissue	Time (hr) of dehydration											
	0 (control)	3	6	9	12	15	18	21	24	27	30	36
Brain	450.50 ±45.63	553.34 ±20.35	606.05 ±33.15	622.36 ±24.60	646.02 ±42.62	635.92 ±25.37	646.06 ±42.62	652.10 ±32.83	656.42 ±37.46	659.22 ±35.42	652.95 ±44.94	664.89 ±30.24
	a	+22.83	+34.53	+38.15	+43.40	+41.16	+43.41	+44.75	+45.71	+46.33	+44.94	+47.59
	b	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	<0.001	<0.001	< 0.001	< 0.001	< 0.001
Liver	55.27 ±6.20	57.56 ±5.30	62.71 ±14.48	69.66 ±11.35	82.59 ±11.84	83.99 ±12.48	84.02 ±8.43	84.65 ±9.45	85.42 ±11.20	88.49 ±9.25	92.32 ±16.58	94.12 ±15.60
	a	+4.14	+13.46	+26.04	+49.43	+51.96	+52.02	+53.16	+54.55	+60.10	+67.03	+70.29
	b	N.S.	N.S.	< 0.05	< 0.005	<0.005	<0.001	< 0.001	< 0.001	<0.001	<0.001	< 0.001
Kidney	60.75 ±5.28	62.66 ±10.25	63.25 ±2.30	69.21 ±4.70	71.22 ±4.20	73.36 ±11.26	72.93 ±4.80	74.65 ±3.70	79.74 ±8.50	78.51 ±5.58	80.42 ±4.90	79.74 ±5.25
	a	+3.14	+4.12	+13.93	+17.23	+20.76	+20.05	+22.88	+31.26	+29.23	+32.38	+31.26
	b	N.S.	N.S.	< 0.05	< 0.025	< 0.05	< 0.01	< 0.005	<0.005	< 0.001	<0.001	< 0.001

a-% change compared to control; b-p values comapred to control.

Table 24. Alteration in the specific activity (units/mg protein)X10 of glutamine synthetase in the brain, liver and kidney of Heteropneustes fossilis during dehydration (Mean ± S.D.)

Tissue	Time (hrs) of dehydration											
	0 (control)	3	6	9	12	15	18	21	24	27	30	36
Brain	30.2 ±2.5	38.4 ±1.2	43.3 ±1.5	43.9 ±1.1	46.4 ±1.9	45.5 ±1.7	45.8 ±1.9	46.1 ±2.0	46.9 ±1.5	47.2 ±1.4	46.7 ±2.0	7.2 ±2.0
	a	+20.00	+35.31	+37.19	+45.00	+42.19	+43.13	+44.06	+46.56	+47.50	+45.94	+47.5
	b	<0.001	< 0.001	< 0.001	<0.001	<0.001	< 0.001	< 0.001	<0.001	<0.001	< 0.001	< 0.001
Liver	2.90 ±0.5	3.07 ±0.5	3.23 ±0.5	3.48 ±0.4	4.33 ±0.5	4.42 ±0.6	4.40 ±0.6	4.33 ±0.5	4.47 ±0.4	4.42 ±0.5	4.65 ±0.4	4.67 ±0.6
	a	+5.86	+11.38	+20.00	+49.31	+52.41	+51.72	+49.31	+54.14	+52.41	+60.34	+61.03
	b	N.S.	N.S.	N.S.	<0.005	< 0.005	< 0.005	< 0.005	<0.001	< 0.001	< 0.001	<0.001
Kidney	3.30 ±0.3	3.41 ±0.5	3.41 ±0.5	3.66 ±0.4	3.73 ±0.4	3.88 ±0.4	3.81 ±0.3	4.06 ±0.4	4.10 ±0.3	4.16 ±0.2	4.13 ±0.2	4.21 ±0.4
	a	+3.33	+3.33	+10.91	+13.03	+17.58	+15.45	+23.03	+24.24	+26.06	+25.15	+27.58
	b	N.S.	N.S.	N.S.	N.S.	<0.05	< 0.05	< 0.01	<0.001	<0.001	< 0.001	<0.005

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

Table 25. Alteration in the total activity (units/g wet wt.) of glutaminase in the brain, liver kidney of Heteropneustes fossilis during dehydration (Mean±S.D.)

Tissue	Time (hr) of dehydration											
	0 (control)	3	6	9	12	15	18	21	24	27	30	36
Brain	72.63 ±10.69	71.90 ±6.74	66.56 ±13.29	61.76 ±5.60	58.18 ±4.64	55.08 ±7.24	52.85 ±7.80	51.28 ±9.32	53.36 ±9.50	53.62 ±7.24	50.25 ±5.26	50.54 ±8.25
	a	-1.0	-8.35	-14.97	-19.90	-24.16	-27.23	-29.39	-26.53	-26.17	-30.18	-30.41
	b	N.S.	N.S.	N.S.	<0.025	< 0.02	< 0.01	<0.005	<0.02	< 0.01	< 0.005	< 0.01
Liver	28.72 ±4.5	27.05 ±4.5	27.30 ±5.40	26.95 ±4.5	25.84 ±7.90	22.95 ±2.60	20.68 ±4.40	19.25 ±4.32	22.11 ±2.20	20.50 ±2.72	18.97 ±2.26	17.97 ±3.2
	a	-5.81	-4.94	-6.16	- 10.03	-20.09	-27.99	-32.97	-23.02	-28.62	-33.95	-37.43
	b	N.S.	N.S.	N.S.	N.S.	<0.05	< 0.025	< 0.01	<0.02	<0.01	< 0.005	< 0.005
Kidney	29.16 ±2.9	27.85 ±4.75	24.76 ±1.8	24.50 ±2.5	24.03 ±3.8	24.25 ±2.0	23.75 ±3.19	23.85 ±1.35	22.25 ±2.50	21.81 ±2.30	21.72 ±3.5	20.10 ±4.06
	a	-4.59	-15.18	-16.07	-17.68	-16.92	-18.64	-18.29	-23.77	-25.28	-25.59	-31.14
	b	N.S.	<0.02	< 0.025	<0.05	<0.02	<0.025	< 0.01	< 0.005	<0.005	<0.01	< 0.005

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

Table 26. Alteration in the specific activity (units/mg protein)X10 of glutaminase in the brain, liver and kidney of Heteropneustes fossilis during dehydration (Mean ± S.D.)

Tissue	Time (hr) of dehydration											
	0 (control)	3	6	9	12	15	18	21	24	27	30	36
Brain	5.50 ±0.4	5.2 ±0.4	5.0 ±0.3	4.3 ±0.4	4.5 ±0.5	4.2 ±0.3	4.0 ±0.2	3.9 ±0.1	3.8 ±0.4	3.7 ±0.4	3.7 ±0.2	3.7 ±0.3
	a	-5.45	-9.09	-10.90	-18.18	-23.64	-27.27	-29.09	-30.90	-32.73	-32.73	-32.73
	b	N.S.	N.S.	N.S.	< 0.025	< 0.001	<0.001	<0.001	< 0.001	< 0.001	< 0.001	< 0.001
Liver	1.40 ±0.05	1.40 ±0.1	1.30 ±0.2	1.25 ±0.3	1.14 ±0.2	1.10 ±0.2	1.02 ±0.3	0.9 ±0.2	0.87 ±0.08	0.84 ±0.1	0.86 ±0.1	0.86 ±0.1
	a	0	-7.14	-10.71	-18.57	-21.43	-27.14	-35.71	-37.86	-40.14	-38.57	-38.57
	b	N.S.	N.S.	N.S.	< 0.02	< 0.005	< 0.025	< 0.001	< 0.001	< 0.001	< 0.001	<0.001
Kidney	1.50 ±0.08	1.50 ±0.07	1.45 ±0.07	1.34 ±0.10	1.30 ±0.1	1.20 ±0.1	1.15 ±0.1	1.12 ±0.2	1.10 ±0.2	0.95 ±0.1	0.95 ±0.2	0.95 ±0.1
	a	0	-3.33	-10.66	-13.33	-20.00	-23.33	-25.33	-26.67	-36.67	-36.67	-36.67
	b	N.S.	N.S.	< 0.025	< 0.01	< 0.05	<0.025	< 0.025	< 0.005	<0.001	< 0.001	<0.001

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

Table 27. Alteration in the ratio of glutamine synthetase/glutaminase activity (total and specific) in brain, liver and kidney of Heteropneustes fossilis during dehydration (Mean ± S.D.)

Activity	Tissue	Time (hr) of dehydration											
		0 Control	3	6	9	12	15	18	21	24	27	30	36
Total (GS/Glnase)	Brain	6.20	7.70 (+24.19)	9.11 (+46.94)	10.07 (+62.42)	11.10 (+79.03)	11.55 (+86.29)	12.22 (+97.10)	12.72 (+105.1)	12.30 (+98.39)	12.29 (+98.22)	12.99 (+109.5)	13.16 (+112.2)
	Liver	1.92	2.13 (+10.94)	2.30 (+19.79)	2.58 (+34.38)	3.20 (+66.67)	3.66 (+90.63)	4.06 (+111.4)	4.40 (+129.1)	3.86 (+101.0)	4.32 (+125.0)	4.87 (+153.6)	5.24 (+172.9)
	Kidney	2.08	2.25 (+8.17)	2.55 (+22.60)	2.82 (+35.58)	2.96 (+42.31)	3.03 (+45.67)	3.07 (+47.60)	3.12 (+50.0)	3.58 (+72.12)	3.60 (+73.08)	3.70 (+77.88)	3.97 (+90.87)
Specific (GS/Glnase)	Brain	5.80	7.38 (+27.24)	8.66 (+49.31)	10.20 (+75.86)	10.31 (+77.76)	10.83 (+86.72)	11.45 (+97.41)	11.82 (+103.8)	12.34 (+112.8)	12.76 (+120.0)	12.62 (+117.6)	12.76 (+120.0)
	Liver	2.07	2.19 (+5.80)	2.48 (+19.81)	2.78 (+34.30)	3.80 (+83.57)	4.01 (+93.72)	4.31 (+108.2)	4.81 (+132.3)	5.14 (+148.3)	5.26 (+154.1)	5.41 (+161.3)	5.43 (+162.3)
	Kidney	2.20	2.27 (+3.18)	2.36 (+7.27)	2.73 (+24.09)	2.87 (+30.45)	3.23 (+46.82)	3.31 (+50.45)	3.63 (+65.0)	3.72 (+69.10)	4.38 (+99.10)	4.35 (+97.73)	4.43 (+101.4)

% change from control are given in parentheses.

Table 28. Purification protocol for glutamine synthetase (GS) from the brain of Heteropneustes fossilis.

Purification steps	Total Protein (mg)	GS activity (units)	Specific activity	Purification fold	% recovery
Homogenate	497.25	1,118.81	2.25	1	100
$(NH_4)_2SO_4$ fractionation (20-30%)	350.50	853.05	2.43	0.93	76.25
Dialysis	340.25	1097.10	3.22	1.43	98.05
DEAE Sephacel	16.5	1064.58	64.52	28.67	95.15
Sephadex G-200	5.36	696.03	129.85	57.71	62.21

Table 29. Molecular weight determination by Sephadex G-200 gel filtration of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis

Reference/Unknown (GS) protein	Total Volume (Vt) ml	Void volume (Vo) ml	Elution volume (Ve) ml	$K_{av} = \frac{Ve-Vo}{Vt-Vo}$	log molecular weight	Molecular weight
Thyroglobulin	188	70	70.5	0.004	5.8254	6,69,000
Ferritin	188	70	79.0	0.076	5.6435	4,40,000
Catalase	188	70	99.0	0.245	5.3655	2,32,000
Aldolase	188	70	110.0	0.330	5.1987	1,58,000
Albumin	188	70	135.0	0.550	4.8261	67,000
Unknown (GS)	188	70	84.0, 82.0 → 83.0	0.11, 0.10 → 0.105	5.5925	3,91,300

Table 30. Stability of the activity (%) of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis under various storage condition.

	Temperature	Days								
		0.5	1	2	4	7	15	30	45	60
GS in purifying buffer (This-HCl 20mM pH-7.4)	0°C	100	64	50	0	0	0	0	0	0
(a)	4°C	100	63	48	0	0	0	0	0	0
Same as above (a) + Glycerol (10%) (b)	4°C	100	100	100	100	100	100	95	90	90
GS in purifying buffer containing 0.25 N NaCl(c)	0°C	100	100	100	100	100	100	100	100	92
	4°C	100	100	100	100	100	100	100	100	90
Same as above(c) + Glycerol (10%)	4°C	100	100	100	100	100	100	110	110	109
Same as above (c) + Albumin (0.25%)	4°C	100	100	100	100	100	98	95	97	95

Table 31. Effect of pH and temperature on the activity of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

pH	Enzyme activity (units)	Temperature °C	Enzyme activity (units)
5	6.90	5	9.85
6	9.57	10	14.78
7	35.05	20	19.00
7.2	35.89	30	30.97
7.5	41.09	40	40.11
8	39.69	45	39.83
8.5	40.68	50	30.40
9	38.99	60	9.15
10	40.11	70	4.22
11	38.99		
12	33.92		

Table 32. *Thermal stability and half life of glutamine synthetase (GS) purified from the brain of* Heteropneustes fossilis *in 20mM Tris-HCl buffer pH 7.4.*

Thermal stability		Half life	
Temperature (°C) for 30 min	Enzyme activity (units)	Incubation time (min) at 45°C	Enzyme activity (units)
5	38.75(100)	5	38.25(100)
10	38.75(100)	10	38.25(100)
20	38.95(100.52)	15	34.80(90.98)
30	38.92(100.43)	20	24.86(64.99)
40	19.76(50.99)	25	19.12(49.99)
45	13.56(34.99)	30	1 3.29(34.75)
50	2.91 (7.51)	40	1.72 (4.49)
60	0.47 (1.21)	50	0 (0)

% activity is given in parentheses.

Table 33. Factors protecting thermal denaturation of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

	Temperature °C						
	0 (Control)	5	10	20	30	40	45
Enzyme (E)	38.75 (100)	38.75 (100)	38.75 (100)	38.95 (100.5)	38.92 (100.4)	19.76 (50.99)	13.56 (34.99)
E+Glutamate	39.05 (100)	39.05 (100)	38.95 (99.71)	38.75 (99.23)	39.02 (99.92)	38.65 (98.97)	38.99 (98.70)
E+$MgCl_2$	38.65 (100)	38.65 (100)	39.02 (100.9)	38.85 (100.5)	36.79 (95.19)	28.98 (74.98)	27.20 (70.37)
E+ATP	37.25 (100)	37.25 (100)	37.25 (100)	37.20 (99.86)	37.30 (100)	33.52 (89.98)	23.46 (62.97)
E+ATP+$MgCl_2$	39.30 (100)	39.30 (100)	39.25 (99.87)	39.35 (100.1)	38.99 (99.21)	39.15 (99.61)	39.30 (100)
E+2 mercapto ethanol	36.95 (100)	36.95 (100)	20.20 (54.67)	15.35 (41.54)	0 (0)	0 (0)	0 (0)
E+Hydroxylamine	37.95 (100)	37.95 (100)	38.05 (100.2)	37.90 (99.86)	32.25 (84.98)	25.80 (67.98)	10.24 (26.99)

% activity is given in parentheses.

Table 34. Requirement of substrate/cofactors for the activity glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

	Enzyme activity (units)	% activity
Complete system (C) [Buffer + Enzyme + Mg^{+2} + 2-mercaptoethanol + Hydroxylamine + ATP + glutamate]	35.37	100
C-Hydroxylamine	0	0
C-Mg^{+2}	0	0
C-Glutamate	0	0
C-ATP	0	0
C-2-mercaptoethanol	35.3	100

Table 35. Factors protecting the activity at 30°C against 2-mercaptoethanol denaturation of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

Sequence of addition of different chemicals	Enzyme activity (units)	% activity
Enzyme (E) (Control)	32.5	100
E + 2-mercaptoethanol	0	0
E + Mg^{2+} + 2-mercaptoethanol	31.85	98
E + ATP + 2-mercaptoethanol	27.95	86
E + ATP + Mg^{2+} + 2-mercaptoethanol	30.87	94.98
E + 2-mercaptoethnol + Mg^{2+} + ATP	31.20	96
E + Glutamate + 2-mercaptoethanol	19.5	60

Table 36. Km and V_{max} of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

Substrates/cofactors	Km (mM)	V_{max}
Mg^{2+}	6.25	40.00
L-glutamate	5.0	40.00
Hydroxylamine	0.5	43.47
ATP	2.5	43.47

Table 37. Effect of metal ion on the activity of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

Metal ion	% GS activity at different concentrations of metal ions[a]	
	5mM	10mM
Na^+	100	100
K^+	100	100
Li^+	100	100
Co^{2+}	72.50	62.39
Cd^{2+}	49.49	0
Cu^{2+}	47.06	14.63
Mn^{2+}	21.91	10.14
Ca^{2+}	58.01	17.51
Zn^{2+}	15.62	4.56
Po_4^{2-}	78.50	6.02

a - GS activity with normal assay condition was taken as 100%.

Table 38. Effect of amino acids on the activity of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

Amino acids	% activity at different concentrations of amino acids[a]		
	5mM	50mM	125mM
L-Glutamine	100	98.07	95.12
L-Aspartic acid	94.90	85.28	64.87
L-Aspargine	97.02	87.77	72.02
L-Ornithine	79.63	65.29	32.45
L-Citrulline	92.80	91.68	69.94
L-Alanine	96.98	69.98	55.02
L-Arginine	86.22	72.23	51.18
L-Glycine	73.93	10.85	0
Carbamylphosphate	69.98	49.76	0

a-activity of GS without any amino acids except the substrate (glutamate) with normal assay condition was taken as 100%.

Table 39. Effect of some nucleotides on the activity of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis.

	Nucleotide (mM)	% activity
Control	0	100
IMP	0.05	100
	0.5	95.87
	2.0	78.81
	10.0	81.55
	20.0	75.30
	40.0	68.22
IMP	0.05	91.72
	0.5	80.25
	2.0	65.32
	10.0	35.30
	20.0	26.39
	40.0	19.07
ADP	0.05	88.43
	0.5	45.19
	2.0	40.36
	10.0	26.41
	2.0	19.34
	40.0	16.25

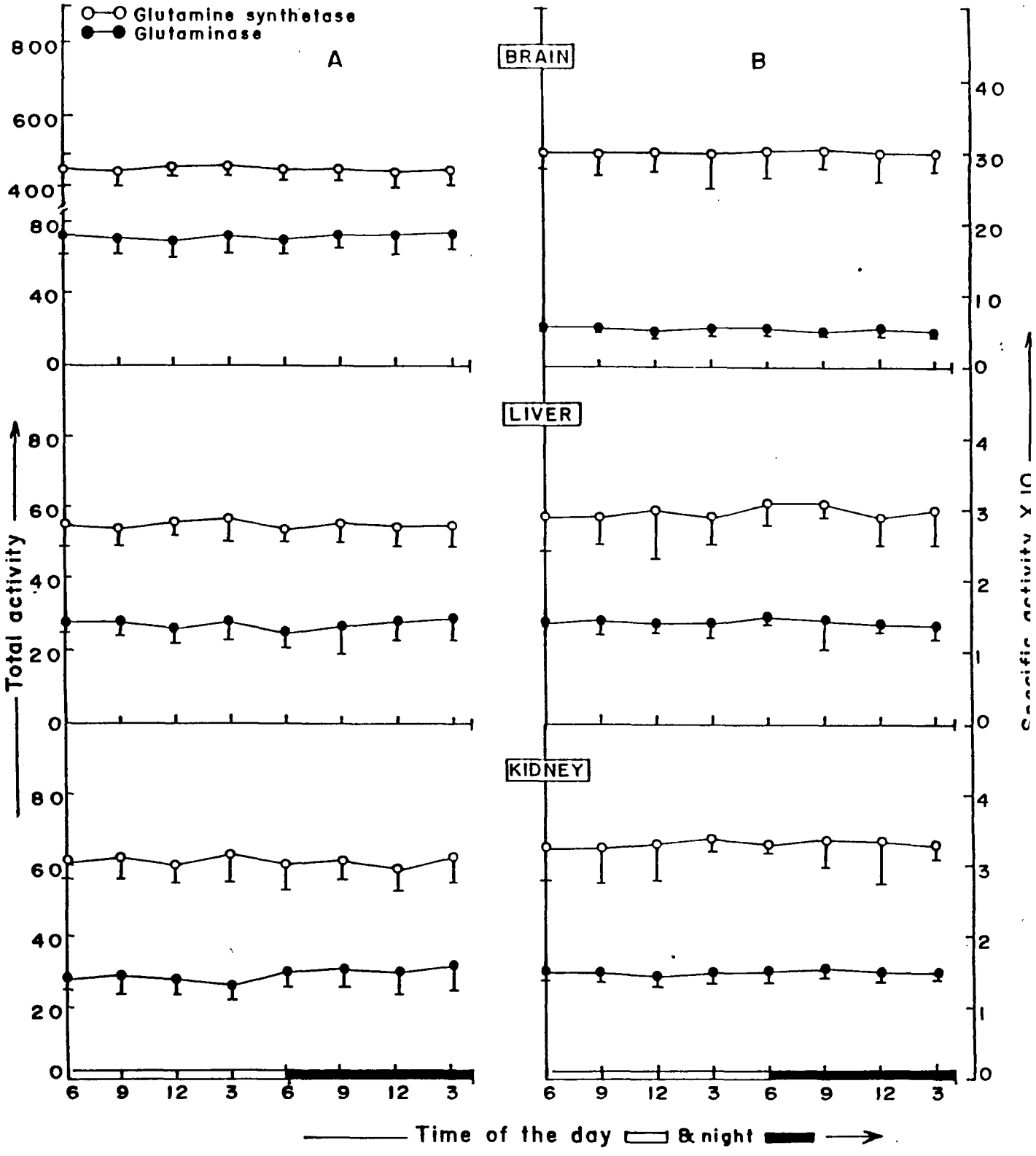


Fig.1 Glutamine synthetase and glutaminase activity in brain, liver and kidney of Heteropneustes fossilis during 24hr cycle.
(A) Total activity (units/g wet wt.), (B) Specific activity (units/mg protein).

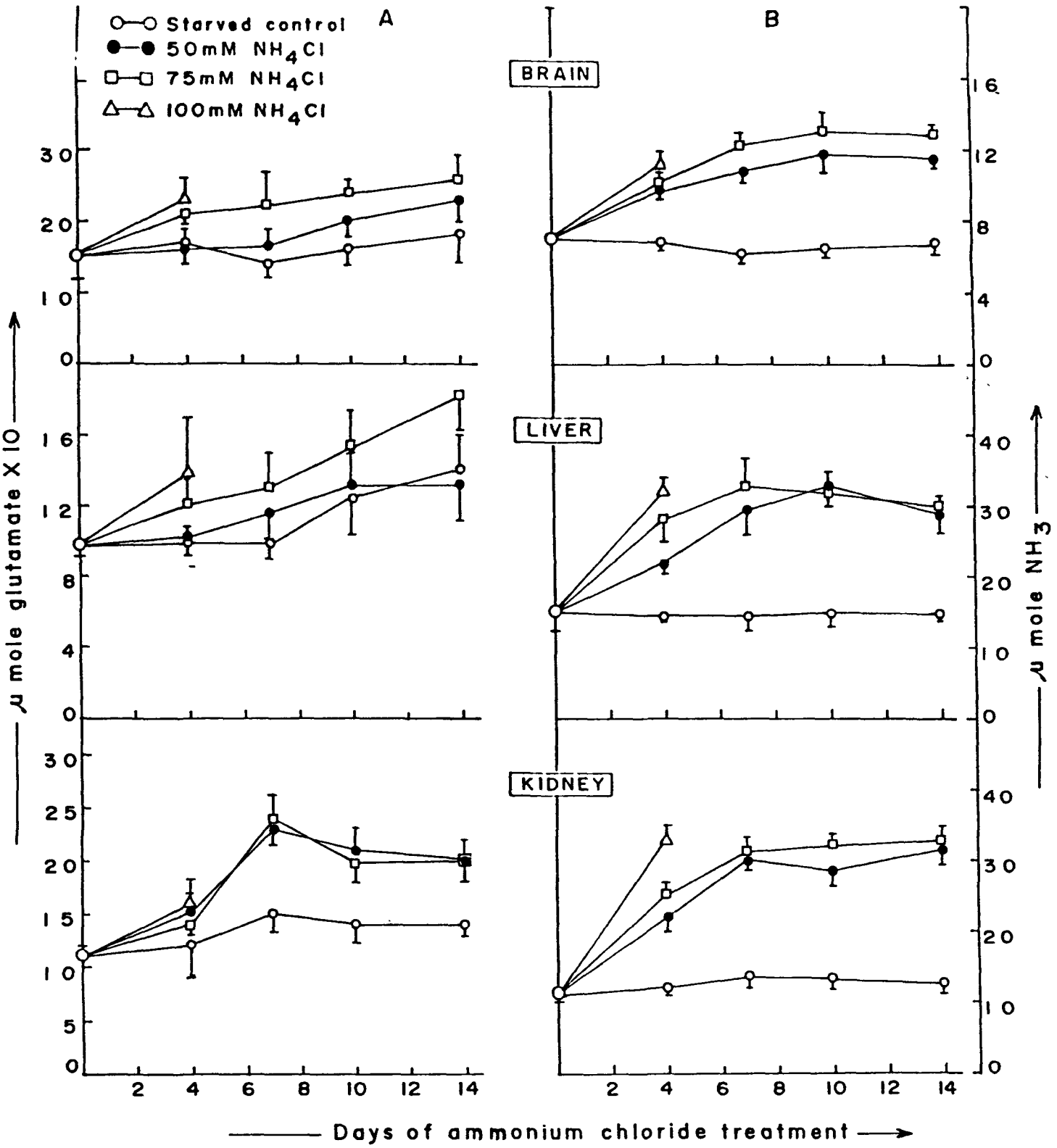


Fig. 2 Alteration in the concentration (μ moles/g wet wt.) of glutamate (A) and ammonia (B) in brain, liver and kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride.

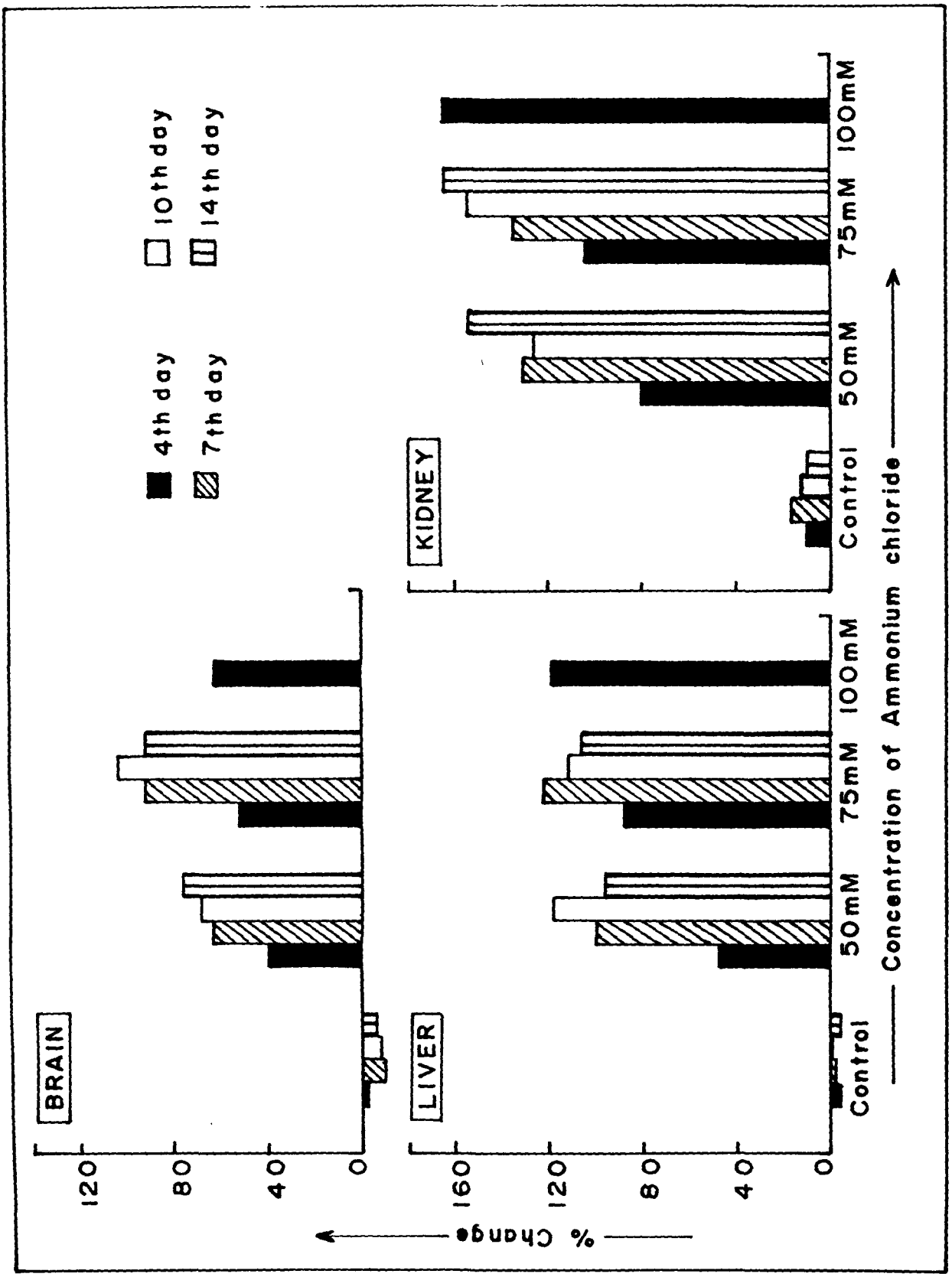


Fig. 3 Percent (%) change in the concentration (μ mole/g wet wt.) of ammonia in brain, liver and kidney of Heteropneustes fossilis during starvation (control) and exposure to various concentrations of ammonium chloride.

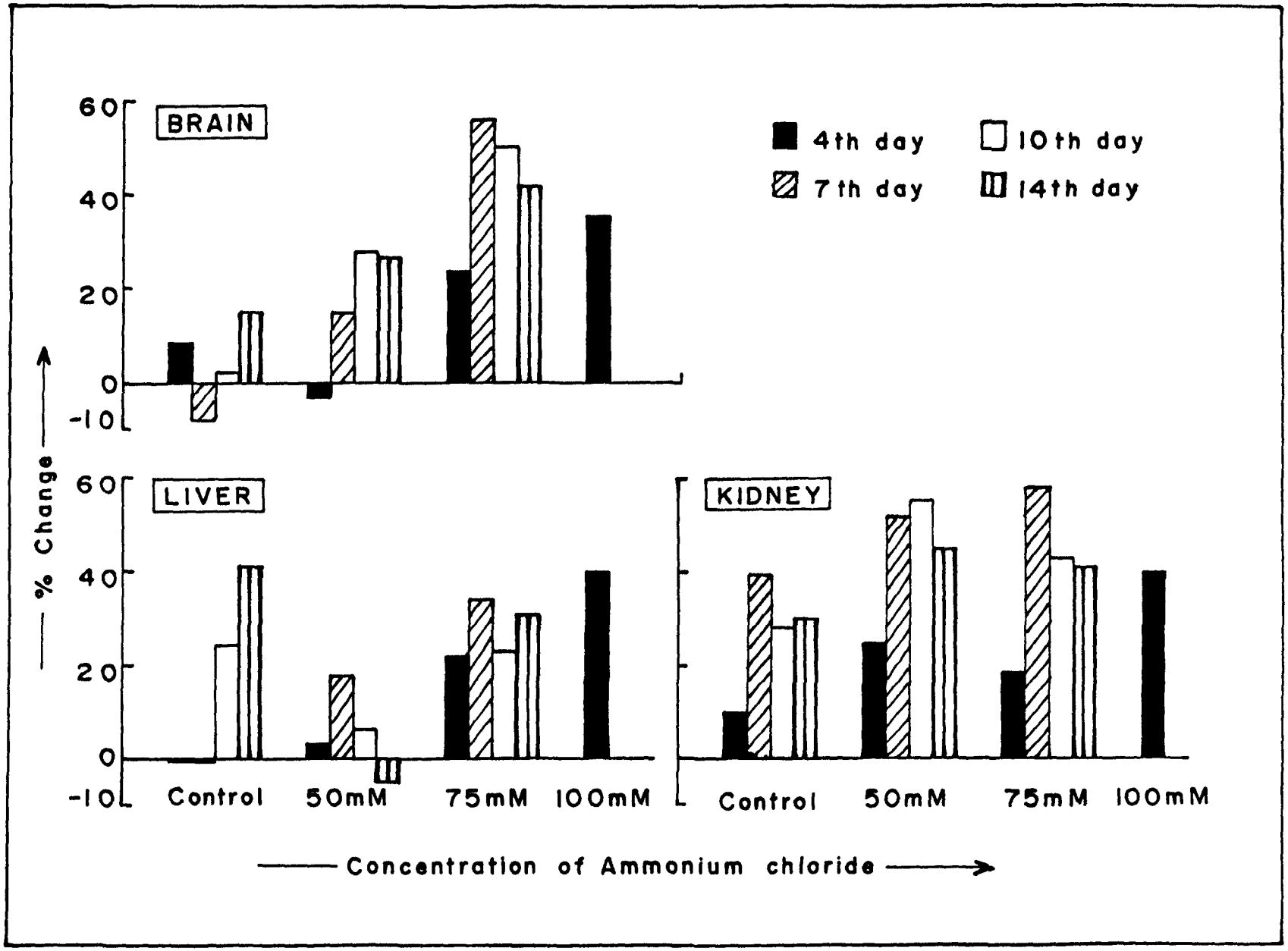


Fig.4 Percent (%) change in the concentration (μmole/g wet wt.) of glutamate in brain, liver and kidney of Heteropneustes fossilis during starvation (control) and exposure to various concentrations of ammonium chloride.

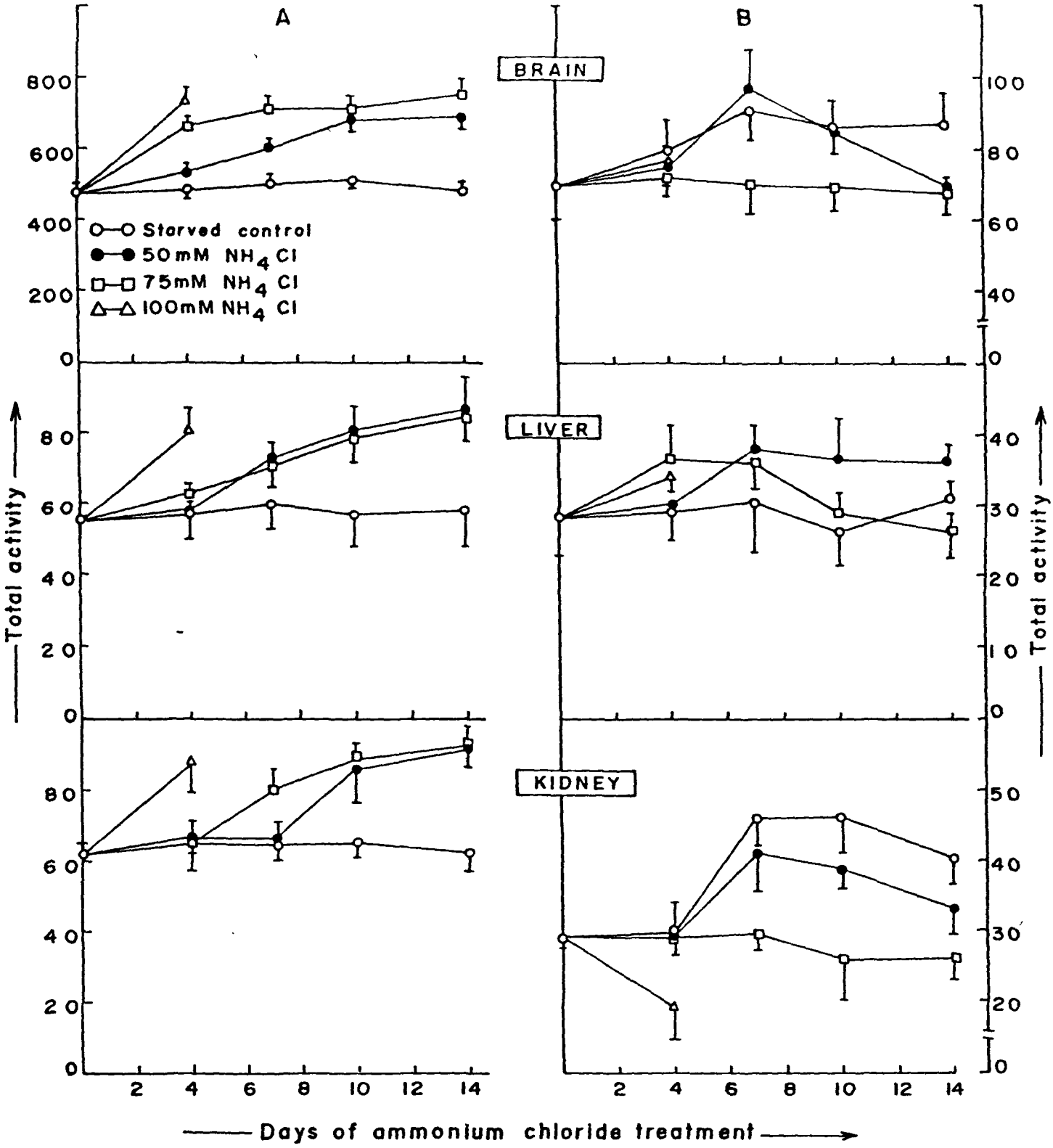


Fig. 5. Alteration in the total activity (units/g wet wt.) of glutamine synthetase (A) and glutaminase (B) in brain, liver and kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride.

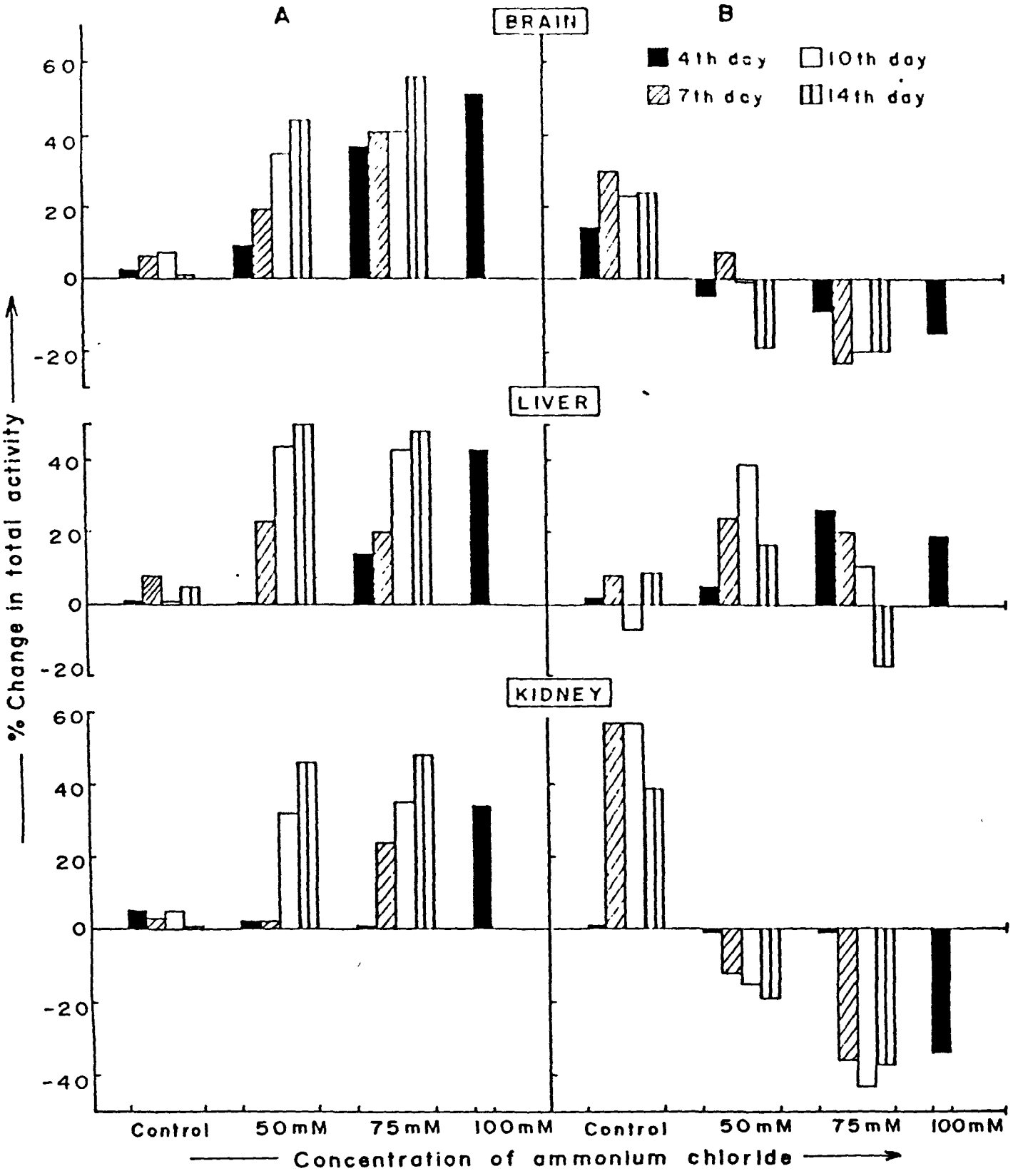


Fig. 6. Percent (%) change in the total activity (units/g wet wt.) of glutamine synthetase (A) and glutaminase (B) in brain, liver and kidney of Heteropneustes fossilis during starvation (control) and exposure to various concentrations of ammonium chloride.

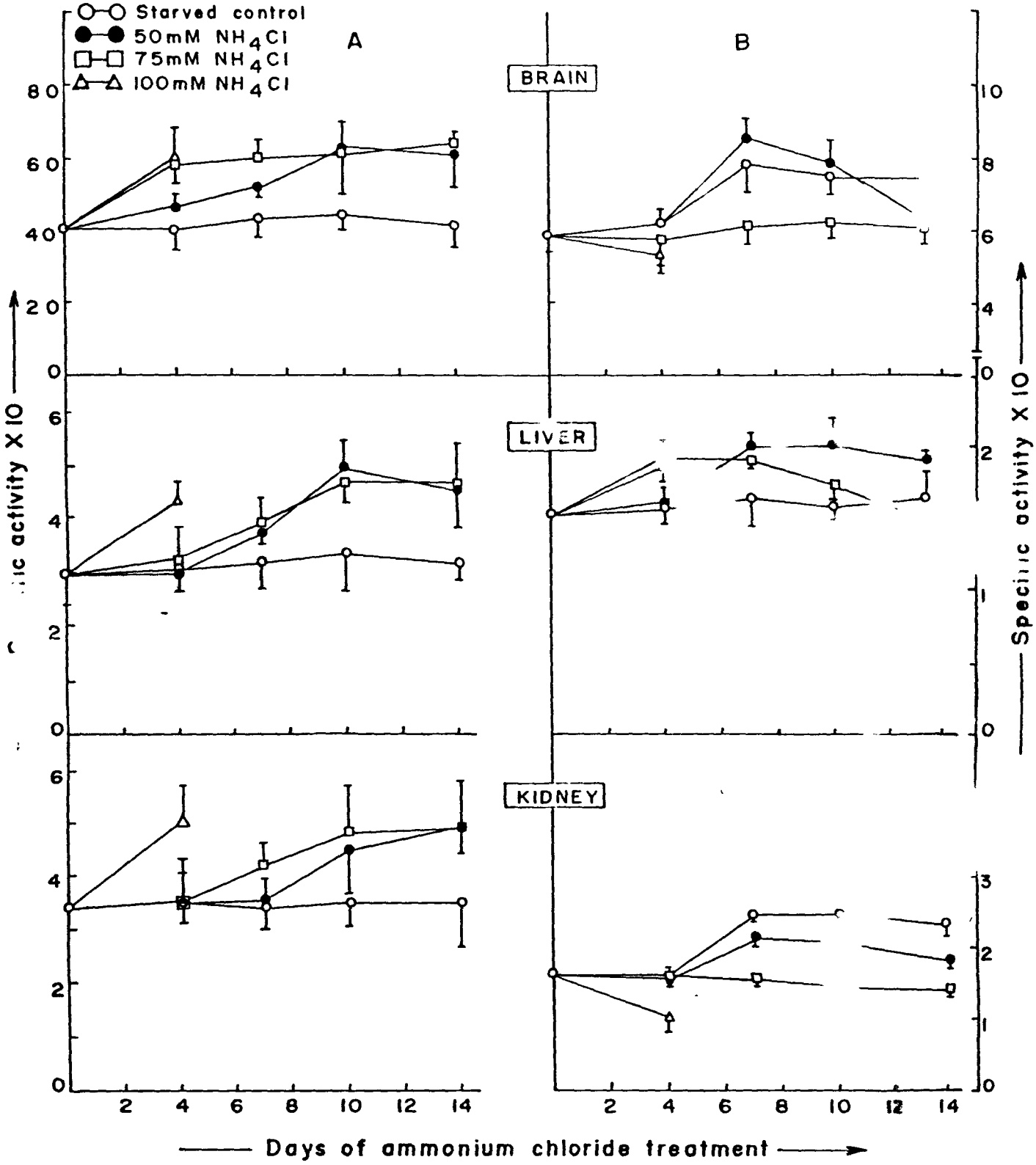


Fig. 7 Alteration in the specific activity (units/mg protein) of glutamine synthetase (A) and glutaminase (B) in the brain, liver and kidney of Heteropneustes fossilis during starvation and exposure to various concentrations of ammonium chloride.

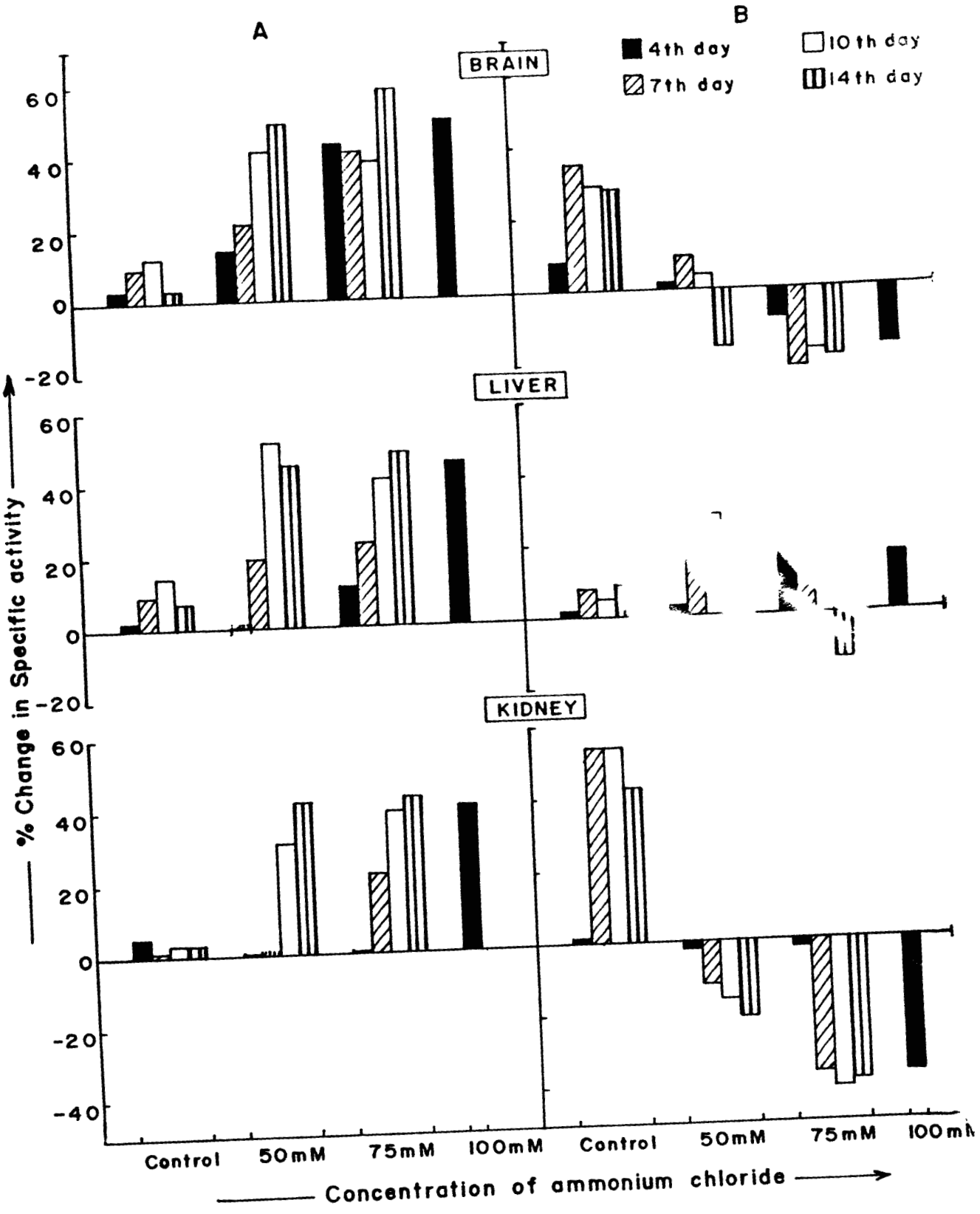


Fig. 8 Percent (%) change in the specific activity (units/mg protein) of glutamine s thetase(A) and glutaminase (B) in brain, liver and kidney of Heteropn fossilis during starvation (control) and exposure to various concentratio s ammonium chloride.

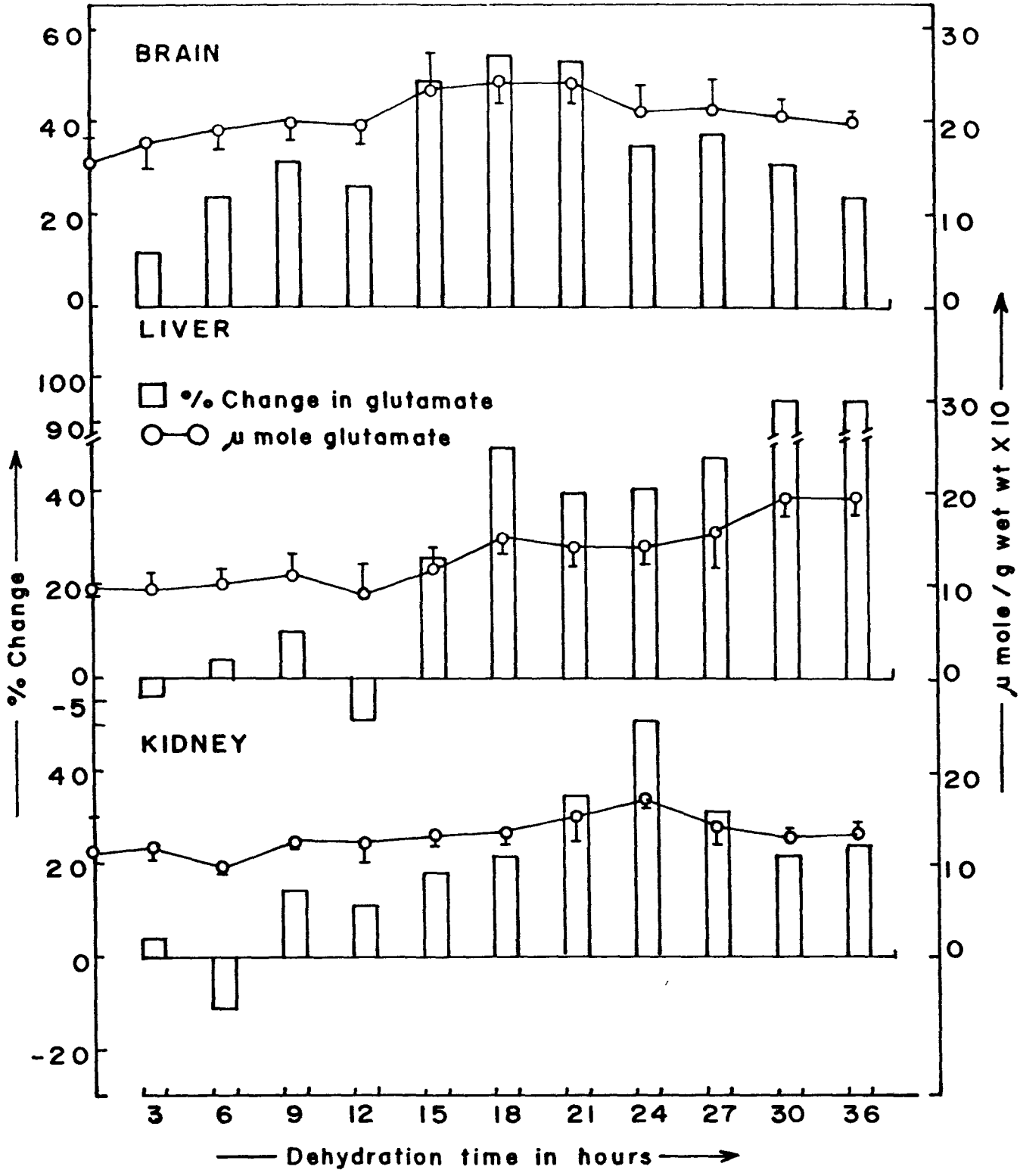


Fig.9 Effect of dehydration on the concentration (mole/g wet wt.) and % change of glutamate in brain, liver and kidney of Heteropneustes fossilis.

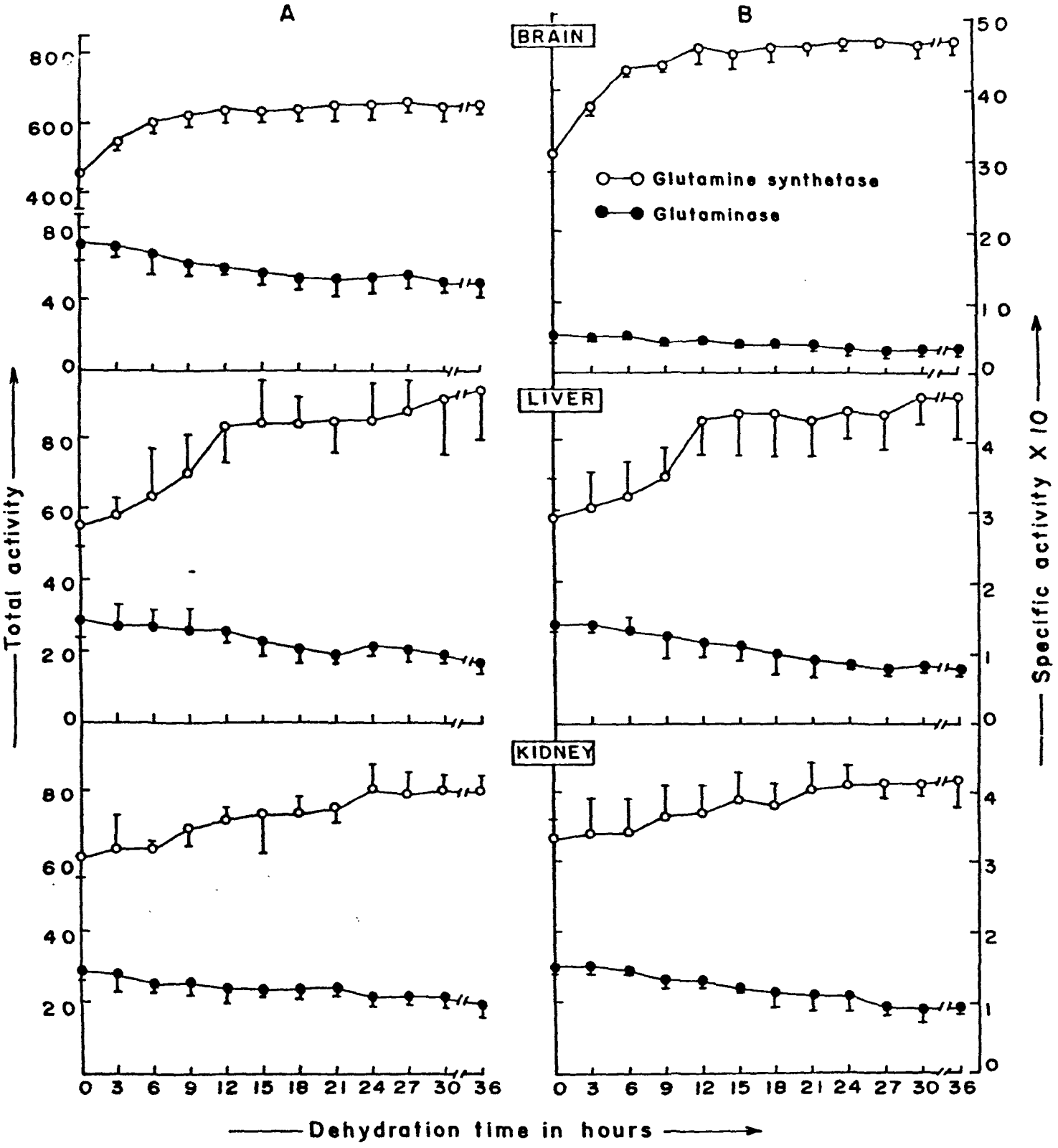


Fig.10 Effect of dehydration on the activity of glutamine synthetase and glutaminase in brain, liver and kidney of Heteropneustes fossilis.
(A) Total activity (units/g wet wt.), (B) Specific activity (units/mg protein).

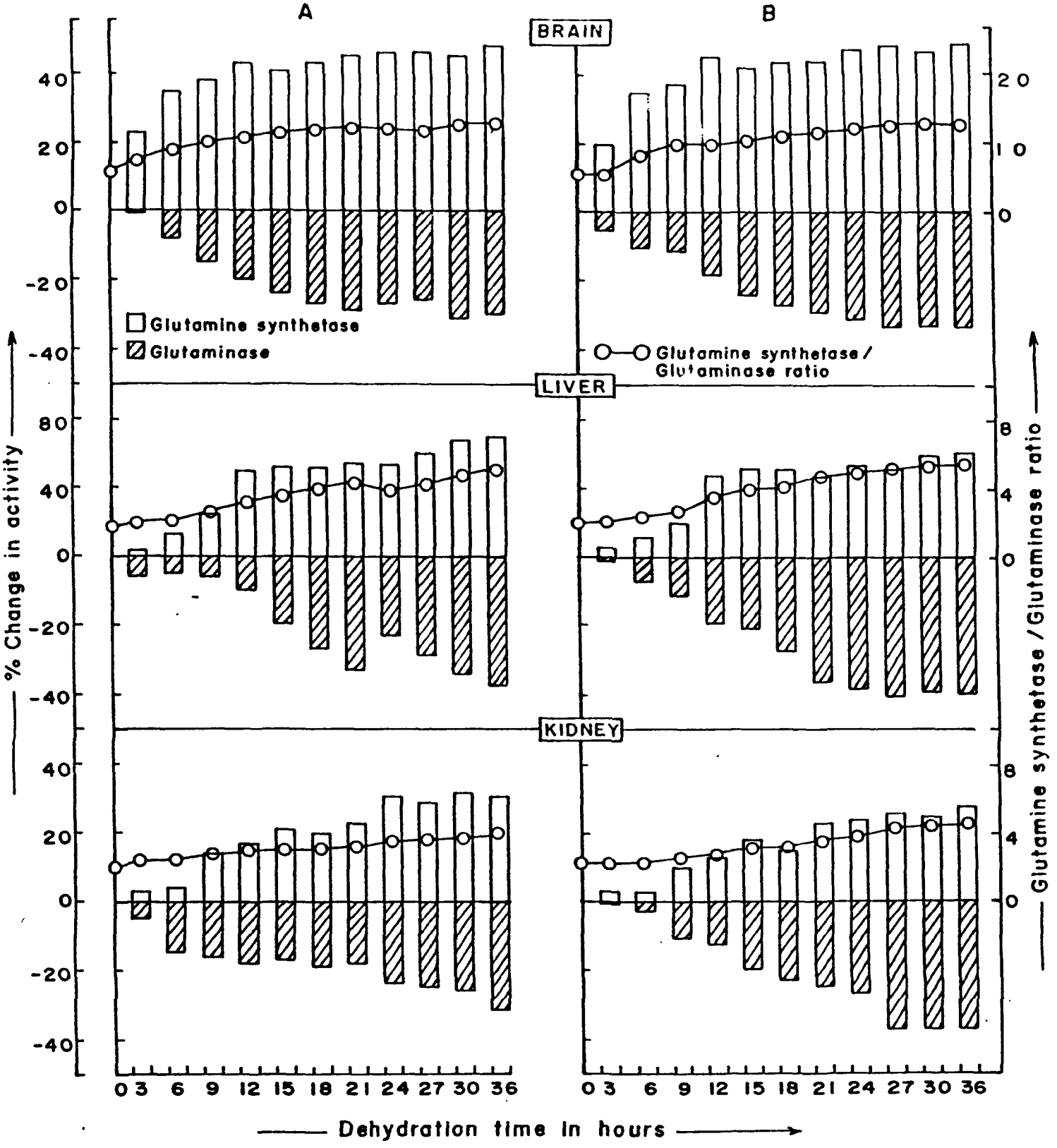


Fig. 11 Effect of dehydration on the % change and the ratio of glutamine synthetase/ glutaminase activity in brain, liver and kidney of Heteropneustes fossilis.(A) Total activity (units/g wet wt.), (B) Specific activity (units/mg protein).

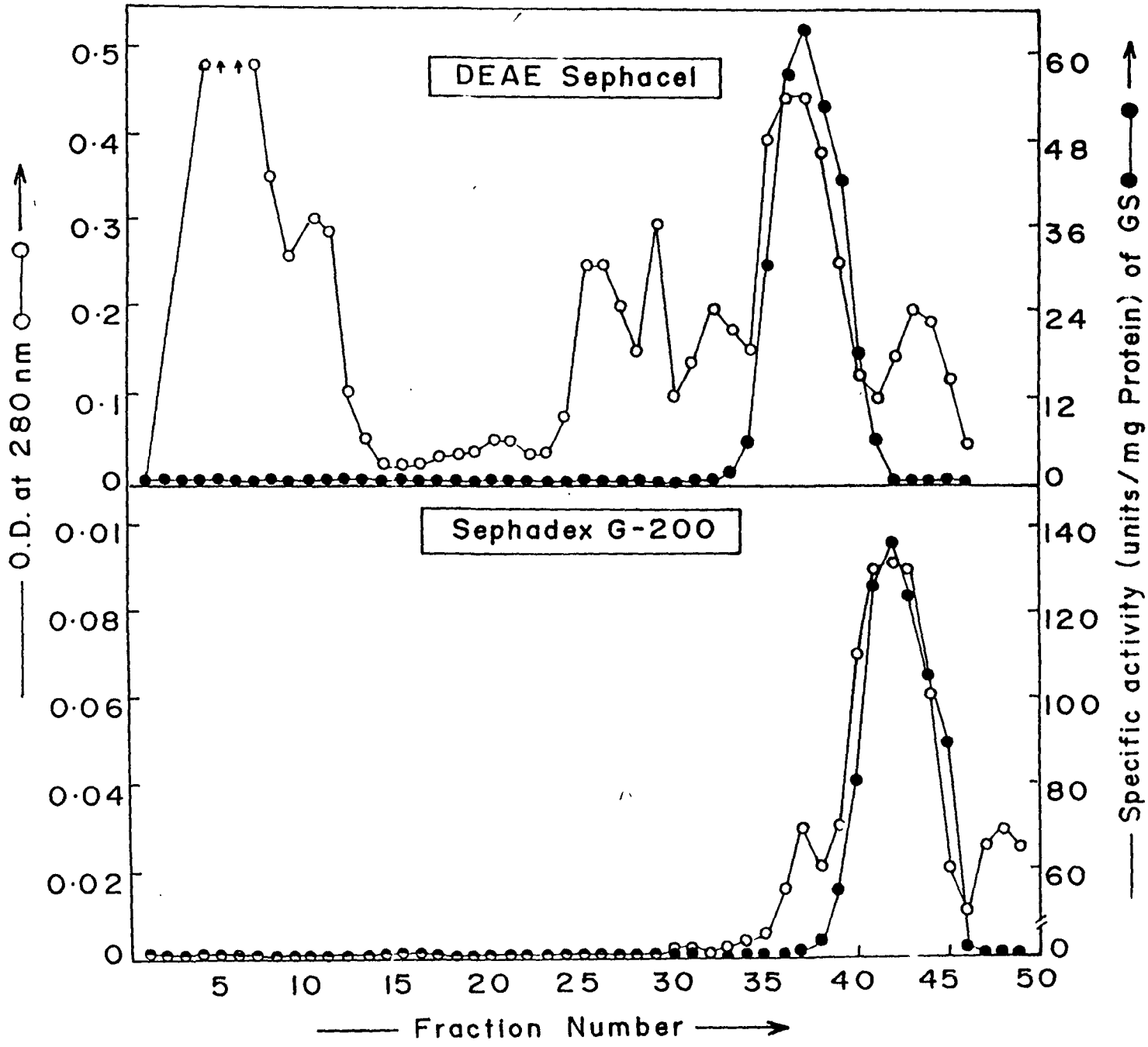


Fig.12 Elution pattern of glutamine synthetase (GS) from DEAE sephacel and sephadex G-200 column during purification from the brain of Heteropneustes fossilis.

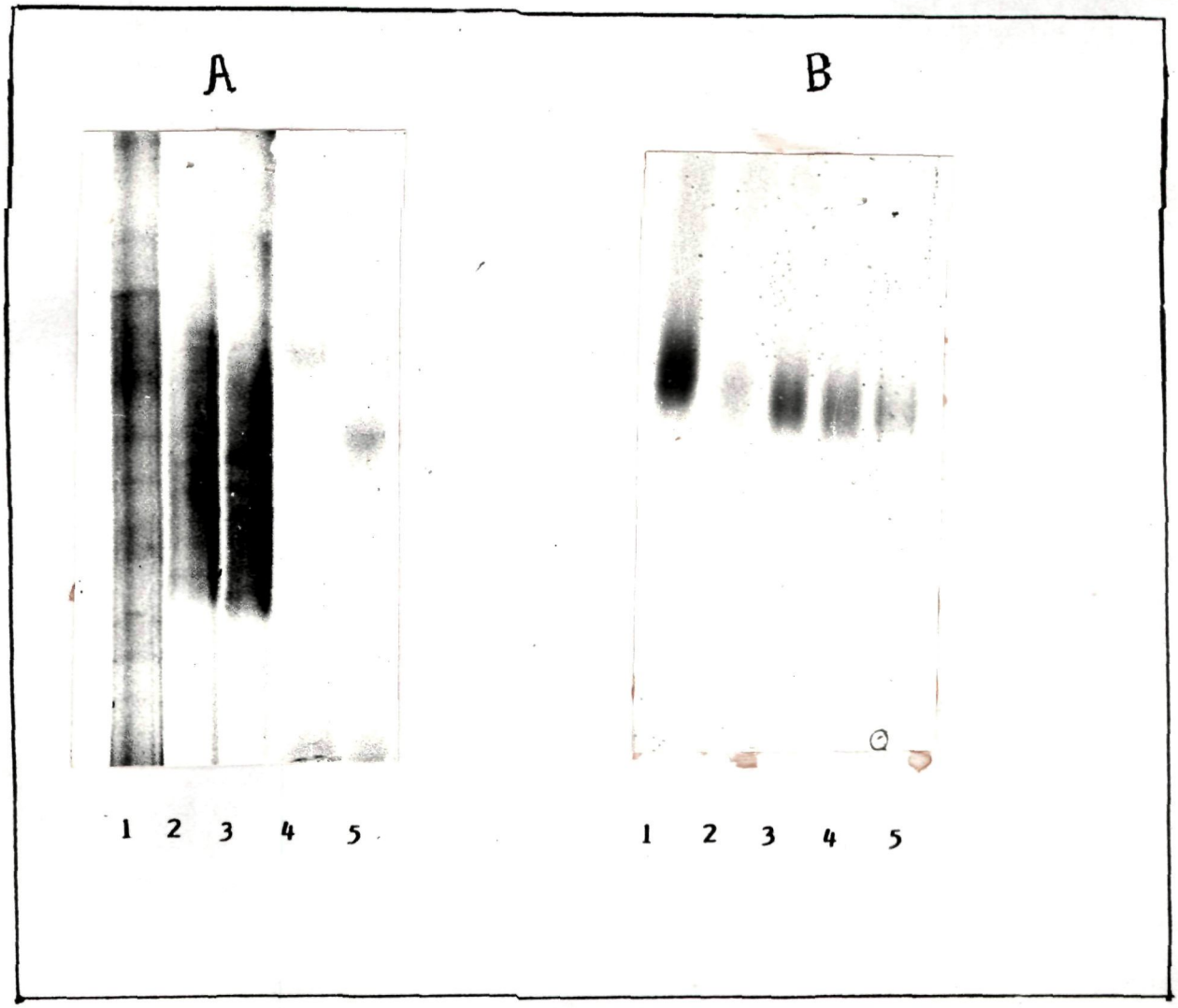


Fig.13 Protein staining (A) and specific staining (B) of GS on Polyacrylamide gel electrophoresis at different stages of purification

1. Homogenate 2. $(NH_4)_2SO_4$ fractionation 3.Dialysis
4. DEAE Sephacel 5. Sephadex G-200.

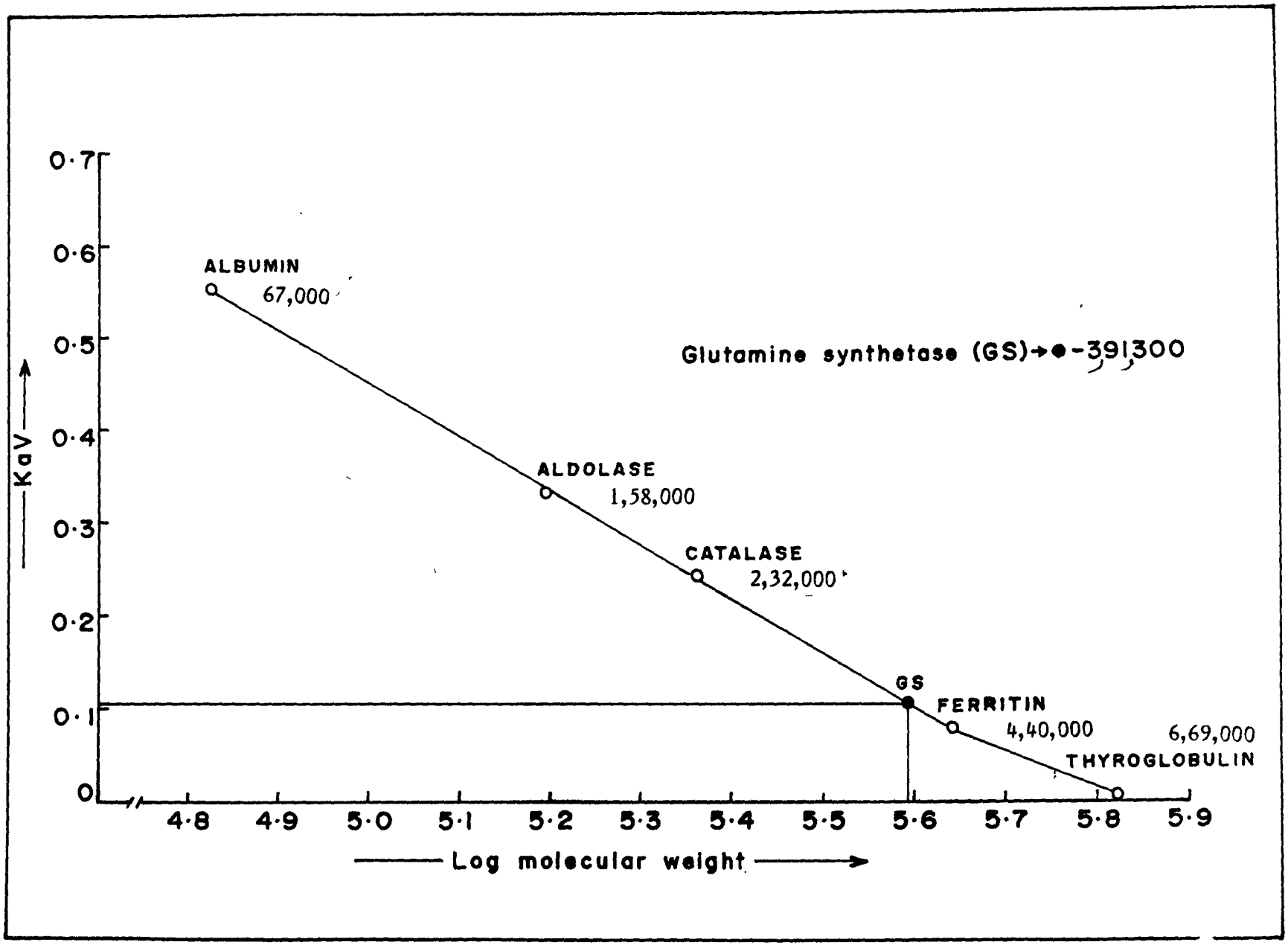


Fig.14 Determination of molecular weight of glutamine synthetase (GS) purified from the brain of Heteropneustes fossilis by SephadexG-200 gel filtration.

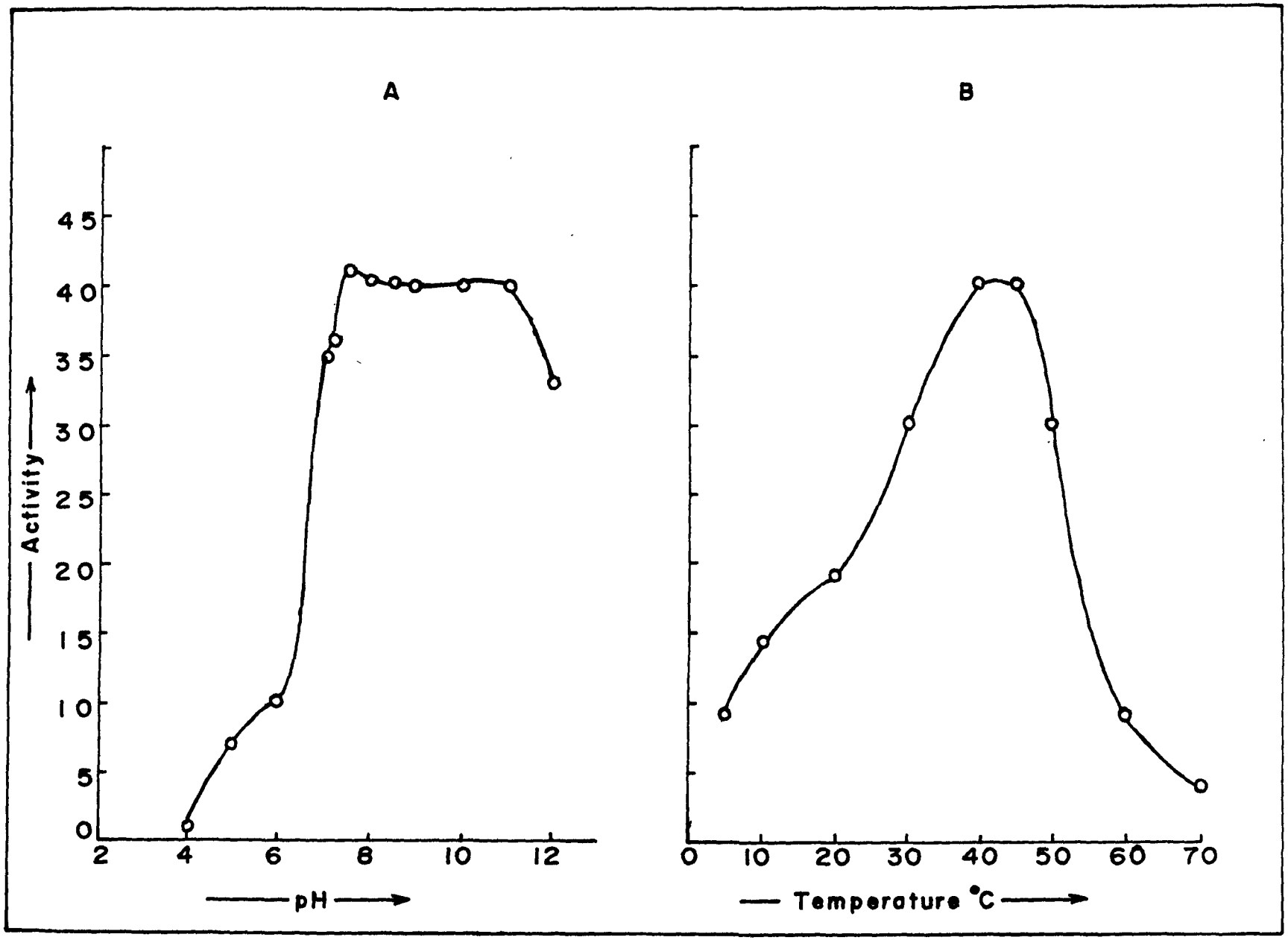


Fig.15 Effect of assay pH (A) and temperature (B) on the activity (μmole γ-glutamyl hydroxamate formed /ml./hr) of glutamine synthetase purified from Heteropneustes fossilis brain.

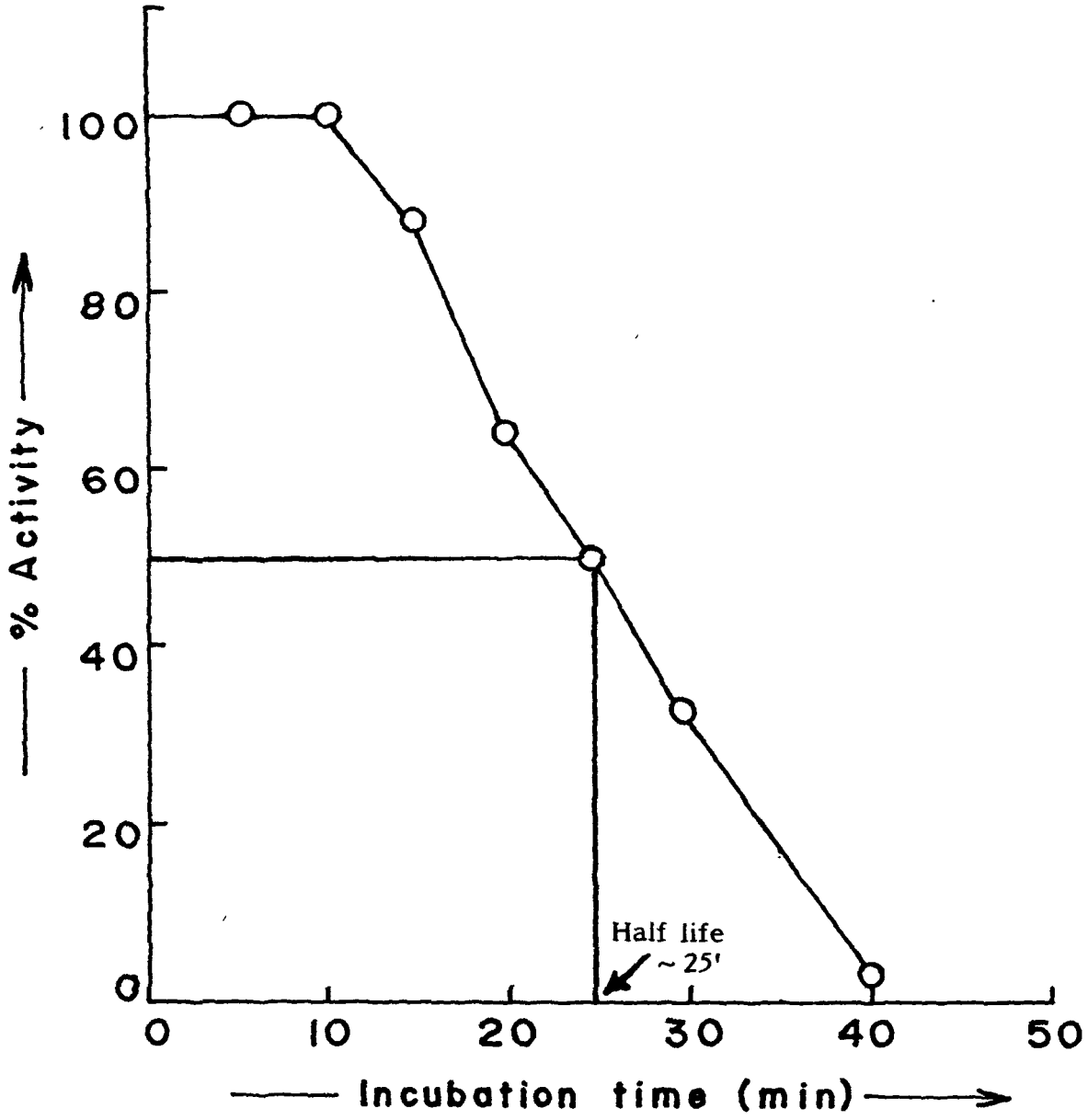


Fig. 16 Determination of half life of glutamine synthetase (purified from Heteropneustes fossilis brain) pre-incubated at 45°C for different periods of time.

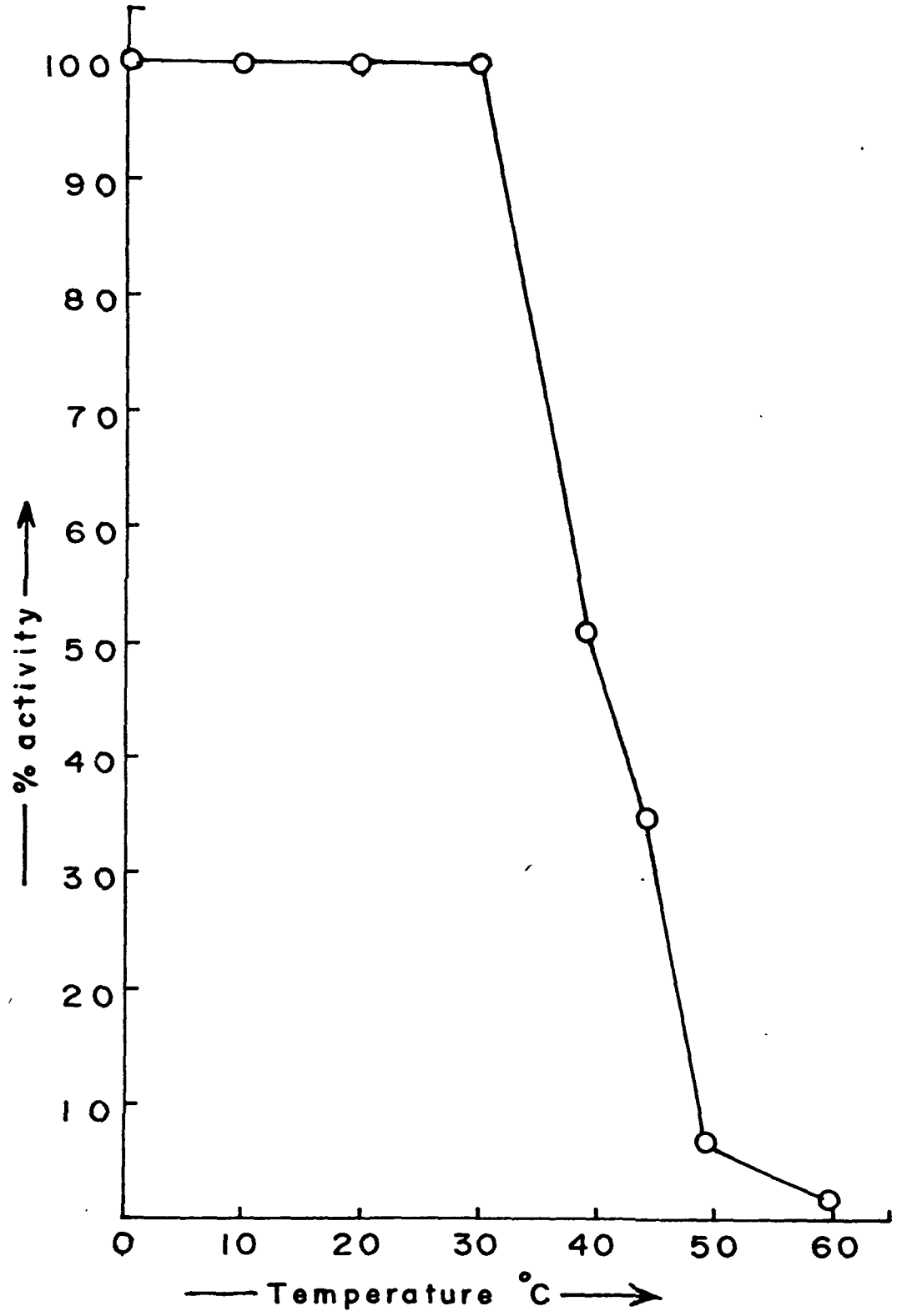


Fig. 17 Percent (%) change in the activity of glutamine synthetase (purified from Heteropneustes fossilis brain) pre-incubated for 30 min. at various temperatures.

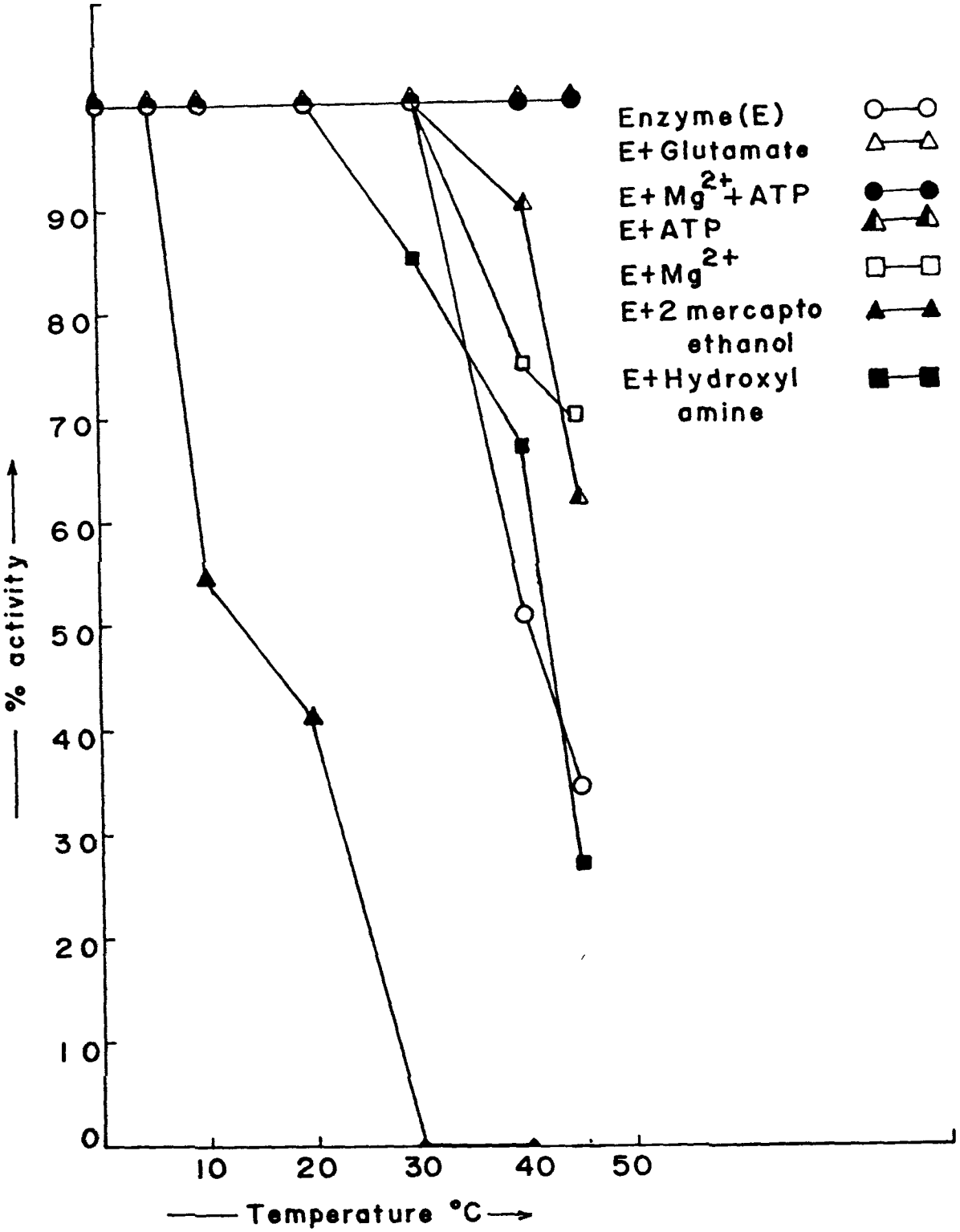


Fig.18 Percent (%) change in the activity of glutamine synthetase (purified from Heteropneustes fossilis brain) pre-incubated at different temperature for 30 min. with various combinations of substrates and cofactors.

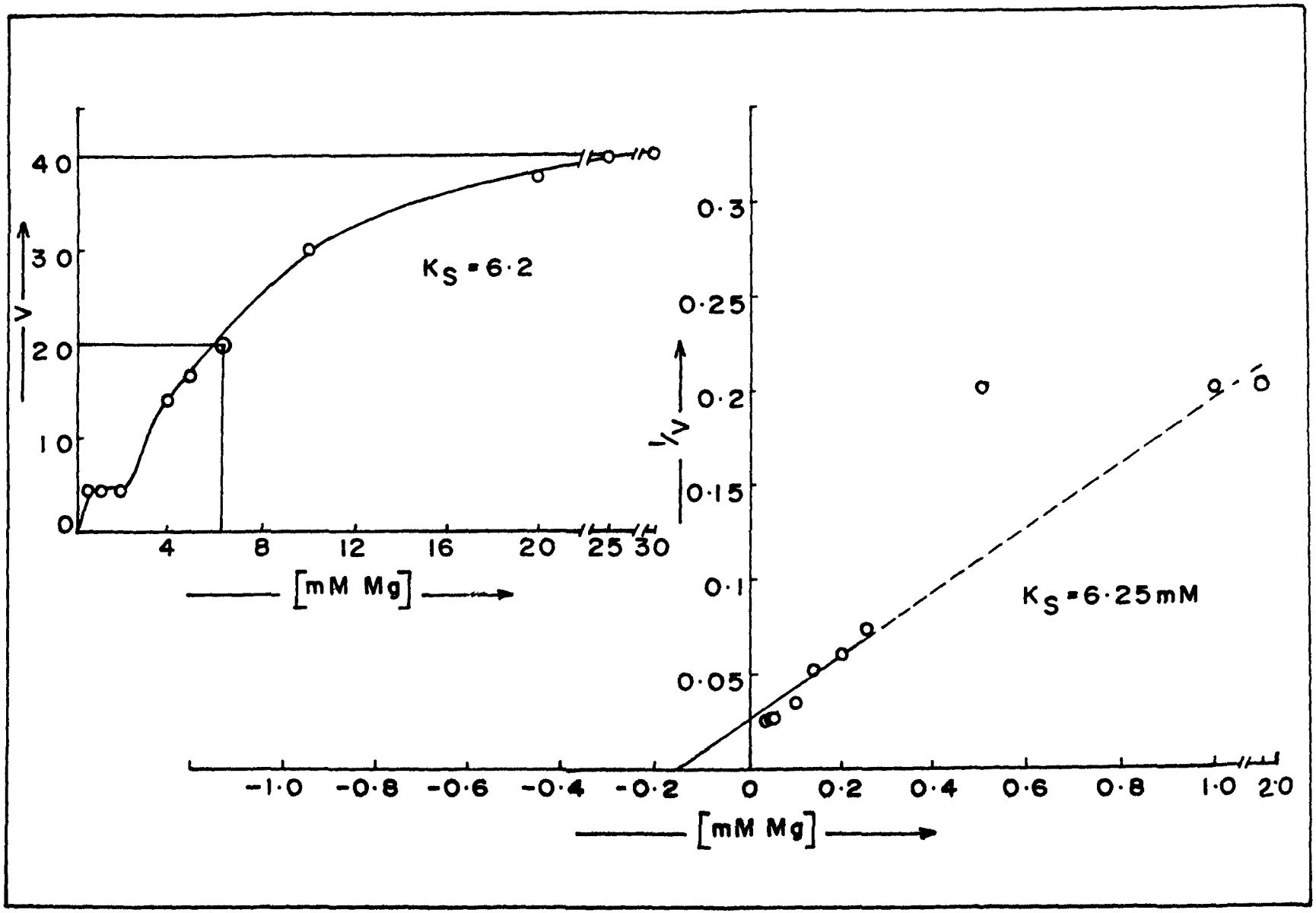


Fig.19 Determination of Ks of glutamine synthetase (purified from Heteropneustes fossilis brain) for Mg^{+2} by Lineweaver-Burk plot. (Inset is Michaelis-Menten plot).

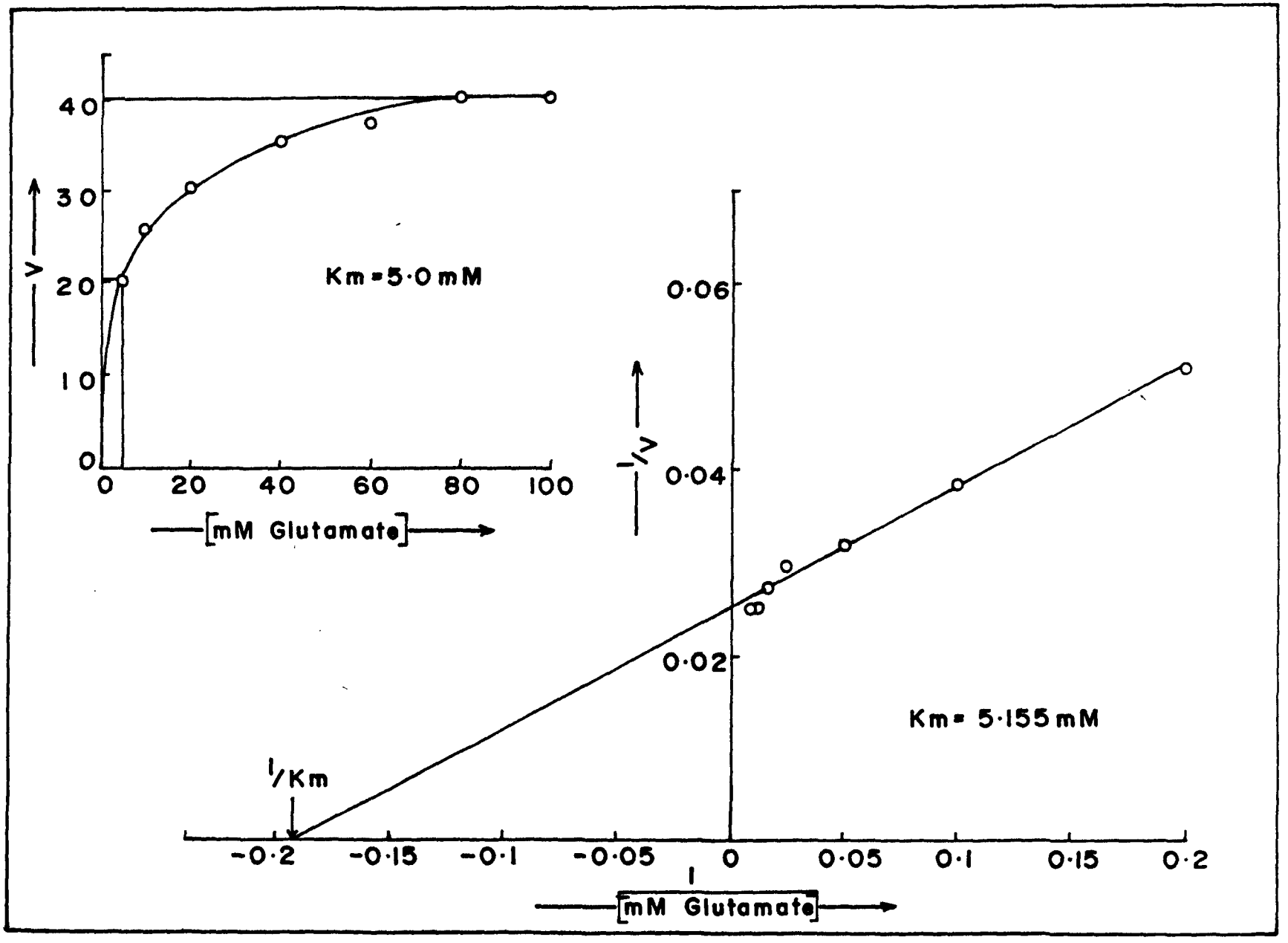


Fig.20 Determination of Km of glutamine synthetase (purified from Heteropneustes fossilis brain) for glutamate by Lineweaver-Burk plot (Inset is Michaelis-Menten plot).

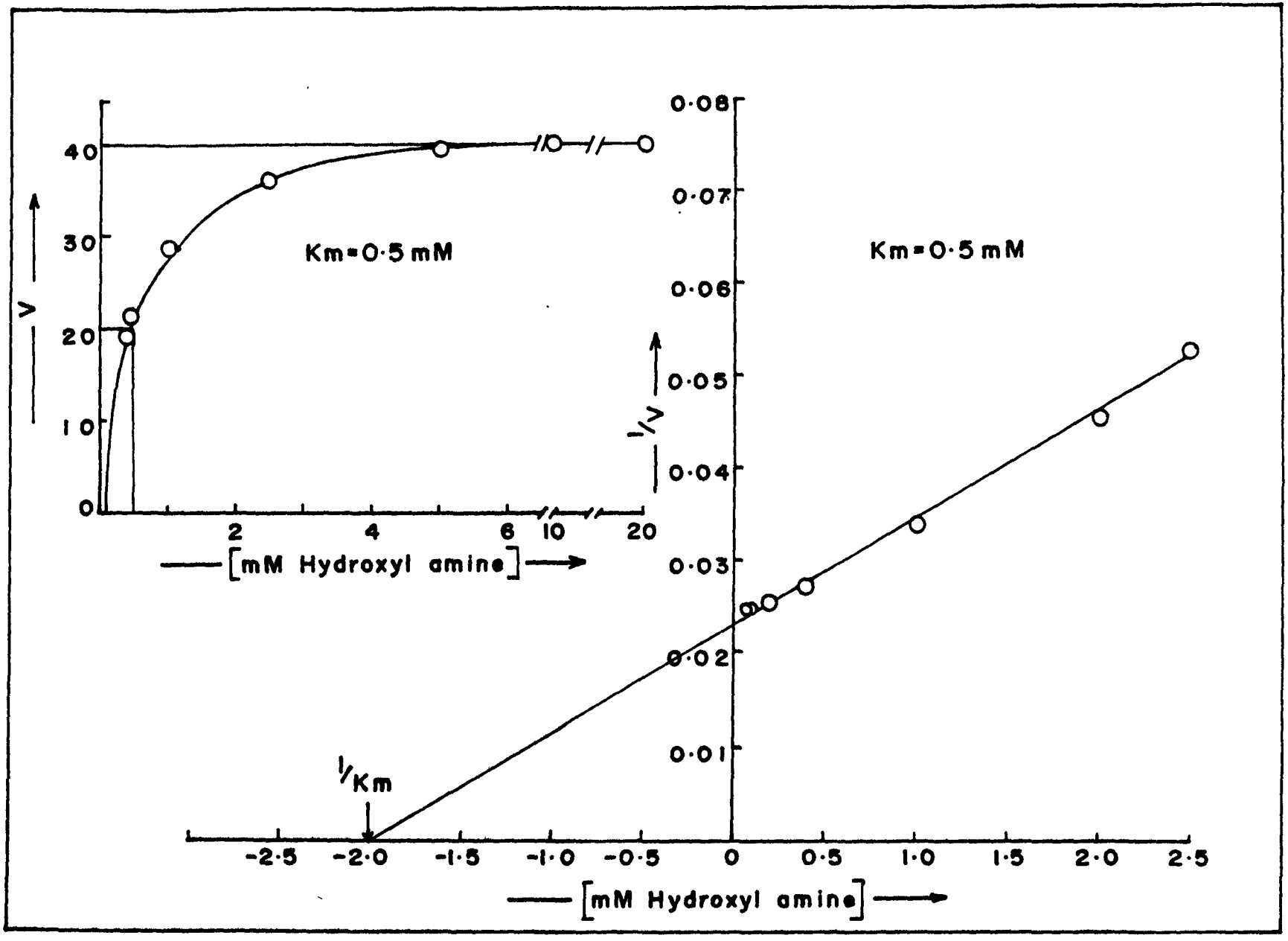


Fig.21 Determination of Km of glutamine synthetase (purified from Heteropneustes fossilis brain) for hydroxylamine by Lineweaver-Burk plot. (In set is Michaelis-Menten plot).

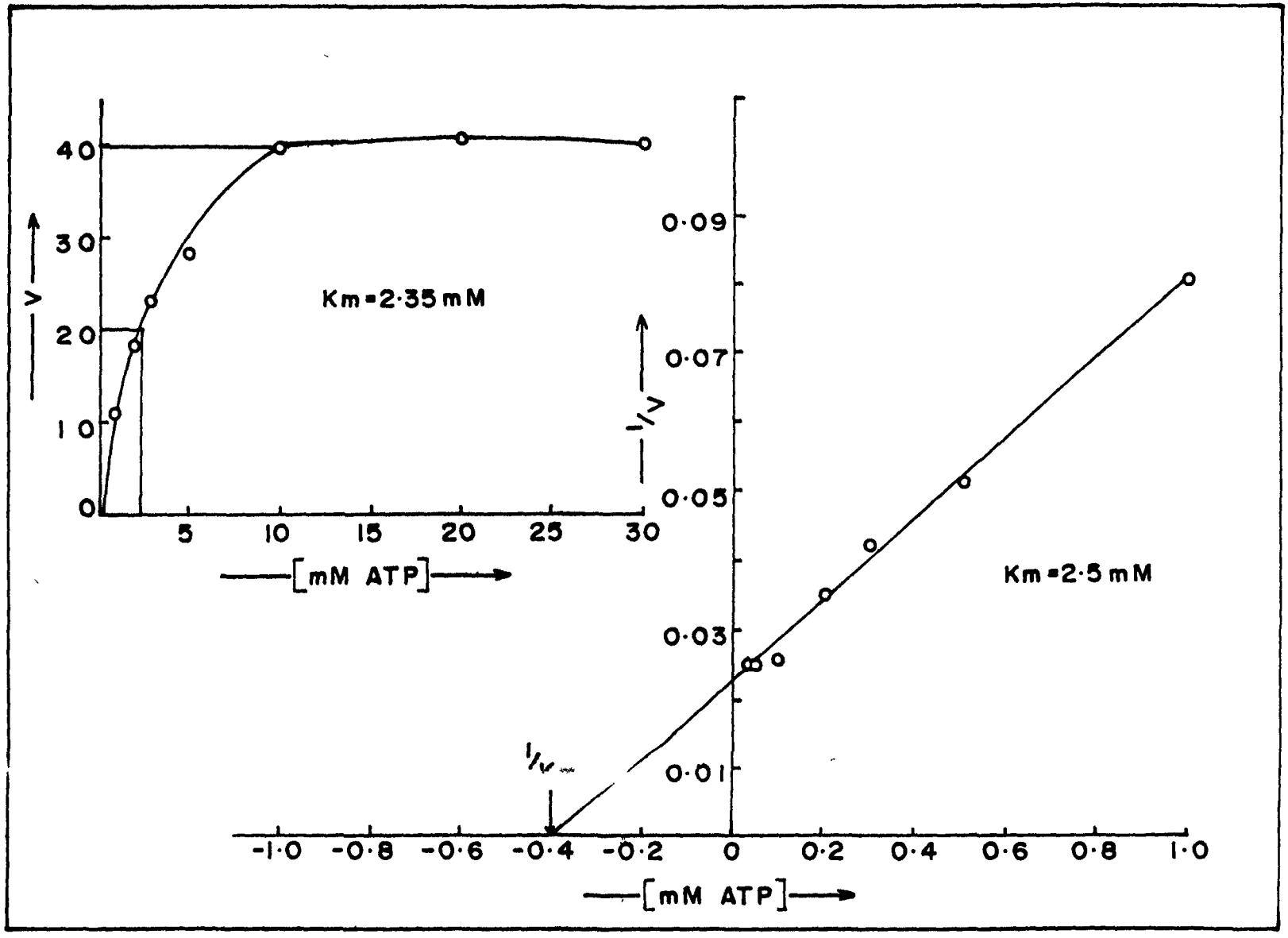


Fig.22 Determination of Km of glutamine synthetase (purified from Heteropneustes fossilis brain) for ATP by Lineweaver-Burk plot. (Inset is Michaelis-Menten plot).

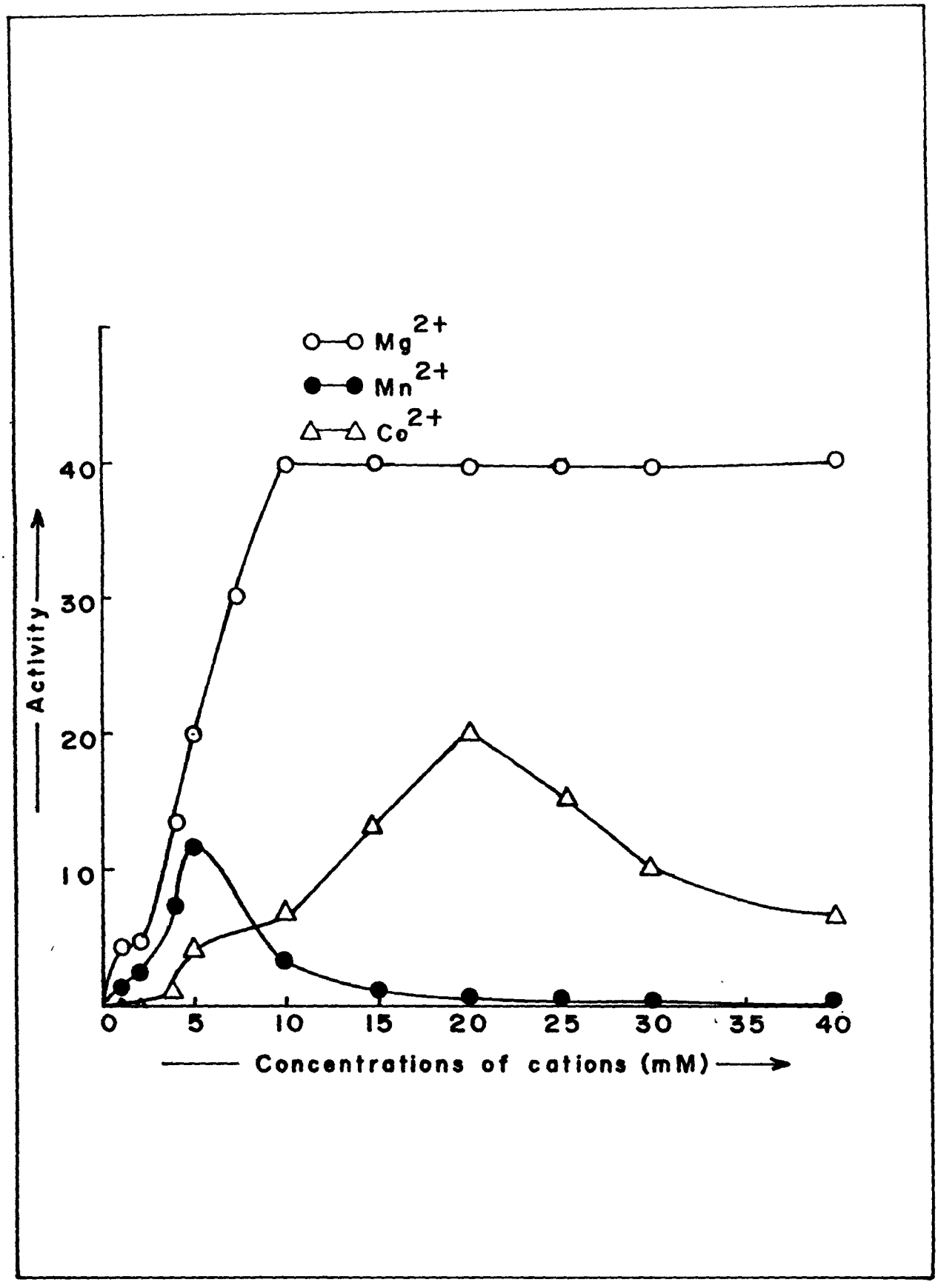


Fig.23 Effect of various concentrations of Mg^{2+}, Mn^{2+} and Co^{2+} on the activity of glutamine synthetase purified from Heteropneustes fossilis brain.

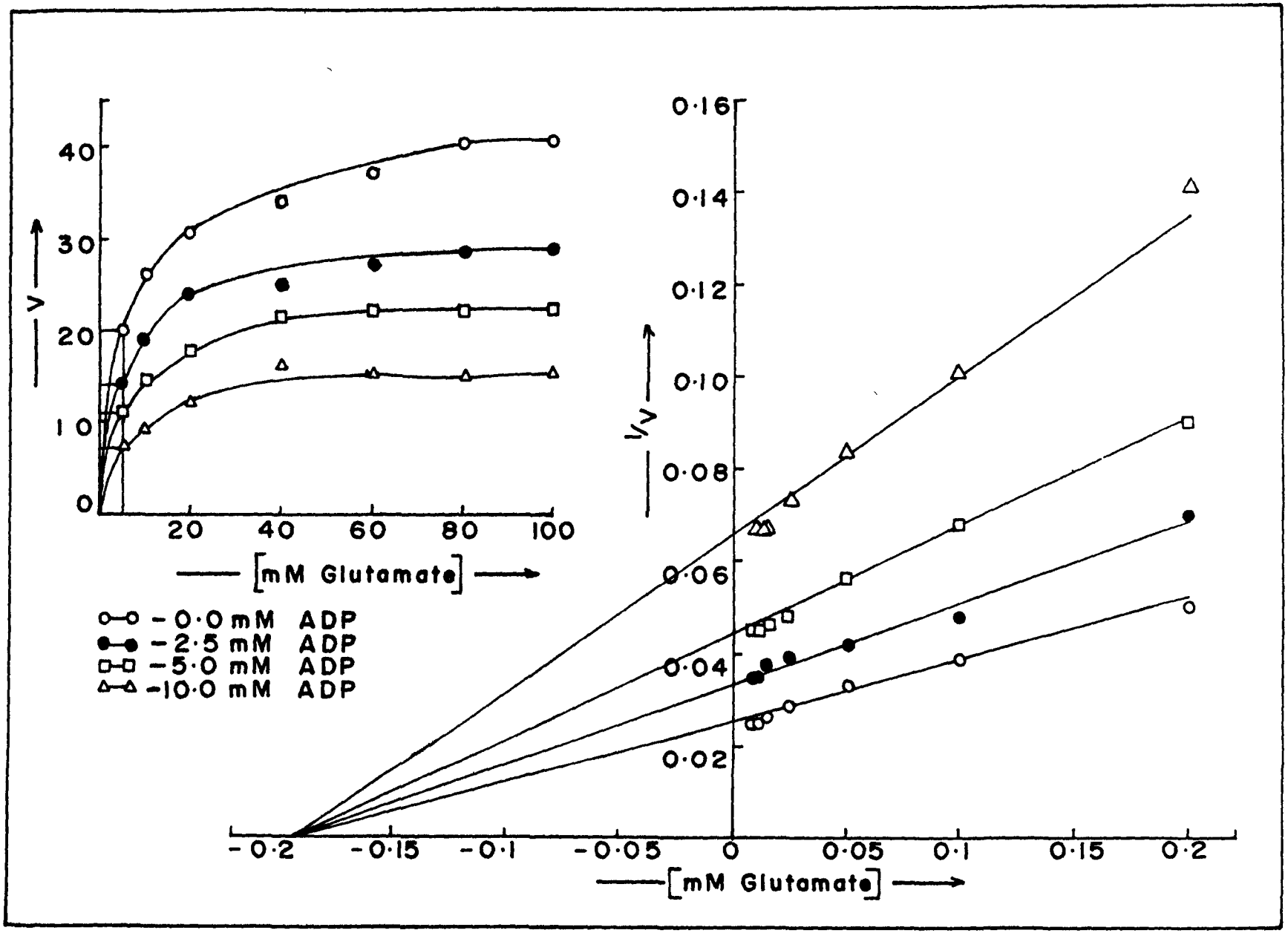


Fig.24 Lineweaver-Burk plot and Michaelis-Menten plot (inset) of the effect of various concentration of ADP on the activity of glutamine synthetase purified from Heteropneustes fossilis brain.

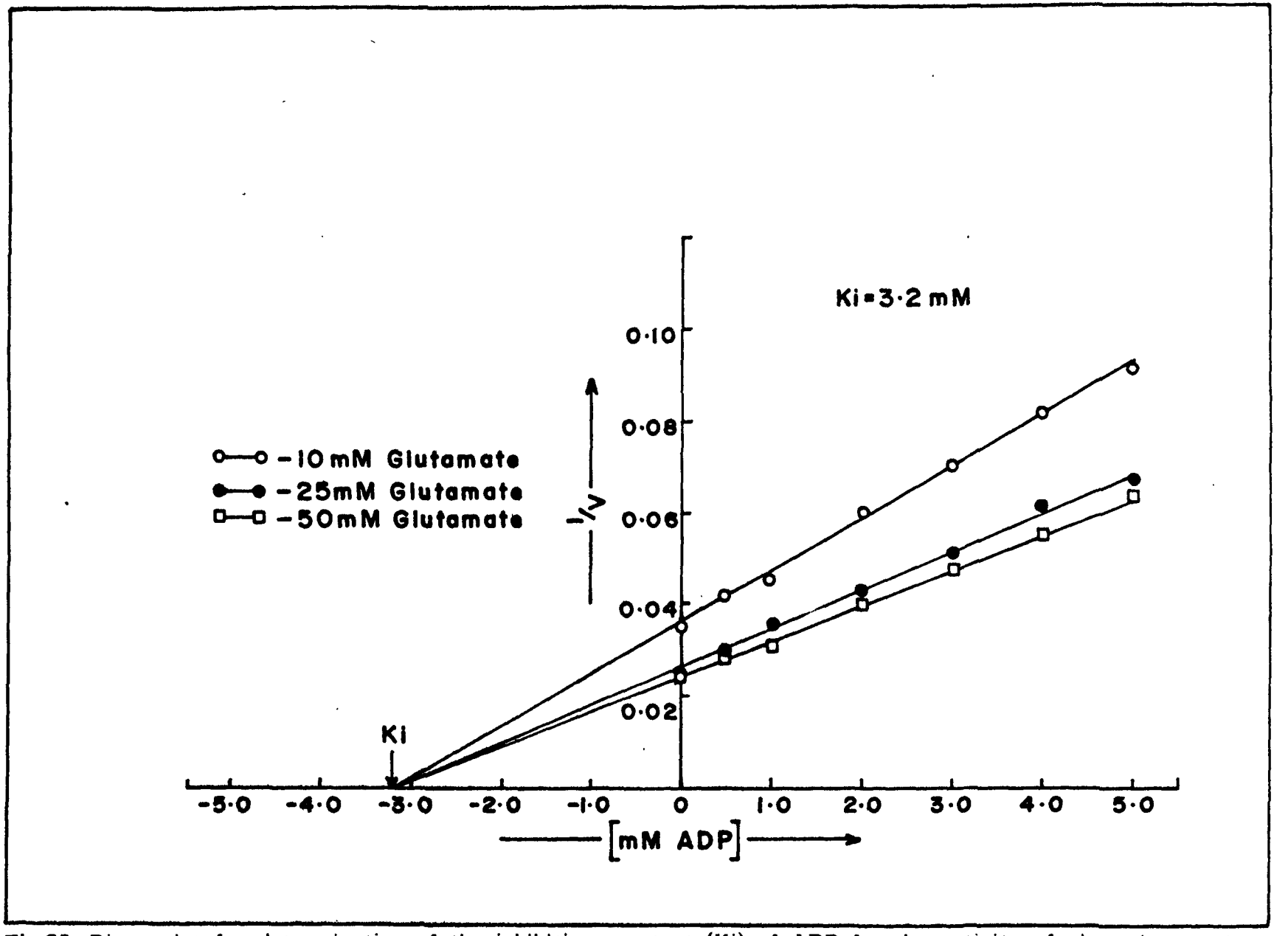


Fig.25 Dixon plot for determination of the inhibition constant (Ki) of ADP for the activity of glutamine synthetase purified from Heteropneustes fossilis brain.

DISCUSSION

Ammonia is a common pollutant in inland water and its toxicity has been studied in fish (Alabaster & Lloyd, 1980; Haywood, 1983). Olson and Fromm (1971) reported a 50% mortality of trout, Salmo gairdneri in 24 hrs at 8 µg ammonia/ml (equivalent to 0.48 mM NH_4Cl). However, gold fish did not show any response at ammonia concentration as high as 25 µg/ml (equivalent to 1.47 mM NH_4Cl). Among teleosts, gold fish was reported to have better tolerance (10% mortality in 24 hrs at 40 µg/ml ≃2.35 mM NH_4Cl) for ammonia toxicity (Schenone et al, 1982). Aquatic frog, Xenopus laevis did not survive beyond 1 hr at 10 mM NH_4Cl (Janssens, 1972). Prolonged exposure to sublethal concentration of ammonia caused histopathological changes in various tissues and decline in growth rate of fish (Smith & Piper, 1975; Robinette, 1976; Colt & Tchobanoglous, 1978; Alderson, 1979; Calamari et al, 1981; Søderberg et al, 1983). Earlier studies from this laboratory have shown that the freshwater airbreathing teleost, Heteropneustes fossilis can tolerate very high ambient ammonia concentration (upto 75 mM NH_4Cl), longer periods (60-70 hrs) of water deprivation and hyperosmolar (300 m osm) ambient medium without any apparent deleterious effect (Saha & Ratha, 1986, 1989b). During

such exposures the fishes suppressed excretion of ammonia and accumulated excess ammonia in their tissues during the early periods of experimentation. The induction of the activities of the enzymes of o-u cycle in liver and kidney was followed resulting in inhanced rate of conversion of accumulated ammonia to urea *in vivo* (Saha & Ratha, 1986, 1989b). Urea first accumulated in all the tissues and then excess of urea was excreted at a higher rate during the later part of exposure to NH_4Cl (Saha & Ratha, 1990). This high ammonia tolerance limit *in vivo* was suggested to be due to the existence of better ammonia management mechanisms including induction of ureogenesis in *H. fossilis* (Saha & Ratha, 1989b). Webb and Brown (1980) showed a direct correlation between the urea production and glutamine synthesis in marine fishes where CPS III utilized glutamine as the amino-donating substrate for urea cycle. The synthesis of glutamine and its degradation has been suggested to play a major role in the regulation of ammonia besides serving as a nitrogen source for the synthesis of various biomolecules *in vivo*. The ammonia produced in different tissues is fixed in the form of glutamine and transported to the target organs for its direct entry to o-u cycle through CPS III or deaminated to release ammonia either for its excretion or utilization by o-u cycle through CPS I. Therefore, the role of glutamine metabolism was evaluated in ammonia management in an ammoniotelic but ureogenic freshwater air-breathing teleost, *Heteropneustes fossilis*. The

levels of the enzyme activity and the level of ammonia and glutamate were studied under physiological condition and under hyper ambient ammonia and dehydration stress. The molecular character of GS was studied after purification from brain of H. fossilis.

Tissue distribution of GS and Glnase

High GS activity could be assayed in all the five tissues studied (Table 1) with brain showing highest level of enzyme activity followed by liver, kidney, muscle and gill of Heteropneustes fossilis. Presence of glutamine synthetase activity at an appreciable level has been reported only in the brain tissue of freshwater teleosts (Wu, 1963a,b; Janssens & Cohen, 1968; Webb & Brown, 1976; Wilson & Fowlkes, 1976; Walton & Cowey, 1977). Either low activity or no activity could be observed in other tissues such as liver, kidney, gill, intestinal mucosa and muscle (Janssens & Cohen, 1968; Pequin & Serfaty, 1968; Pequin et al, 1969; Wilson & Fowlkes, 1976; Walton & Cowey, 1977; Webb & Brown, 1980; Waarde & Kesbeke, 1982; King & Goldstein, 1983; Chew & I_p, 1987). H. fossilis was maintained in the laboratory mainly on pork liver (high protein) diet which might have caused a constant endogenous ammonia load. Schultz and Lowenstein (1978) showed endogenous extra ammonia load in the brain also from purine nucleotide cycling (from active neurons). High GS activity in the brain of H. fossilis might

be helping in prompt detoxification of ammonia besides maintaining glutamate-glutamine pool.

The role of GS activity associated with liver, kidney, gill and muscle is unclear in this ammoniotelic fish. The synthesis of glutamine has been associated with either purine synthesis in uricotelic birds (Hartman, 1970; Vorhaben & Campbell, 1972, 1977) or pyrimidine synthesis through CPS II in E. coli (Robin et al, 1989), mammals (Ratner, 1973); elasmobranch (Anderson, 1989) or urea production via CPS I or CPS III as found in mammals and amphibians (Ratner, 1973), elasmobranch (Anderson, 1980, 1981; Casey & Anderson, 1983) and a species of tilapia (Randall et al, 1989). The GS activity in H. fossilis liver and kidney was 2-3 times higher than those reported in ammoniotelic freshwater fish (Janicki & Goldstein, 1969; Wilson & Fowlkes, 1976; Webb & Brown, 1976; Webb, 1980) but lower compared to ureo-osmotic elasmobranch, marine fishes and a tilapia, O. a grahami (Webb & Brown, 1976; Webb, 1980; Smith et al, 1987; Randall et al, 1989). Thus, H. fossilis showed an intermediate position between the ammoniotelic freshwater fishes and ureo-osmotic marine fishes with respect to the level of GS activity.

Webb and Brown (1976) suggested that ammoniotelic teleost did not require a significant amount of glutamine for either excretion or osmoregulation, whereas elasmobranch

utilised glutamine for both the functions. Urea retaining marine fishes showed high GS activity in their liver and kidney compared to very low levels of enzyme activity in non-urea retaining species (Webb & Brown, 1980). Presence of a different isoenzyme (CPS III) which utilizes glutamine instead of ammonia for carbamyl phosphate synthesis during ureogenesis has been reported in the liver and kidney of some urea retaining marine elasmobranchs (Anderson, 1980). Primarily ammoniotelic H. fossilis showed a higher tissue level and excretion of urea and the transition from ammoniotelism to ureotelism during exposure to hyper ambient ammonia (Saha & Ratha, 1986, 1989b), water deprivation and hyperosmotic stress (Saha, 1986). The ambient ammonia tolerance limit (75 mM) was quite high (Saha & Ratha, 1989b). GS might be playing some role in the detoxification of excess ammonia in the liver and kidney of this primarily ammoniotelic carnivorous fish. Relatively high activity of GS in the liver of H. fossilis along with all the isoenzymes of CPS (unpublished observations from this laboratory) might indicate the capability of this fish for the production of pyrimidine or urea. However, the possibility of use of glutamine as a direct amino-donating substrate for urea synthesis needs further confirmation.

The activity of glutaminase observed in brain, liver, kidney and gill of H. fossilis was lower than those reported

in various tissues of fresh water fish (Makerewicz, 1963; Walton & Cowey, 1977), amphibians (Makerewicz & Zydowo, 1962) and mammals (Janicki & Goldstein, 1969; Linder-Horowitz, 1969; McGivan et al, 1980), but higher than those reported in the liver of Squalus acanthias (Casey & Anderson, 1985), mudskipper (Chew & Ip, 1987), neotenous newt (Fanelli & Goldstein, 1964) and chicken (Vorhaben & Campbell, 1977). However, the enzyme level in H. fossilis was comparable with those reported in different tissues of freshwater goldfish (Waarde & Kesbeke, 1982).

The physiological significance of highest level of glutaminase activity in brain compared to other tissues is not clear. High glutaminase activity was accompanied by high activity of glutamine synthetase (which is about 6 times higher than that of glutaminase activity) in the brain. This might lead to substantial production of glutamine as a non-toxic carrier form of ammonia and minimum utilization of glutamine to produce ammonia. Besides, high activity of both glutaminase and glutamine synthetase in H. fossilis might maintain a balance in glutamate - glutamine conversion for the availability of glutamate as a neurotransmitter in the brain. Kvamme (1984) suggested glutaminase as the major glutamine metabolizing enzyme in the brain of mammals.

Glutaminase in kidney and gill have been shown to be mostly involved in the excretory ammonia production ir

higher vertebrates (Kovacevic & McGivan, 1983) and to a lesser extent in lower vertebrates (Makerewitz & Zydowo, 1962). Liver glutaminase activity was also suggested to be associated with pH regulation besides with the synthesis of urea and ammonia (Goldberg & Chang, 1978). Pequin (1967) suggested that liver of carp fixed the exogenous ammonia as glutamine and then deaminated this glutamine to glutamate and free ammonia before the ammonia reached the gill for its rapid excretion.

The role of glutamine in NH_3 excretion by teleost has not yet been clearly understood. Some suggested that glutamine played a minor role (Goldstein et al, 1964; Walton & Cowey, 1977) whereas others have shown it playing a major role in transport and the formation of NH_3 (Vellas & Serfaty, 1974). In aquatic animals the excreted ammonia, produced by catabolism of nitrogenous bio-molecules, is carried partly as ammonia and partly as other precursors such as amino acids to the gill or other body surface (Kormanik & Cameron, 1981). Glutaminase found in gill tissue might either produce NH_3 for excretion as in fish (Goldstein & Forster, 1961) or produce NH_4^+ if required for ion regulation after glutamate utilization (Walton & Cowey, 1977). However, in H. fossilis gill both GS and Glnase activity could be detected indicating that glutamine in gill tissue might play some role in release of ammonia as suggested for channel catfish (Wilson & Folkes, 1976).

The ratio of the activity of GS/Glnase in various tissues of H. fossilis, in general, favoured detoxification of ammonia for the production of metabolically important glutamine. However, the activity levels and ratio were very high in the brain compared to other tissues indicating their significance in maintaining glutamate and glutamine pool in the brain in addition to ammonia detoxification. The absence of glutaminase activity in the muscle might be for the utilization of glutamine synthesized by glutamine synthetase for gluconeogenesis and energy metabolism (Goldberg & Chang, 1978) as well as a source of nitrogen for other biosynthetic processes.

Sub-cellular distribution of GS and Glnase (Table 2-9)

The distribution of GS and Glnase in different sub-cellular fractions was studied in brain, liver and kidney tissues of H. fossilis. The marker enzymes LDH as cytosolic and GDH as a soluble mitochondrial matrix enzyme were separated well in their respective fractions indicating that the mitochondria remained fairly intact during fractionation. The activity of microsomal enzyme G-6-Pase in brain was found primarily in the microsomal (53%), besides in nuclear 12%, in mitochondrial (19%) and in cytosolic (16%) fractions indicating its trapping in different sub-cellular fractions. Smith and Campbell (1983) also faced similar problems and suggested that the difficulty in sub-cellular fractionation of brain

tissue was due to its considerable myelin membrane content. This suggestion seems to be valid also for H. fossilis brain, as the separation of sub-cellular fractions indicated by marker enzyme activities was better in liver and kidney compared to brain (Table 2-4).

Most of the GS activity (70%) was localized in the cytosolic fraction of the brain of H. fossilis and 11, 14 and 5% of total GS activity in mitochondrial, microsomal and nuclear fractions respectively. These values were similar to the sub-cellular distribution of GS reported in rat brain (Sellinger & DeBalbain Verster, 1962; Dennis et al, 1980). The heterogeneous sub-cellular distribution of GS has been reported in different groups of animals and also in different organs of the same animal. The GS activity has been cytosolic in the brain of all groups of animals reported and has been related to ammonia detoxification by glutamine formation. Ratner (1973) suggested the involvement of cytosolic GS in pyrimidine synthesis in ureotelic mammals. Anderson (1989) reported cytosolic glutamine synthetase in spleen of ureo-osmotic elasmobranch and suggested its involvement in pyrimidine synthesis. In ureocotelic birds, the glutamine formed due to glutamine synthetase in mitochondria moved to cytosol for purine and uric acid synthesis (Vorhaben & Campbell, 1977 This indicated that the glutamine synthetase or glutamine in the cytosol was either meant for ammonia detoxification or purine or pyrimidine synthesis. This might be true for

cytosolic glutamine synthetase in the brain of H. fossilis.

In contrast to the cytosolic localization in brain, GS was found primarily (75%) in mitochondrial fraction of liver and kidney in H. fossilis. The mitochondrial localization of GS in H. fossilis was a unique observation for any freshwater teleost. Glutamine synthetase activity in liver and kidney was reported to be cytosolic in ureotelic mammals, amphibians and catfish (Sellinger & DeBalbain Verster, 1962; Wu, 1963a,b; Katunuma et al, 1970; Vorhaben & Campbell, 1977; Casey et al, 1983) and mitochondrial in urecotelic birds and reptiles (Vorhaben & Campbell, 1972, 1977 ;Smith & Campbell, 1983) and ureo-osmotic marine elasmobranchs (Webb & Brown, 1976, 1980; Anderson, 1980, 1981; Casey & Anderson, 1982; Smith et al, 1987). In these animals ammonia, originated intra-mitochondrially during transdeamination in the liver and kidney was promptly detoxified by converting to glutamine by GS. Glutamine then came out of mitochondria for further conversion to purine in cytosol of birds and reptiles (Vorhaben & Campbell, 1972). In ureo-osmotic marine elasmobranchs a unique N-acetyl glutamate and glutamine dependent isoenzyme of carbamyl phosphate synthetase (CPS III) has been reported to be present in the liver mitochondria (Anderson, 1980, 1981). Glutamine, therefore, instead of leaving mitochondria could be utilized there for urea synthesis (Casey & Anderson, 1985).

Primarily ammoniotelic H. fossilis showed relatively high tissue level and excretion of urea and the transition from ammoniotelism to ureotelism during exposure to hyper ambient ammonia, water deprivation and hyperosmotic stress (Saha, 1986; Saha & Ratha, 1986, 1987, 1988, 1989a,b, 1990) and tolerated a very high (75 mM) ambient ammonia concentration (Saha & Ratha, 1986). Mitochondrial glutamine synthetase might be playing an important role in the detoxification of excess ammonia in the liver and kidney of this primarily ammoniotelic but ureogenic fish, H. fossilis by using glutamine for ureogenesis through CPS III (which is now detected in the liver of this fish in our laboratory).

Triton X-100 treatment and sonication (Table 5-6) could increase glutamine synthetase activity in the crude homogenate of brain, liver and kidney indicating that GS was membrane bound in all the three tissues. However, GS activity was enhanced by only about 26% in brain homogenate compared to 90% in liver and kidney. This indicated that binding of GS was almost complete in liver and kidney but partial in brain of H. fossilis. Sonication could solubilize GS to a lesser extent (75-98%) than Triton X-100 treatment (92-96%) (Table 5). Triton X-100 was shown to be good releasing agent for GS than sonication in catfish, I. punctatus (Wilson & Fowlkes, 1976).

Glutaminase was mostly confined (68-72%) to mitochondrial

fraction in all the tissues studied i.e. brain, liver and kidney in H. fossilis (Table 2-4). Mitochondrial localization of Glnase has been reported in mammals (Carter & Greenstein, 1947; Greenstein & Carter, 1947; Errera & Greenstein, 1949; Otey et al, 1958; Hird & Murginson, 1968; Guha, 1971; Beaken & Hird, 1972; Kovacevic, 1971, 1976), birds (Vorhaben & Campbell, 1977), mudskipper (Chew & Ip, 1987) and elasmobranch (Vorhaben & Campbell, 1977).

Like GS, Glnase could also be released from crude homogenates (Table 8) by both sonication (65-78%) and Triton X-100 (65-72%) treatment. Kalra and Brosnan (1973, 1974) and Kovacevic (1976) made similar observations in rat liver and kidney glutaminase. Inactivation of glutaminase by Triton X-100 has been reported in rat brain and avain liver mitochondria (Salganicoff & De Roberties, 1965; Vorhaben & Campbell, 1977). Vigorous sonication was found to cause inactivation of Glnase activity in all the tissues of H. fossilis, the enzyme in liver homogenate being maximally affected. This was in contrast to the observation in rat liver Glnase where vigorous sonication solubilized the enzyme (McGiven et al, 1980). Sonicated extract of both brain and kidney homogenates of H. fossilis maintained the enzyme activity upto 48 hrs when preserved at 4°C as in the case of rat brain and kidney (Haser et al, 1985). However, in liver, glutaminase activity started decreasing by 20% after 8 hrs and 56% after 36 hrs (Table 9). Patel and McGivan (1984) reported such inactivation

in rat liver. Triton X-100 treated liver homogenate lost its activity earlier than that with sonication. After 4 hrs of Triton X-100 treatment, the enzyme activity decreased by about 18%. This reduction in glutaminase activity might be due to some protease activity causing destruction or inactivation of the enzyme after its release from the membrane. Though the enzyme was sensitive after its release from membrane by both sonication and Triton X-100 treatment, it was found to be comparatively stable after sonication at least in liver.

Diurnal variation of GS and Glnase

Glutamine synthetase and glutaminase did not show any significant variation during 24 hrs cycle in brain, liver and kidney of H. fossilis. In some plant species such as barley leaves, sunflower (Knight & Weissman, 1982; Lillo, 1984) and some species of cyanobacteria (Florencio & Ramas, 1985) glutamine synthetase activity showed diurnal variation which has been correlated witn the inactivation and reactivation of the enzyme through the energy supply (ATP or ferredoxin). However, there has been no report on the diurnal variation of GS or Glnase in any animal species. Autotrophs which trap solar energy during day time is expected to have diurnal variation in most of their metabolic process. Absence of diurnal variation of these two enzymes in H. fossilis

indicated that the glutamine metabolism normally continued at the same rate throughout the 24 hr cycle in different tissues of this freshwater teleost.

Effect of starvation and NH_4Cl treatment

The fish did not take any food and continued to starve during their exposure to different concentrations of NH_4Cl. Starvation was reported to influence amino acid metabolism (Prosser, 1973; Bever et al, 1981). Therefore, it was necessary to monitor the effect of starvation simultaneously during the treatment with NH_4Cl. It was reported earlier that the ammonia level in different tissues of H. fossilis exposed to various concentrations of ambient NH_4Cl reached highest level by 14th day and did not alter thereafter (Saha & Ratha, 1989b). Therefore, NH_4Cl treatment during the present studies was confined to 14 days.

Starvation: During starvation the concentration of metabolic ammonia and the activity of GS did not change significantly in any of the tissues studied upto 14 days (Table 12, Fig.2 & 3). Prosser (1973) suggested induction of GS activity during starvation mainly for ammonia detoxification which is produced via transdeamination process. Induction of GS activity during starvation was reported in birds (Katunuma et al, 1970) and mammals (King et al, 1983). Saha and Ratha (1989b) did not observe any accumulation of ammonia in any of the three tissues

studied in H. fossilis maintained without food upto 28 days, indicating no extra ammonia production or accumulation in vivo. GS activity, therefore, might have remained unchanged during starvation as there was no hyper-ammonia stress in vivo. The glutaminase activity which was induced in brain and kidney of H. fossilis during starvation (Table 17-19; Figs.5-8), might have produced sufficient glutamate and ammonia. Significant accumulation of glutamate was observed in liver and kidney of H. fossilis (Table 13; Figs.2 & 4). The accumulation of glutamate during starvation was reported in liver of rainbow trout (Walton & Cowey, 1977), channel cat fish (Campbell et al, 1983), muscle of rat (Goldstein et al, 1983). The rise in glutamate accumulation with unchanged ammonia and GS activity during starvation indicated that ammonia was either converted to glutamate through GDH or utilised else where in H. fossilis. Induction in GDH activity (reductive amination) in liver and kidney of H. fossilis during starvation has been observed in our laboratory (unpublished observation). Glutamate being a neurotransmitter, H. fossilis maintained glutamate level unchanged during the 14 days of starvation for proper functioning of the brain. The glutamate which might have been produced in excess due to induction of Glnase activity in brain might have been utilized for energy production. The induction of Glnase activity for production of glutamate for energy production during starvation has been suggested by Ardawi and Newsholme (1984). Due to the induction of Glnase in brain and kidney

GS/Glnase activity decreased significantly in both the tissues with higher reduction in kidney. This condition did thus favour indirectly the glutamate formation probably for its use in energy production during periods of starvation.

NH_4Cl treatment: Exposure of H. fossilis to various concentrations of NH_4Cl resulted in significant accumulation of ammonia within 4 days in different tissues such as brain, liver and kidney. Maximum level of accumulation of ammonia was found to be different in different tissues indicating that each tissue had its own specific ammonia tolerance limit. Highest accumulation of ammonia was found in liver and kidney (~ 30 µmole/g) followed by brain (12 µmole/g). However, the percentage accumulation was maximum in kidney (164%) followed by liver (~ 120%) and brain (~ 103%). Similar pattern of accumulation of ammonia in H. fossilis has been reported by Saha and Ratha (1989b) at 7, 14, 21, 28 days of exposure to different concentrations (25, 50, 75 mM) NH_4Cl. Brain has been known to be highly susceptible to ammonia toxicity (Campbell, 1973; Copper & Plum, 1987). Hence, accumulation of ammonia in brain tissue was minimum. Liver and kidney have been the main organs for ammonia formation in fish (Pequin & Serfaty, 1963; Vellas & Serfaty, 1974), and therefore, the accumulation of ammonia was more in them. Suppression of ammonia excretion and absorption of ambient ammonia into the body in favour of concentration gradient have been suggested to cause

accumulation of excess ammonia *in vivo* (Saha & Ratha, 1986, 1989b). Induction of o-u cycle leading to the transition from ammoniotelism to ureotelism in *H. fossilis* during hyper-ammonia stress to avoid ammonia toxicity was reported by Saha and Ratha (1987; 1989b). The induction of o-u cycle was observed between 7-14 days of exposure to hyper-ammonia stress and that might not have been the only mechanism to control the accumulated ammonia at a non-toxic level in this fish. Higher physiological level of GS in *H. fossilis* tissues compared to other fresh water teleosts (Janicki & Goldstein, 1969; Wilson & Fowlkes, 1976; Webb & Brown, 1976; Webb, 1980) and its mitochondrial localization in liver and kidney (Chakravorty *et al*, 1989) strongly suggested its involvement in detoxification of excess ammonia.

GS activity was induced significantly in brain, liver and kidney tissues during exposure of *H. fossilis* to different concentrations of NH_4Cl (Table 14-16; Figs.5-8). Brain showed early induction (within 4 days) of GS activity followed by liver and kidney. Brain being highly sensitive to ammonia toxicity and also the o-u cycle being absent in the brain (Saha & Ratha, 1987), early induction of GS activity might have controlled the ammonia level in brain by converting it to glutamine. GS activity was also very high in brain. In addition to the induction of o-u cycle in both liver and kidney tissues (Saha, 1986; Saha & Ratha, 1987, 1989b) significant

induction of GS activity observed in both the tissues must have helped accelerating the ammonia detoxification process under hyper-ammonia stress in H. fossilis. Besides converting toxic ammonia to non-toxic glutamine, the induced GS activity in the liver and kidney of H. fossilis might be actively feeding glutamine for urea production through carbamoyl phosphate synthetase III (CPS III) (Glutamine and N-acetyl glutamate dependent) in the liver and kidney mitochondria of H. fossilis like elasmobranchs (Anderson, 1980, 1981). The unusual localization of GS in the mitochondria of liver and kidney of this freshwater ammoniotelic and ureogenic teleost also suggested such a situation. The role of glutamine contributing its amide group for urea synthesis during hyper-ammonia stress via induced o-u cycle (Saha & Ratha 1986, 1989b) has not yet been established. However, the indirect evidences are suggestive and studies to clarify this is in progress.

Although there was significant induction of GS activity indicating excess utilization of glutamate and ammonia for glutamine synthesis during exposure to NH_4Cl solution, significant acumulation of glutamate in all the three tissues of H. fossilis was also observed (Table 13, Figs.2 & 4). In another study from this laboratory the activity of GDH (reductive amination) has been shown to be induced significantly in different tissues of H. fossilis exposed to different concentration of NH_4Cl (unpublished observation). The glutamate synthesis for ammonia detoxification via GDH activity might

be higher than the rate of conversion of glutamate to glutamine by GS activity resulting on the accumulation of glutamate during hyper-ammonia stress. Significant accumulation of glutamate besides some other non-essential amino acids during accumulation of ammonia *in vivo* have been reported in carp, *C. carpio* (Dabrowska & Wlasow, 1986) and mudskipper *P. cantonensis* (Iwata *et al*, 1981; Iwata, 1988) during their exposure to NH_4Cl solution and dehydration respectively.

Exposure to higher ambient ammonia resulted in immediate accumulation of ammonia *in vivo* followed by induction of GDH (unpublished observation) and GS activity (present observation) which led to more synthesis and accumulation of glutamate and glutamine. Amination of keto acids and transamination reactions are likely to produce different non-essential amino acids. Some preliminary observations in this laboratory support this suggestion of synthesis and accumulation of various non-essential amino acids for detoxification of excess ammonia *in vivo* in *H. fossilis* exposed to higher ambient ammonia.

The enzyme glutaminase which has been reported in all the three tissues such as brain, liver and kidney of *H. fossilis* was suspected to contribute to the accumulation to toxic ammonia *in vivo*. But interestingly Glnase activity was significantly inhibited in brain and kidney of *H. fossilis* exposed to 75 and 100 mM NH_4Cl. There was no change of activity at 50 mM ambient NH_4Cl (Table 17-19, Fig. 5-8). In liver,

Glnase activity significantly increased only at early stages of exposure and decreased more significantly during later periods of exposure when ammonia accumulation reached maximum tolerance limit. The inhibition pattern was tissue specific. The inhibition of Glnase activity might be an adaptation to decrease addition of metabolic ammonia in various tissues under ammonia stress and hence to tolerate higher ambient ammonia.

The ratio of GS/Glnase activity in all the three tissues of H. fossilis was found to increase at different concentrations of NH_4Cl. It also indicated more synthesis of glutamine rather than hydrolysing it to glutamate and ammonia under hyper-ammonia stress.

Effect of dehydration

The nature of nitrogen excretory products in animals have been correlated with the availability of water in the surrounding environment (Needham, 1938; Balinsky et al, 1961; Gordon, 1970). Hence, the intermediary nitrogen metabolism has significantly altered during the evolution of vertebrates (Campbell, 1973; Cohen, 1976; Hoar, 1983). Aquatic animals are ammoniotelic and excrete mainly ammonia by simple diffusion to the ambient aquatic medium (Forster & Goldstein, 1969; Campbell, 1973). However, in amphibians and terrestrial animals major excretory products are either urea or uric acid which are being formed from ammonia via different enzymatic

pathways utilizing metabolic energy. Some groups of animals such as aquatic amphibians show dual characters being ammoniotelic in water and ureotelic on land mainly to avoid ammonia toxicity during its stay outside water (Goldstein, 1972; Cohen, 1976). They have a functional urea cycle which gets usually induced during their transition from water to land (Balinsky, 1970; McClanahan, 1967, 1972). Transition from ammoniotelism to ureotelism has been reported during aerial exposure of mudskippers (Gordon et al, 1969, 1970, 1978) and in African lungfish, Protopterus during aestivation (Janssens, 1964).

The freshwater air-breathing teleost, H. fossilis could survive outside water for 60-70 hrs (Saha & Ratha, 1989b). On exposure to air H. fossilis decreased by 75 to 85% of its ammonia excretion within 24 hrs probably due to non-availability of water. There was significant accumulation of ammonia in various tissues within 3 hrs followed by further increase at later stages of aerial exposure (Saha, 1986). This was accompanied by the induction of ureogenesis, accumulation and excretion of excess of urea. This transition from ammoniotelism to ureotelism was also suggested to be a adaptation to avoid ammonia toxicity (Saha, 1986). The induction of ureogenesis in H. fossilis might not be the only mechanism to control the ammonia level at a non-toxic level during its stay outside water.

Significant accumulation of glutamate during water deprivation for 36 hrs has been noticed in the present study in all the three tissues studied such as brain, liver and kidney of H. fossilis (Table 22; Fig.9). Glutamate accumulated in brain significantly within 12 hrs reaching the highest level by 18 hrs whereas in liver and kidney significant accumulation of glutamate was noticed from 15 hrs onwards. They increased further at later stages of emersion. GDH activity (reductive amination), where physiological level was already high, got induced in this fish during its stay outside water (unpublished observation). This might have converted excess ammonia to glutamate using α-keto-glutarate.

GS activity was significantly induced in all the three tissues such as brain, liver and kidney of H. fossilis during its stay outside water (Table 23, 24; Fig. 10,11) when the level of both glutamate and ammonia was high. Induced GS activity might have detoxified the exces ammonia by converting it to glutamine. Significant increase of glutamate level in all three tissues indicated that the rate of formation of glutamate via the induced GDH activity (reductive amination) (unpublished observation) was higher than the utilization of glutamate to form glutamine by the induced GS activity. Higher accumulation of glutamate, glutamine and other non-essential amino acids in mudskipper, P. cantonensis during their stay outside water has been suggested to be synthesized

from the accumulated ammonia (Iwata et al, 1981; Iwata, 1988). In another preliminary investigation in our laboratory, it has also been found that there was accumulation of glutamate, glutamine and some other non-essential amino acids in different tissues of H. fossilis when the fish was exposed to the air.

Exposure to air resulted in immediate accumulation of ammonia in vivo (Saha, 1986) followed by induction of GDH (unpublished observation) and GS activity (present observation). This indicated more synthesis and accumulation of glutamate and glutamine. Synthesis of some other non-essential amino acids might have taken place via transamination process which has to be verified.

Glutaminase activity in all the three tissues in H. fossilis was found to decrease gradually with increasing time of aerial exposure (Table 25, 26; Fig. 10,11). This was again a favourable adaptation in this fish under dehydration stress. Due to reduction of Glnase activity, formation of ammonia by hydrolysis of glutamine was reduced which otherwise had already accumulated significantly within the body.

The ratio of GS/Glnase activity in all the three tissues of H. fossilis was found to increase with increasing time of emersion (Table 27, Fig.11). It indicated more synthesis of glutamine rather than hydrolyzing it to glutamate and ammonia under dehydration stress. This has raised the question

to investigate the utilization pathways of this excess of glutamine formed.

Hyper ambient ammonia and dehydration caused high accumulation of ammonia due to the suppression of its excretion. Ammonia accumulation resulted in the increase in glutamate level and the induction of GS with simultaneous inhibition of Glnase leading to the synthesis of excess glutamine in various tissues. Active glutamine synthesis in addition to ureogenesis gave better adaptive capacity to H. fossilis to control the accumulated ammonia in vivo.

Glutamine has been known to be a safe carrier of ammonia. It carried ammonia either to the site of its excretion or to liver for urea synthesis where ammonia was released by glutaminase activity (Meijer, 1985; Welbourne & Joshi, 1986). Glutaminase activity was reported to be induced under hyper ammonia stress (Vanslyke et al, 1943; Davies & Yudkin, 1952; Latspeich, 1959; Welbourne et al 1972; Pitts, 1973; Rector et al, 1955; Curthoys & Lowry, 1973; Goldstein, 1967, 1976; Ardawi & Newsholme, 1984) to provide ammonia in the target tissues for its conversion via o-u cycle. However, in H. fossilis Glnase activity was significantly inhibited in the two ureogenic tissues (liver and kidney). Interestingly the ureogenesis was also significantly induced in H. fossilis under hyperammonia stress (Saha & Ratha, 1986; 1989b).

Mitochondrial localization of GS in liver and kidney tissues like other ureogenic animals indicated possibility of glutamine directly entering the urea cycle by the enzyme CPS III (Glutamine dependent CPS). This question needs to be verified.

Purification of brain glutamine synthetase and its physico-chemical properties

Purification and molecular character: The protocol of purification followed for GS from brain tissues of H. fossilis involving $(NH_4)_2SO_4$ precipitation, DEAE-Sephacel and Sephadex G-200 column chromatography yielded better result with 62% recovery of activity and about 58 fold of purification (Table 28) compared to 30 fold of purification and about 20% of recovery of GS activity from the liver of S. acanthias following phenyl sepharose, DEAE Sephadex and Sephacryl-S 300 chromatography (Shankar & Anderson, 1985). The purified GS was eluted as a single peak from both the DEAE-Sephacel and Sephadex G-200 columns (Fig.12). It showed a single band on protein staining and specific staining for GS activity on polyacrylamide gel after electrophoresis (Fig.13). These results suggested that the enzyme was a single species of protein like the GS purified from other sources (Deuel et al, 1970; Seyama et al, 1972; Vorhaben et al, 1982; Dowton & Kennedy, 1985; Shankar & Anderson, 1985; Krishnan et al, 1986; Hatanaka et al, 1987; Tholey et al, 1987).

The molecular weight of the brain GS from H. fossilis

(Table 29, Fig.14) was found to be 3.91×10^5 by gel filtration. This was similar to the molecular weight of GS reported from brain of chick 3.64×10^5 (Tholey et al, 1987), pig 3.7×10^5 (Stahl & Jaenicke, 1972) and sheep 3.92×10^5 (Ronzio et al, 1969), retina of chick 3.4×10^5 (Sarkar et al, 1972), liver of rat, 3.6×10^5 (Deuel et al, 1978) and liver of S. acanthias 4.0×10^5 (Shankar & Anderson, 1985). However, it was very low compared to bacterial GS which was between 6.0×10^5 - 1.0×10^6 (Krishnan et al, 1986; Kimura et al, 1989; Ojha & Kantengwa, 1989).

Stability: The purified GS from H. fossilis brain was unstable (Table 30) when stored in purification buffer. It lost 35% of its activity by 24 hrs and total loss of activity by 96 hrs when stored at 0-4°C. However, in the presence of 10% glycerol the enzyme could be stored without loss of any activity for more than a month at 0-4°C temperature. Glycerol has been reported to stabilize the enzyme (GS) activity purified from bacterial, C. pasteurianum (Krishnan et al, 1986) and S. acanthias liver (Shankar & Anderson, 1985). Infact, in all these cases, there was an increase in activity upto 10% when stored in glycerol. The mechanism of stabilizing effect by glycerol and related polyhydric compounds has not yet been clearly understood. Jaraback et al (1966) suggested that the protective effect of various organic solvents might be due to their common property of stabilizing network of structural water molecule (i.e. water-glycerol

structure) for the maintenance of proper spatial configuration of the protein in its native state. Besides glycerol, NaCl also showed stabilizing effect on H. fossilis brain GS for more than a month. Shankar and Anderson (1985) also reported such protective role of NaCl on GS purified from S. acanthias liver. Sodium ions probably binds to the enzyme to stabilize its natural configuration. Albumin showed weak stabilizing effect. However, glycerol and NaCl together gave better stability for longer time and was easy to handle the purified enzyme.

Effect of pH: The pH profile (7.2-11) obtained for purified GS from brain of H. fossilis (Table 31, Fig.15A) was broader compared to GS from S. acanthias liver (Shankar & Anderson, 1985), sheep brain (Elliot, 1951) avian liver and brain (Vorhaben et al, 1982) where the enzyme was active only at a narrow pH range. However, E. coli and Archeobacterium (Shapiro & Stadtman, 1970; Bhatnagar et al, 1986) showed comparatively broader pH profile. The physiological role of this broader pH profile is not yet known. It only indicated that the brain GS of H. fossilis would have better resistance to any fluctuation in pH. This might be one of the physiological adaptive reasons for better survival of H. fossilis in sewage fed water bodies, where there are chances of fluctuation of pH in addition to prevailing hyper ammonia condition. Meister (1985) suggested that pH optima of GS varies from

4.8 to 8.5 depending upon the nature and concentration of divalent cations present. In the present study the pH effect was studied only at 20 µmole Mg^{2+}.

Effect of temperature: The temperature maxima (Table 31, Fig.15B) of catalytic activity of purified GS was found out to be 45°C. However, the purified enzyme alone when incubated at 45°C, started loosing its activity (Table 32, Fig.16). The prevention of loss in activity of pure enzyme in presence of the reaction mixture suggested that the enzyme might be getting stabilized against thermal denaturation by one or more of the substrate/product/cofactor. The stabilising effects of the GS activity by preincubation of the pure enzyme at 45°C for 30 min with the substrates individually and in combination have been presented in Table 33 and Fig.18. Among the combination of substrates cofactors studied Mg^{2+} alone and combination of Mg^{2+} and ATP were found to stabilize best the H. fossilis brain enzyme. Pamiljan et al (1962) reported the protection of GS activity purified from sheep brain by ATP and Mg^{2+} from thermal denaturation at 60°C. The results indicated that Mg^{2+} in the presence or absence of ATP could bind to the enzyme and to protect it against thermal denaturation. Pamiljans et al (1962) suggested for sheep brain GS, that the combination of enzyme with Mg^{2+} ATP complex was the first step in GS reaction and the cleavage of ATP did not occur in absence of glutamate. Glutamate attached to the enzyme only when ATP and Mg^{2+} were present

(Krishnaswamy et al, 1962). H. fossilis brain GS was protected from thermal denaturation by glutamate unlike sheep brain GS where glutamate could not protect the enzyme from thermal denaturation (Pamiljans et al, 1962). However, in the bacterial (C. pasteurianus) GS the protective role of glutamate was reported (Krishnan et al, 1986). H. fossilis brain GS like the bacterial GS might have separate binding sites for glutamate to bind in absence of nucleotide and/or divalent ions and protect the enzyme from thermal denaturation (Meister, 1985).

Effect of 2-mercaptoethanol: The purified enzyme preincubated with 2-mercaptoethanol resulted in complete inhibition of GS activity (Table 34, 35). The 2-mercaptoethanol denaturation of GS has also been found in case of bacterial GS (C. pasteurianum) (Krishnan et al, 1986). It has been shown that sulfhydryl reagent (Buchanan, 1960) and compounds having a sulfur atom in compound (Fushiya et al, 1988) exhibited a strong inhibitory effect on GS activity. H. fossilis GS maintained its activity in presence of 2-mercaptoethanol when in combination with substrate or cofactor in the reaction mixture. Bacterial (C. pasteurianum) GS also showed its complete activity when it was with EDTA and α-keto-glutarate along with 2-mercaptoethanol.

Kinetics: The Km value of purified H. fossilis brain GS (Table 36) for ATP (2.2 mM) was comparable to that of sheep brain (2.3 mM) (Rowe et al, 1970) and rat liver

(2.3 mM) (Meister, 1985). It was higher compared to chicken liver (0.7 mM) (Seyama et al, 1972), pig heart (1.6 mM) and rat muscle (1.1 mM) (Rowe, 1985). The Km value observed for glutamate (4.5 mM) was lower compared to S. acanthias liver (11 mM) (Shankar & Anderson, 1985) and fleshfly flight muscle (12.8 mM) (Dowton & Kennedy, 1985), but was higher compared to sheep brain (2.5 mM) (Rowe, 1985). It was similar to rat liver (5.0 mM) (Meister, 1985) and pig heart (5.3 mM) and rat muscle (5.8 mM) (Rowe, 1985). The Km value for hydroxylamine (0.5 mM) which is very high compared to rat liver (0.02 mM), sheep brain (0.18 mM) (Rowe et al, 1970) and rat muscle (0.17 mM), pig heart (0.15 mM), Rowe (1985). Generally higher Km value of H. fossilis brain GS for glutamate and hydroxylamine indicated its low affinity for glutamate and ammonia compared to the result from other ureogenic species such as rat liver and muscle, sheep brain and pig heart. This high Km values in this ureogenic teleost, H. fossilis might have been compensated by the presence of high enzyme protein (specific activity, 3.5) in the brain which was 2-60 times higher than the specific activity (unit/mg protein) reported for other ureotelic animal tissues such as rat liver (1.88), muscle (0.06), sheep brain (0.176) and pig heart (0.12).

Modulation: Purified GS activity has been shown to be modulated by variety of metabolites (Tate & Meister, 1971; Iqbal & Wu, 1971; Meister, 1974; Deuel et al, 1978). The

induction and inhibition effects of certain divalent cations have shown to regulate GS activity *in vivo* in different groups of higher and lower organisms. Since in *H. fossilis* brain GS there was no enzyme activity in absence of Mg^{2+} (Table 34), it was interesting to determine if any other divalent cations could activate the enzyme in absence of Mg^{2+}. It was found that Co^{2+} and Mn^{2+} could activate the enzyme to a lesser extent in absence of Mg^{2+}. The order of activating ability was $Mg^{2+} > Co^{2+} > Mn^{2+}$. However, Co^{2+} and Mn^{2+} inhibited the enzyme at higher concentrations even in the presence of Mg^{2+} (Fig.23). Cation may bind for activatory or inhibitory effect. Similar activation of enzyme was observed in chick brain (Tholey *et al*, 1987) by Mg^{2+}, Mn^{2+}, Co^{2+}, in sheep brain (Elliot, 1951) by Mg^{2+} and Mn^{2+} and in *Anabaena* (Ip *et al*, 1983) by Mg^{2+}, Co^{2+}. Tholey *et al* (1987) suggested for chick brain GS, that the enzyme possesed more than one type of cation binding site. Two metal ion binding sites could be detected in *E. coli* at a distance of 6 A° (Wedler & D'Aurora, 1974; Martin & Richardson, 1979). Maurizi *et al* (1986) found that bovine brain GS contained two divalent cations bound to the active site of each subunit. It needs further investigation before coming to any conclusion for *H. fossilis* brain GS about the number and nature of binding sites for metal ions.

Though Mn^{2+} and Co^{2+} could replace Mg^{2+} to certain extent to activate GS, the enzyme was inhibited by Cu^{2+}, Zn^{2+} Po_4^{3-} and Cd^{2+}, Ca^{2+} (Table 37). The inhibitory effect

of different metal ions on GS has been reported in sheep brain (Elliot, 1951), fleshfly flight muscle (Dowton & Kennedy, 1985), rat muscle, kidney and liver and chinese hamster liver (Cimino & Tramonti, 1973). H. fossilis brain GS might be regulated by activation and inhibition effects of various divalent cations in vivo and more than one type of cations might be involved in this regulation. However, the interaction of these regulatory ions for H. fossilis brain GS, if any, has to be found out.

The purified enzyme was inhibited by several amino acids (Table 38). Woolfolk and Stadtman (1967) reported that E. coli glutamine synthetase was inhibited by end products of glutamine metabolism, such as CP, Ala, Gly, Ser. Stadtman (1973) and Krishnan et al (1986) suggested that inhibition of GS by metabolites constituted a feedback regulation system in which each end product inhibited the first common enzyme in the pathway. The inhibitory effect of different amino acids on GS activity has been reported in other eukaryotic species (Woolflock & Stadtman, 1967; Tate et al, 1972; Rao & Kanungo, 1972; Cimino & Tramonti, 1973). Ala, Gly, CP, Asp, Asn, Orn, Arg, Cit inhibited the GS purified from H. fossilis brain among all the amino acids studied. This enzyme might have similar feedback or product inhibition mechanism for its regulation. It was completely inhibited by CP at higher concentrations compared to other amino acids. Tate et al (1972) proposed that inhibition of GS by CP may serve

to control the amount of glutamine available for pyrimidine biosynthesis by glutamine dependent CPS pathway. In addition to the inhibition by CP, purified GS was seen to be inhibited by Cit and Arg. These three amino acids i.e. CP, Cit and Arg components of o-u cycle and were expected to be present in the brain of H. fossilis as their corresponding enzymes for their synthesis have been reported. This could be an additional regulatory mechanism for GS in brain of H. fossilis.

In addition to metal ion and amino acid regulation of GS some nucleotides also have been found to regulate GS activity of H. fossilis brain. Among the nucleotides studied inhibition by ADP was maximum followed by AMP and IMP (Table 39). The inhibition by AMP was reported in E. coli, Bifidobacteria, S. platensis, chinese hamster liver, rat kidney. It did not inhibit the enzyme from rat muscle, rat liver and sheep brain (Cimino & Tramonti, 1973; Shapiro & Stadtman, 1970; Hatanaka et al, 1987; Dang et al, 1989). ADP gave significant inhibition at all concentrations and inhibited the enzyme non-competitively with respect to glutamate having the Ki value of 3.15 mM (Fig.24, 25). Similar results were reported for the enzyme from human brain (Yamamoto et al, 1987) and avian liver (Vorhaben et al, 1982). However, Ki value for ADP (3.15 mM) in H. fossilis brain was much higher compared to pig heart (1.1 mM) and rat muscle (0.8 mM) (Rowe, 1985). The inhibition of GS by ADP is of physiological significance. It has been known that the metabolic disorder of brain has been associated with decreased ATP with accumulation of ADP. Decrease in the

energy charge could cause disorders in ammonia metabolism. Ammonia, although is a normal by-product of metabolism, could be toxic to nervous system at moderate concentrations. Glutamine formation has been one of the principal pathways for ammonia elimination in the brain. ADP inhibition would aggravate taxaemic conditions at lower energy level.

From the physico-chemical properties it could be suggested that the GS played a major role in ammonia management in brain of H. fossilis. The fish, therefore, has a better adaptation to different adverse environmental conditions mainly involving higher ammonia level.

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