# STUDY ON ADAPTIVE STRATEGIES AGAINST AMMONIA TOXICITY IN THE AMPHIBIOUS MUD EEL (*Amphipnous cuchia*)

**JAMESTEWORD L. KHONGSNGI**

**DEPARTMENT OF ZOOLOGY**

**NORTH-EASTERN HILL UNIVERSITY**

**SHILLONG-793 022**

**2010**

*Dedicated*

*To*

*My beloved Parents*

# ***DECLARATION***

*I, Mr. Jamesteword L. Khongsngi, hereby declare that the subject matter of this thesis is the record of the work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research in any other University/Institute.*

*This thesis is being submitted to the North-Eastern Hill University, Shillong for the degree of Doctor of Philosophy in Zoology.*

(Mr. Jamesteword L. Khongsngi)
*Candidate*

Head,
*Deptt. Of Zoology*

(Prof. N. Saha)
*Supervisor*

# Acknowledgement

I would like to convey my sincere gratitude to my respected guide, Prof. N. Saha, Department of Zoology, NEHU, Shillong, without whose able guidance, persistent encouragement, constructive and critical analytic abilities, valuable advices and comments all through my research period, would not have been made this investigation a successful one.

I am also thankful and grateful to the former Heads of the Department, Prof. B. B. P. Gupta, Prof. S. B. Prasad and the present Head of the Department Prof. (Mrs) R. N. K. Hooroo for providing the departmental facilities to carry out my research works, thereby, enabling me to complete my research works without any hitches or setbacks.

I would also like to extend my sincere thanks and appreciation to all my Lab-mates, Lucy, Mahua, Manas, Gitalee and Liani for their valuable assistance, encouragement and co-operation and also Marshal for his help and assistance. My special thanks are due to my seniors Dr. Zaiba, Dr. Arundhati, Dr. Shritapa and Dr. Kuheli for their generous help at various stages of my work, for which I shall remember and appreciate always.

I extend my thanks to all the non-teaching staffs and members of the Department for their kindness and encouragement, whenever I needed.

This acknowledgement would fail, if I am not expressing my sincere thanks and gratitude to all my relatives especially my parents and to all my family members, friends far and near for their constant support and encouragement.

*Last but not the least; I greatly acknowledge and appreciate the Council for Scientific and Industrial Research (CSIR), New Delhi for awarding the Research Fellowship (JRF and SRF) and Contingency Grants 2004-2009 with gratefulness.*

*Date:* 17.05.2010

*Place: Shillong* *(Jamesteword L. Khongsngi)*

# INTRODUCTION

Air breathing and nitrogen metabolism are the two but different physiological adaptations that have to evolve side by side, especially in those fishes, which have adopted the amphibious way of life style. Air breathing is one of the adaptive responses utilized by those fishes dwelling in habitats where the quantity of dissolved $O_2$ is extremely small. It is an ancient vertebrate specialization, thought to have appeared in fishes during the late Silurian Period (438-408 million years before present, mybp), long before the evolution of amphibians and the invasion of land tetrapods (Gardiner, 1980; Gordon and Olson, 1994; Long, 1995). Modern air-breathing fishes occur in a variety of freshwater and marine habitats that are subjected to seasonal drought. Marine air-breathers occur in tropical coral reefs, in rocky intertidal zones, marshes and bays. A variety of factors, from the adoption of an amphibious behavior to exploit resources at the air-water interface, to the periodic aerial exposure imposed by low tides and even aquatic hypoxia in certain habitats have all played a role in selection for air-breathing among marine species (Gibson, 1969; 1982; Sayer and Davenport, 1991). In freshwater fishes the impetus for air-breathing seems to have come about primarily from the effects of aquatic hypoxia (Carter, 1957; Dunn, 1983). The lack of rain and rise of temperature of water bodies causing evaporation resulting in water area shrinkage and often leads to crowding of fishes in isolated pools. Photosynthesis by the aquatic vegetation is not sufficient to maintain the normoxia because they are usually shaded by surface-growing macrophytes or dense stands of trees. Thus in these types of habitats, where $O_2$ production is limited, heating and unusually large biological $O_2$ demand imposed by crowding and decomposing organic materials can lead to hypoxia and even anoxia, that natural selection has worked repeatedly, among diverse tropical freshwater fish fauna, to bring about a number of successful and strikingly different specializations for air-breathing.

As a rule, fishes are so adapted to the aquatic habitat that under normal conditions it is not possible for them to emerge unto land. The direct cause of emergence into dry land cannot be either search for food material, intense competition or flight from pursuing predators. None of these or other factors could have led to positive changes if there had not been some form or organizational preconditioning in the form of adaptations for aerial respiration and locomotion on the ground. A fundamental cause is found in contrast of ecological conditions by which aerial respiration as well as temporary emergence from water was accomplished in modern fishes (Schmalhausen, 1968). If we paraphrase this saying, we can say that structural and biochemical changes (i.e. genes evolution) have to evolve to equip the organism to adapt to the present mode of living. Structural adaptations for aerial respiration in tropical air-breathing fishes are more specialised and exhibit greater diversity of air-breathing organs and adaptations due to their regular exposure to seasonal drought and hypoxia (Munshi and Hughes, 1992; Graham, 1997a). However, on land, fishes face another major problem, i.e., the accumulation of toxic ammonia inside their body, for which they have to equip themselves with mechanism(s) or various biochemical adaptations that would facilitate the invasion of the terrestrial habitat by fishes during evolution. The intricate association between environmental water availability and nitrogen metabolism in lower vertebrates has influenced theories about the habitat and evolution of the early vertebrate terrestriality (Hochachka and Somero, 1984; Griffith, 1991). The presence of the ornithine-urea cycle (OUC) in species such as the African (*Protopterus*), South American (*Lepidosiren*) lungfishes and tetrapods has historically provided the rationale for an evolutionary link between the OUC and the invasion of land by vertebrates (Graham, 1997a). The OUC is ideally suited for solving the problems of nitrogen metabolism, desiccation and hypercapnia imposed by air breathing and terrestriality. Therefore, urea formation by these amphibious fishes would enable them to avoid ammonia toxicity, conserve water and reduce hypercapnia due to consumption of bicarbonate by urea synthesis (Atkinson, 1992) maintaining the acid-base balance due to aerial exposure. The OUC has evolved from a straight-chain arginine biosynthetic pathway found in primitive prokaryotes, and hence carbamyl phosphate synthase (CPS) (the key enzyme in OUC) has a very long evolutionary history (Paulus, 1983). The existence of the OUC in fishes has been dated back to fossil anadromous agnathans. With the appearance of arginase (ARG) to convert arginine to urea and regenerate ornithine, the straight-chain arginine biosynthetic pathway of the primitive prokaryotes became cyclical (for review, see Saha and Ratha, 2007a). Several modifications in the structure, function, and distribution of CPS have occurred inside the cell during evolution from primitive prokaryotes to the most modern evolved eukaryotes (Hong *et al*., 1994; Anderson, 1995). At present, three different CPS isoenzymes (CPS I, II and III) have been demonstrated on the basis of differences in terms of substrate specificity, activators, inhibitors, subcellular localization, and physiological functions in higher vertebrates (Anderson and Walsh, 1995).

Urea biosynthesis via the OUC for both ammonia detoxification and osmoregulation in the embryonic stages probably evolved with evolution of larger cleidoic eggs and prolonged embryonic development in gnatostomes (Saha and Ratha, 2007a). Embryos utilizing the yolk proteins as an energy source produce a large amount of ammonia during catabolism, which needs to be detoxified. It has also been suggested that ureo-osmotic regulation was a common strategy in Paleozoic marine gnatostomes. It has further been suggested that ureo-osmotic regulation evolved in freshwater anadromous fishes that gradually became established in the marine habitat (Griffith, 1991). Ureotelic evolution in vertebrates has been considered to be monophyletic trait on the basis of the enzymatic profiles found in ammoniotelic fishes, ureo-osmotic marine elasmobranches, ureogenic lungfishes and ureotelic amphibians (Mommsen and Walsh, 1989; Walsh, 1997). The profile of the enzymes involved in urea synthesis from ammonia is different, particularly with reference to glutamine synthetase (GS), mitochondrial CPS and ARG. They have shown a pattern of changes during evolution of ureotelism. In fishes and ureo-osmotic elasmobranch glutamine serves as the nitrogen-donating substance for the mitochondrial CPS III isozyme. The GS and ARG are co-localized in the mitochondria along with CPS III. In ureotelic amphibians and mammals, the mitochondrial CPS III was replaced by CPS I, which utilizes ammonia directly instead of glutamine as nitrogen-donating substrate along with shifting of GS and ARG from mitochondria to cytosol.

When amphibious fishes leave water, they move between media of differing properties. The lack of water in the surrounding medium presents many challenges to fishes, especially with the nitrogen excretion. Ammonia is the major nitrogenous excretory product of teleosts and its high solubility in water ensures that it is disposed off rapidly by the process of simple diffusion. However, on land this excretory pathway is not possible or severely reduced, and continuous elimination of nitrogen in the form of ammonia during emersion might result in undesirable loss of water. But amphibious fishes must avoid the accumulation of ammonia concentration to near toxic levels; alternatively by active conversion of ammonia to a less toxic form such as urea and other compounds, for storage and subsequent elimination on re-immersion (Ip *et al.*, 2004a). Hence, the nature of the nitrogenous excretory products in animals has changed with the evolution of vertebrates from the aquatic to the land habitat (Campbell, 1991; Wright, 1995; Saha and Ratha, 1998; 2007a). Thus, the major factor that determines the type of nitrogen excretion in animal is the availability of water in the animal’s habitat that has resulted in various adaptations to nitrogen metabolism with an intention to lessen the demand on water for the purpose of nitrogenous waste excretion as animals moved on land. In terrestrial animals, where diffusion of ammonia is difficult due to limited availability of water, toxic ammonia is converted to soluble urea for temporary storage and excreted out in concentrated form through urine using less amount of water (Cohen, 1976; Campbell, 1991; Wood, 1993; Anderson, 1995). Mammalian and amphibian vertebrate species and the lungfishes are ureotelic, and they maintain blood levels of ammonia below ≈0.03 mM by converting toxic ammonia to urea via the classical OUC in the liver (Krebs and Hanseleit, 1932; Cohen, 1976; Anderson, 2001). Insoluble uric acid in the form of solid pellets is the excretory product of those animals where conservation of metabolic water is highly essential due to their arid environment (Hoar, 1983; Nenr, 1988; Powers and Meister, 1988; Campbell, 1991; Wood, 1993). Insects, reptiles and birds are uricotelic, converting toxic ammonia into uric acid (Campbell, 1991). Taking these all-together animals can be divided into three major groups such as ammoniotelic, ureotelic and uricotelic. However, many animals exhibit mixed patterns of nitrogen excretion depending upon their physiological and environmental conditions. For example, amphibians are ammoniotelic in water and ureotelic on land. The tadpole living in water excretes its nitrogenous waste as ammonia, but on metamorphosis, its nitrogen metabolism changes and the adult frog, which spends much of its life on land, excretes mainly urea.

In response to normal aquatic habitat, the major nitrogenous excretory product of the vast majority of teleost fishes is ammonia (for reviews, see Wood, 1993; Saha and Ratha 1998). This overall mechanism implies that ammonia formed in the cytosolic and/or mitochondrial compartments of liver and other tissues simply diffuses down an $NH_3$ gradient across the boundary membranes of various body compartments. It is usually assumed that most nitrogenous excretion (≈90%) takes place across the gills, predominantly in the form of un-ionised ammonia ($NH_3$). Although this is true of many freshwater teleosts, this is not the case for marine and/or amphibious fishes. Marine fishes excrete more nitrogen via skin, gut and/or gills (Sayer and Devenport, 1991). In general, however, ammonia formed in the liver, as well as in other tissues, is cleared from the blood through gills (Heiler, 1990; Wilkie, 1997). The rate of $NH_3$ excretion is determined by the magnitude of the $NH_3$ gradient between the blood and water (Wilson *et al.*, 1994) or partial pressure gradient of $NH_3$ ($\Delta P_{NH3}$). However, under high environmental ammonia, there is a net influx of exogenous ammonia from the environment, due to reverse partial-pressure gradient of $NH_3$ ($\Delta P_{NH3}$) and also in waters with high pH values excretion of ammonia is difficult. Hence at these environmental conditions, fishes are confronted simultaneously with retention of endogenous ammonia and uptake of exogenous ammonia. All these conditions lead to accumulation of ammonia inside their system. Elevated ammonia is toxic to aquatic animals, including fishes.

Therefore, some tropical air-breathing fishes exhibit various biochemical adaptations to ameliorate environmental ammonia toxicity at branchial and/or epithelial surfaces.

Most teleost fishes depend mainly on amino acids for their energy sources. Therefore, ammonia is being continuously produced inside the body of fishes, with the catabolism of amino acids through the process of transdeamination mainly in the liver (Wilkie, 1997). Normal dietary intake of proteins provides excess of amino acids than the amount required to sustain protein turnover. Elevated levels of amino acids in the plasma have been reported in fishes with high protein diets (Cowey *et al*., 1977). Unlike carbohydrates and lipids that can be stored inside the body of animals as glycogen and fats respectively, excess amino acids cannot be stored. Vitellogenin is the only special storage protein in the egg of oviparous animals (Driedzic and Hochachka, 1978). Therefore, the excess amino acids are deaminated to produce ammonia, and the resulting carbon residues are either funneled into gluconeogenic pathway or to tricarboxylic acid (TCA) cycle for generation of metabolic energy. In carnivorous fishes, the natural diet is rich in proteins and low in carbohydrates. Cells utilize amino acids as energy source during starvation either directly by oxidation of carbon skeleton or indirectly by the conversion of carbon skeleton to glucose via the process of gluconeogenesis (Bever *et al*., 1981). Proteins and other nitrogen-containing biomolecules are regular and essential dietary components of fishes, digestion and metabolism of these compounds result in excess production of nitrogenous wastes.

## **Ammoniogenesis:**

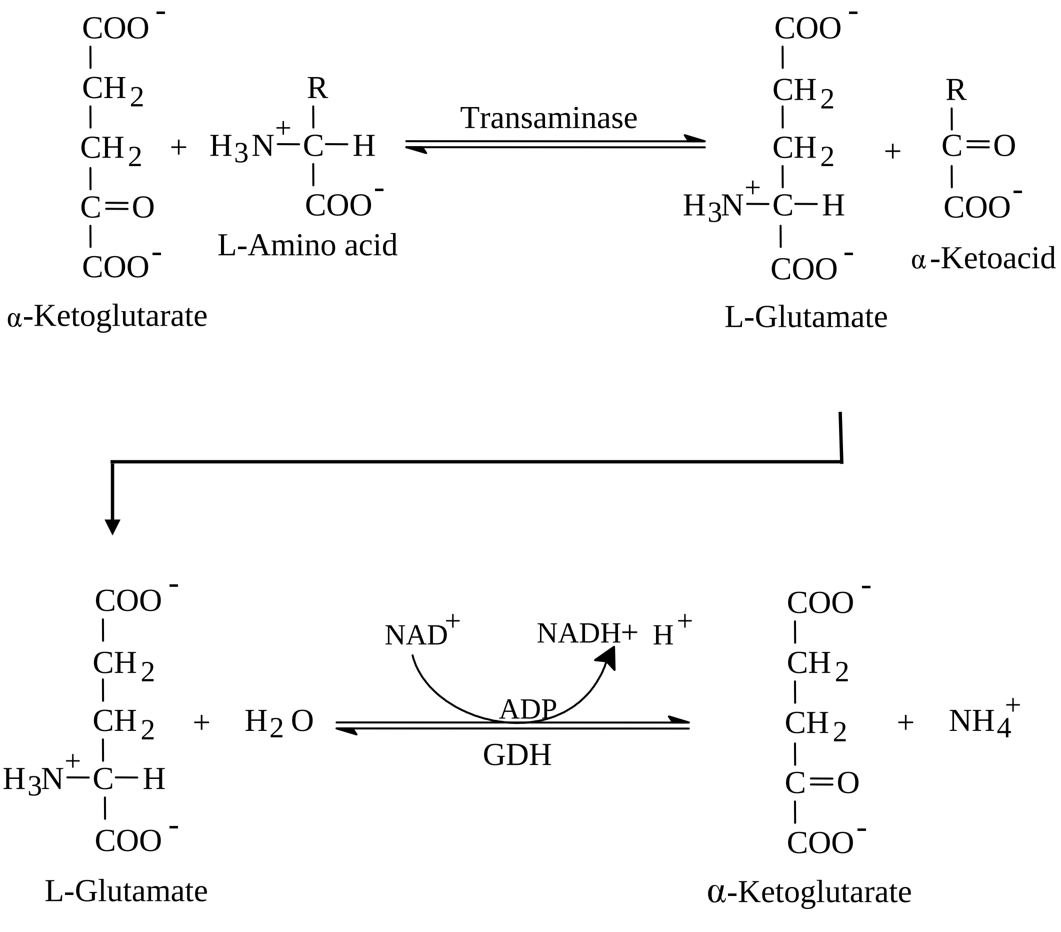
The major pathway of ammoniogenesis or ammonia formation in fishes, as in other vertebrates, is via transdeamination of amino acids besides deamination of amides, purines, pyrimidines and hexosamines (Cohen and Brown, 1960; Foster and Goldstein, 1969; Watts and Watts, 1974; Walton and Cowey, 1977, 1982; Randall and Wright, 1987). The primary site for ammonia production is probably the liver (Pequin and Serfaty, 1963), but the necessary enzymes have also been located in kidney, gill, and skeletal muscle tissues (Goldstein and Foster, 1971;

McBean and Golstein, 1967). Ammonia is also produced by deamination of adenylates in fish muscle (Driedzic and Hochachka, 1976).

**Transdeamination:**

In the cytosol of hepatocytes, amino groups from most amino acids are transferred to α-ketoglutarate by transamination reaction, catalyzed by enzymes call aminotransferases or transaminases to form glutamate. Glutamate is then transported from the cytosol into liver mitochondria where it undergoes oxidative deamination catalyzed by the enzyme, glutamate dehydrogenase (GDH) to form ammonia. The combined action of an aminotransferase and GDH is referred to as *transdeamination*.

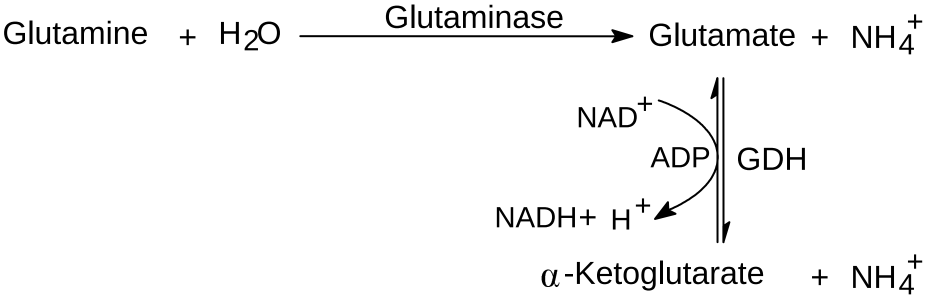
The overall reaction is summarized as follows:



Transdeamination has been reported as the major pathway for ammonia formation in the liver of freshwater teleosts (Janssens, 1964; Campbell, 1983; Campbell, 1991; Wilkie, 1997) and in the mudskippers (*Boleopthalmus boddaerti* and *Periophthalmodon schlosseri*) (Chew and Ip, 1987).

**Deamination:**

Few amino acids bypass the transdeamination pathway and undergo direct oxidative deamination. Glutamine is a non-toxic transport form of ammonia. In animals, glutamine in excess of that required for biosynthesis is transported through the blood to the liver and kidney for processing. In liver and kidney mitochondria, glutamine is deaminated through hydrolytic removal of secondary amino group by the enzyme glutaminase, which converts glutamine to glutamate and $NH_4^+$. Glutamate is further processed in the liver by the enzyme GDH, releasing more ammonia. The reaction is summarized as follows:



Direct deamination of serine, glutamine, aspartate, asparagine, cysteine and histidine catalyzed by the respective amino acid deaminases also contribute to the production of ammonia (Salvatore *et al*., 1965; Janicki and Lingis, 1970; Watts and Watts, 1974).

**Nucleodeamination:**

Deamination of different nucleosides and nucleotides catalyzed by nucleodeaminases is another source of ammonia (Walsh, 1998).

$$\text{Guanylic acid} + H_2O \xrightarrow{\text{Guanylic acid deaminase}} \text{Xanthylic acid} + NH_3$$

$$\text{Adenosine} \xrightarrow{\text{Adenosine deaminase}} \text{Inosine} + NH_3$$

Hydrolysis of particularly adenosine monophosphate (AMP) by AMP deaminase has been shown to play an important role in ammonia production in some fishes (Makarewicz and Zydowo, 1962; Makarewicz, 1963).

**Ammonia toxicity:**

Ammonia is an unusual toxicant in that it is produced by as well as being poisonous to animals. In solution, ammonia exists both as $NH_3$ and $NH_4^+$. The $NH_3/NH_4^+$ ratio varies with pH, the pKa of the reaction being about 9.5 in distilled water at 15 °C (Cameron and Heisler, 1983). In addition to the water pH, which has a marked effect on both the $NH_3/NH_4^+$ equilibrium and on ammonia toxicity, temperature, pressure, ionic strength, dissolved oxygen, salinity and carbon dioxide also affect the $NH_3/NH_4^+$ equilibrium (Alabaster and Herbert, 1954; Alabaster *et al*., 1979; Hillaby and Randall, 1979; Thurston *et al*., 1981; Ip *et al*., 2001a). Increasing the pH value by 1 unit (e.g. pH 7-8) at 10 ºC produces 10-fold increase in $NH_3$ concentration while increasing the temperature by 10 ºC (10-20 ºC) approximately doubles the $NH_3$ concentration (Eddy, 2005). Although $NH_3$ in water is considered the major toxic pollutant in the environment, $NH_4^+$ is probably the major toxic element within the body (Ip *et al*., 2001b). Body or the physiological pH is around 7.0-7.8, varying with the tissue. Most biological membranes are permeable to unionized ammonia ($NH_3$) but relatively impermeable to ionized ammonia ($NH_4^+$). As a result, most ammonia entering the body as $NH_3$ will be rapidly converted to $NH_4^+$. Thus, more than 95% of total ammonia in the body exists as $NH_4^+$.

Ammonia as a common pollutant in inland waters and its toxicity to fishes has been a subject of extensive laboratory studies and reviews (Alabaster and Lloyd, 1982; Haywood, 1983; Randall and Wright, 1987; Wood, 1993; Saha and Ratha, 1998; 2007a; Ip *et al.*, 2001c). Ammonia is excreted by many aquatic animals and is also continually produced as a degradable product of micro- and macro-vegetation, the decomposition of dead organisms and excreted wastes of living organisms. Anthropogenic activities also produce increasing amount of nitrogenous material and significant proportion is in the form of ammonia or compounds that can yield ammonia. Significant amounts of ammonia are generated from the production and use of fertilizers, biomass burning, from domestic sewage, intensive animal husbandry, agricultural run off and industrial effluents (Alabaster and Lloyd, 1982; Eddy, 2005). All these lead to increase the level of ammonia concentration in the ambient environment and have shown to cause serious damage to the aquatic fauna. In general, however, aquatic animals can tolerate more elevated levels of blood ammonia than the terrestrial animals. Plasma total ammonia ($NH_3$ + $NH_4^+$) normally remains between 0.05 and 2 mM in most teleost fishes (Campbell and Anderson, 1991; Wood, 1993; Saha and Ratha, 1998), with the exception of singhi catfish (*Heteropneustes fossilis*), where it has been reported at a level of up to 4 mM in high ambient ammonia concentrations (Saha and Ratha, 1990, 1994). In contrast, blood ammonia level greater than 0.05 mM can be toxic to the central nervous system of most mammals (Copper and Plum, 1987; Meijer *et al.*, 1990).

Elevated body ammonia levels are toxic, and have both acute and chronic impacts that vary with the species. Acute ammonia toxicity in fishes leads to decrease in oxygen carrying capacity of haemoglobin (Sousa and Meade, 1977), increased oxygen consumption, respiratory rate and heart beat (Smart, 1978; Chen and Nan, 1993) and disturbance of acid-base balance (Maetz, 1973; Cameron and Heisler, 1983; Cameron, 1986; Paley *et al.*, 1993). Elevated water ammonia levels inhibit feeding and growth in Dover sole (*Solea solea* L.) (Hampson, 1976; Alderson, 1979). Chronic ammonia toxicity results in the reduction of growth rate, gill hyperplasia, thickening of the gill epithelium, fusion of lamellae, swollen gills, haemorrhaging (Burrows, 1964; Reichenbach-Klinke, 1967; Smart, 1976; Thurston *et al*., 1978; Ip *et al.,* 2001b) and changes in mucous production, growth and stamina (Lang *et al*., 1987). Elevated water ammonia level reduces swimming ability in Coho salmon (*Oncorhynchus kisutch*) (Randall and Wicks, 2000), probably because of the fact that increased ammonium ion levels in fishes cause muscle depolarization (Taylor, 2000). Ammonia can interfere with energy metabolism through impairment of the TCA cycle (Sousa and Meade, 1977; Arillo *et al*., 1981). The suppression of TCA cycle is through depletion of α-ketoglutarate, which is converted to glutamate to remove ammonia and also due to inhibition of some key enzymes, including isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, as in mammals (for review, see Cooper and Plum, 1987; Ip *et al*., 2001b). Ammonia stimulates glycolysis by activation of phosphofructokinase in the muscle (Kloppick *et al*., 1967). Elevated ammonia level leads to increase in pyruvate and lactate levels in the plasma of rainbow trout (*Salmo gairdneri*) (Fromm and Gillette, 1968). An increase of acid metabolites from glycolysis and Krebs cycle lowers the blood pH due to accumulation of pyruvate and lactate (Campbell, 1991; Ip *et al*., 2001b). The resulting acidemia would shift the oxygen saturation of haemoglobin and cause death by suffocation. Elevated ammonia levels have also been shown to impair glutamate and aspartate metabolism in mammals (Hindfelt *et al*., 1977) and interfere with amino acid transport (Mans *et al*., 1983). Ammonia affects the ionic balance in fishes at both cellular and organismal levels, reducing $Na^+$ influx and $K^+$ loss through substitution of $NH_4$ for $K^+$ in $Na^+$-$K^+$ -ATPase and/or $Na^+/K^+/2Cl^-$ cotransport (Wilkie, 1997; Person-LeRuyet *et al*., 1988) and/or the substitution for $H^+$ in $Na^+/H^+$ exchanger (Randall *et al*., 1999).

### **Effect of ammonia toxicity in brain:**

Ammonia acts on the central nervous system of vertebrates, including fishes, causing hyperventilation, hyperexcitability, coma, convulsions and finally death (Hillaby and Randall, 1979; McKenzie *et al*., 1993). Ammonia can cross the blood-brain barrier in mammals (Sears *et* *al*., 1985) and high ammonia levels modify many aspects of the blood brain barrier (Cooper and Plum, 1987). Exposure to ammonia may lead to the depletion of cerebral glutamate, and thus remove an important neurotransmitter (Hindfelt *et al*., 1977). The uncoupling of oxidative phosphorylation by $NH_4$ ion is another adverse effect of ammonia to inhibit ATP production (Smart, 1978). Furthermore, the increased ATP demand for the GS reaction may account for the decrease in cerebral ATP as noted in ammonia-exposed rainbow trout (*S. gairdneri*) (Arillo *et al*., 1981).

Glutamate is the principle excitatory neurotransmitter in brain. Inhibition of glutamate uptake (Oppong *et al*., 1995) or increased glutamate release from neurons (Rose, 2002) and/or astrocytes can cause an increase in extra-cellular glutamate (Michalak *et al*., 1996) that will initiate a series of deleterious effects. Glutamate can be released from astrocytes by three different mechanisms out of which two are $Ca^{2+}$-independent (reverse- glutamate transport and induced swelling) and one is $Ca^{2+}$-dependent on intra cellular calcium concentration (vesicular release). First, glutamate-release can result from astrocyte swelling (brain edema) in acute liver failure (Takahashi *et al*., 1991; Blei *et al*., 1994; Rose *et al*., 1999; Brusilow, 2002). Brain edema is a consequence of glutamine accumulation (synthesized through GS) in the astrocytes, possibly because of the inhibition of glutaminase (Bradford *et al*., 1989; Brusilow, 2002). An increase in extra-cellular brain glutamate could also lead to glu-induced astroglial swelling (Hansson *et al*., 1997) and release of glutamate, which can in turn aggravate astroglial swelling due to the accumulation of glutamine. Secondly, glutamate release can also result in reversed glutamate transport. Energy demand increases when intracellular ammonia concentrations increased, by the stimulation of glycolysis through activation of phopsphofructokinase I (Sugden and Newsholme, 1975). As the energy demand increases there is less energy available for the maintaining the membrane potential. To establish the membrane potential, the reversal of glutamate transport takes place, with a glutamate and two $Na^+$-ions are transported out of, and a $K^+$- ion transport into the cell (Szatkowski *et al*., 1990). Thirdly, $Ca^{2+}$–dependent glutamate release can occur in acute liver failure. In a recent review, it was demonstrated that astrocytes play an active role in synaptic transmission (Vesce *et al*., 2001). Glutamate binding to receptors stimulates an increase in an intra-cellular level of $Ca^{2+}$ and the subsequent release of glutamate (Pasti *et al*., 2001). Therefore, astroglial swelling can be the consequence of increase of extracellular glutamate concentration (Hansson *et al*., 1997) and accumulation of glutamine (Bradford et al., 1989; Brusilow, 2002). Extracellular glutamate binds with and activates NMDA-type glutamate receptors (Marcaida *et al*., 1992; Hermenegildo *et al*., 1996), which are coupled with NO-cyclic GMP signal transduction pathway in the brain (Hermenegildo *et al*., 2000). Activation of NMDA-receptors can also be the result of membrane depolarization leading to removal of the $Mg^{2+}$ block on the NMDA-receptor (Fan and Szerb, 1993). Activation of NMDA-receptors causes a rise in the intra-cellular $Ca^{2+}$ and $Na^{+}$ levels. In order to maintain homeostasis, $Na^{+}$ is extruded from neurons by $Na^{+}$-$K^{+}$-ATPase, which is activated by decreased protein kinase-C (Konsenko *et al*., 1994; Marcaida *et al*., 1995), causing a net loss of ATP content in the brain (Kosenko et al., 1993; 1994). The rise in the intracellular $Ca^{2+}$ activates various $Ca^{2+}$- dependent enzymes such as nitric oxide synthase (NOS), leading to the subsequent increase in NO, which is a stimulant of guanylyl cyclase and also NO stimulates the L-arginine uptake by ammonia (Roa *et al*., 1997). Arginine is the obligatory precursor for NO production. NO affects neuronal function by increasing cGMP formation, producing both inhibitory and excitatory effects on the neurons (Kosenko *et al*., 1994; 1995). Intracellular $Ca^{2+}$ also stimulates the release of active oxygen ($O^{+}$) from mitochondria that could combine with NO to form peroxynitrite. Peroxynitrite, a highly toxic anion, acts by oxidizing various intracellular proteins and lipids, and eventually leads to the destruction of cell membrane. $Ca^{2+}$ is also capable of activating proteases for protein-degradation (Kosenko *et al*., 1997; 1999; 2000). This is coupled with tyrosine nitration of intracellular proteins, which alters enzyme activities and confers a predisposition on proteins for protein degradation, which could lead to extensive destruction of protein in the astrocyte. Elevated $Ca^{2+}$ levels appear to reduce the cortisol response to stressful situation (Yesaki and Iwama, 1992). Cortisol stimulates protein catabolism, thus reduced cortisol levels will reduce ammonia production by the fish and this may be the major way in which elevated $Ca^{2+}$ ameliorates ammonia toxicity (Wilson *et al.*, 1998).

Ammonia apart from inducing stress response at the biochemical levels, it has various effects at the physiological levels, particularly threatening the homeostasis of the fish such as ionic balance, electrophysiological etc. Response to stress ranges from induction of certain genes and proteins at the cellular level to behavioral response at the organism level. At the cellular level stress response is characterized by the induction of certain class of highly conserved proteins, known as heat shock proteins (hsps) (Welch, 1993). Many hsps have been shown to be induced in fish by various stressors, indicating a protective role for hsps during times of stress (Iwama *et al.*, 1999). Stress is an energy demanding process (Barton and Schreck, 1987; Vijayan *et al.*, 1997) and amino acids are the main source of energy for the teleost fish (Waarde, 1982). Milligan (1997) showed that cortisol rapidly elevates the plasma amino acids and acts through the enhanced proteolysis of tissue proteins mainly in the liver. Vijayan *et al.* (1996) showed that cortisol increased the hepatic GS activity in sea raven suggesting the link between hormone and CPS III in ureogenesis. The hepatic GS activity was not elevated in acutely stressed ureogenic Gulf toadfish treated with metyrapone (Hopkins et al., 1995), which according to them high GS activity probably requires the initial surge in cortisol level. Basu *et al.* (2001), demonstrated that high concentration of cortisol reduces/ attenuates the stress-induced levels of hsp70 in liver and gill tissues of trout and gills of tilapia which is probably by downregulation of glucocorticoid receptor (GR) complexes (Pottinger, 1990) releasing the bound hsp70 to the GR and this freed hsp70 participates in the negative regulation of heat shock gene expression via an autoregulatory loop (Craig and Gross, 1991; DiDomenico et al., 1982). But the study done by Yoshitaka *et al.* (2006) in human found that Gln helps in the inducing the levels of hsp70 proteins.

## Ammonia excretion:

Due to these wide ranging toxic effects, ammonia is either excreted out immediately or converted to less toxic compounds such as urea, uric acid or amino acids for temporary storage *in vivo*. Gills are the major sites of ammonia excretion in fishes, but smaller quantities of ammonia may also be eliminated by kidney (for review see Eddy, 2005) and skin (Morii *et al.*, 1978). The excretion of ammonia by fishes is variable, depending on the state of the animal, the environmental conditions, and the species. Ammonia excretion tripled in sockeye salmon (*Oncorhynchus nerka*), following daily feeding but remained low or unchanging during starvation (Brett and Zala, 1975). In freshwater fishes, ammonia excretion increases in response to exercise (Sukumaran and Kutty, 1977: Holeton *et al.*, 1983), and $NH_4Cl$ infusion (Hillaby and Randall, 1979). In contrast, short-term exposure to acid or alkaline water (Wright and Wood, 1985; Randall et al., 1989; Saha *et al.*, 2002a) and increased levels of ammonia in the environment (Saha *et al.*, 2002b) cause a decrease in ammonia excretion. Although fishes excrete primarily ammonia as the major excretory products, urea could also be the major excretory product in certain fishes, under certain environmental constraints such as habitat desiccation. When fishes are out of water, urea storage and subsequent excretion upon re-immersion in water have been demonstrated, which is usually achieved through the possession of an active OUC (Saha and Ratha, 1987; 1989; Ip *et al.*, 2004d). Continuous nitrogenous excretion during emersion may not be an option in the more harsh environments where dehydration is the severe threat and survival out of water depend on reducing all routes of water loss. Studies in amphibious fish species which are highly adapted to significant periods of aerial exposure have suggested that the storage of nitrogenous waste is the primary option while emersed out of water and the fishes would be expected to switch to ureotelism while storing these end-products (Saha and Ratha, 1989; Saha *et al.*, 2001; Sayer, 2005). However, on re-immersion, it is a common phenomenon that the rates of nitrogenous excretion increased instantaneously. But elimination of the stored ammonia component in the form of urea may take longer time upon re-immersion (Sayer, 2005).

## Strategies to ameliorate ammonia toxicity:

The main reason in adopting strategies for ameliorating ammonia toxicity in animals including fishes is to prevent accumulation of ammonia to a toxic level in various tissues. At low concentration of environmental ammonia and during aerial exposure, ammonia excretion is impeded; and at high concentrations of environmental ammonia, there is retention of endogenous ammonia and uptake of exogenous ammonia, thereby leading to an accumulation of toxic ammonia *in situ* (Ip *et al.*, 2004b; Saha and Ratha, 2007a). In air-breathing tropical fishes, a variety of mechanisms/biochemical adaptations have been evolved to deal with the increase of body ammonia loading as a result of retention and/or reduction in ammonia excretion. These mechanisms can be broadly classified into nine different strategies in defence against ammonia toxicity *in situ*: (1) active excretion of $NH_4^+$ (2) lowering of environmental pH (3) low $NH_3$ permeability of the epithelial surfaces (4) volatilization of $NH_3$ (5) reduced ammonia production (6) high ammonia tolerance at cellular and sub-cellular levels (7) urea synthesis (8) up-regulation of glutamine synthetase activity and (9) partial amino acid catabolism. The first four strategies deal with ammonia toxicity at the branchial and/or epithelial surfaces, and the remaining ameliorate ammonia toxicity at cellular and sub-cellular levels. However, fishes show a mix pattern of responses to avoid ammonia toxicity depending on their physiological and environmental conditions.

### 1) Active excretion of $NH_4^+$:

Active transport of $NH_4^+$ against the inwardly directed electrochemical gradient of ammonia was observed in the mudskipper (*Periophthalmodon schlosseri*) (Randall *et al.*, 1999) and in the African sharp-tooth catfish (*Clarias gariepinus*) (Ip *et al.*, 2004b). Even though tissue levels were only around 0.2 mM, the mudskipper exhibits no increase in tissue ammonia concentrations after 6 days of exposure to 8 mM $NH_4Cl$ at pH 7.0. This indicates that either ammonia is not entering the fish or ammonia that entered is being removed. When the fish were exposed to 30 mM $NH_4Cl$ for 6 days the ammonia excretion was maintained low despite the concentrations of both $NH_4^+$ and $NH_3$ levels in the external media were higher than those in the blood, therefore indicating that storage plays only a minor role and active excretion plays a major role. Because the excretion rate of ammonia is constant and independent of the external ammonia concentration, it can be assumed that the efflux of ammonia increases to offset the increase in ammonia influx (Randall *et al*., 1999; Ip *et al*., 2001c). In nature, burrows of *P. schlosseri* are constructed above the normal tidal zones and consequently, are subjected to tides only 2-3 days per year (Clayton, 1993), but the ability to excrete ammonia against the concentration gradient allows this fish to survive in a "closed" environment where ammonia can build up to high concentrations, especially during breeding seasons when eggs are developing (Ip *et al*., 2004d). The African sharp-tooth catfish is ammoniotelic in water and does not detoxify ammonia to urea or amino acids during hyper environmental ammonia. The levels of ammonia accumulated in the tissues of this fish exposed to 100 µmol/ml $NH_4Cl$ for 5 days remain significantly low. This catfish is apparently able to maintain the steady-state level of plasma ammonia by excreting ammonia against a concentration gradient of ammonia (Ip *et al*., 2004b). Substitution of $NH_4^+$ for $K^+$ in $Na^+$-$K^+$-ATPase has been proposed as a method of branchial ammonia excretion in fish (Claiborne *et al*., 1982; Evans and Cameron, 1986). Indeed, the gill of *P. schlosseri* has very high activity of branchial $Na^+$-$K^+$-ATPase, 3-fold higher than that of *Boleophthalmus boddaerti* and approximately 10-fold higher than that of other fishes (Peng, *et al*., 1998; Randall *et al*., 1999). Inhibition of ammonia excretion and accumulation in the plasma of *P. schlosseri* were observed when ouabain was added to seawater containing 2 mM $NH_4Cl$; and also $Na^+$, $K^+$-ATPase has been localized by immunohistochemistry on the baso-lateral membrane of the mitochondrial rich cells of *P.*

*schlosseri* gills (Wilson *et al.*, 2000). Addition of amiloride to seawater results in inhibition of ammonia excretion by *P. schlosseri*, indicating that a $Na^+/H^+$ exchanger is involved in ammonia excretion. In further support of $Na^+/NH_4^+$ exchanger and $Na^+$-$NH_4^+$-ATPase as the major transporters in branchial ammonium ion excretion during high environmental ammonia levels, there is an increased accumulation of $Na^+$ in the plasma of *P. schlosseri* in relation to increase in the external ammonia concentration (Wilson *et al.*, 2000) because the $Na^+$-$NH_4^+$-ATPase is pumping $Na^+$ into the blood in exchange for $NH_4^+$. This mechanism would result in the accumulation of $Na^+$ in the blood and facilitate the excretion of ammonia out of the body across the gill epithelium. Active pumping of $NH_4^+$ in absence of back diffusion of $NH_3$ is energetically more efficient than turning ammonia into urea and glutamine.

**2) Lowering of environmental pH**:

In water at pH 7.0 less than 1% of any ammonia will be present as $NH_3$, and $NH_3$ excreted by the fish into water of this pH will be converted to as $NH_4^+$. This conversion of $NH_3$ to $NH_4^+$ will maintain the $NH_3$ gradient across the gills and enhance ammonia excretion is commonly known as 'ammonia trapping'. Lowering the environmental pH has advantage for dealing with elevation of environmental ammonia levels by reducing the concentration of $NH_3$; the more permeate species of ammonia, constituting "environmental ammonia detoxification" (Chew *et al.*, 2003c). Carbon dioxide and/or proton excretion via a gill epithelium apical proton pump do acidify gill water (Randall and Wright, 1989; Wilkie and Wood, 1991, 1994, 1996). There is no evidence, however, of manipulation of acid excretion to enhance ammonia excretion in fish, except possibly in the mudskipper (*P. schlosseri*). This fish has the capacity of adjusting the pH of small volumes of seawater (Hong, 1997). In one study, the pH of the water in a canal that supply water to a mud flat was 7.84, yet the pH of the water sampled from burrows of the giant mudskipper was close to 7.0 (Ip *et al.*, 2003a). This would maintain a low pH in the boundary water layer of the branchial epithelia, preventing the excreted $NH_4^+$ from dissociation into $NH_3$ and $H^+$, thus avoiding the back diffusion of $NH_3$ into the body down the inwardly directed $\Delta P_{NH3}$. Indeed, both active $NH_4^+$ excretion and $H^+$ excretion take place in the head region of *P. schlosseri*, where the gills and the opercular membranes are located (Ip *et al*., 2003a). It is essential for these two mechanisms to be located together because the branchial and opercular surfaces have important functions of allowing passage of gases and other ions.

**3) Low $NH_3$ permeability of the epithelial surfaces**:

The branchial epithelial surface of aquatic teleosts has a higher permeability to $NH_3$ due to its major function in gaseous exchange. Air-breathing fishes depend largely on the accessory organs for respiration, since their gills are usually degenerated (Graham, 1997a). They usually hold air in their buccal cavities during immersion, which means their gills would not be exposed to ammonia even in high environment ammonia. Such an adaptation helps to reduce the influx of exogenous $NH_3$ through the gills during ammonia loading. However, many tropical air-breathing fishes substitute branchial respiration by having highly vascularised skins. Hence it would essential for these fishes to reduce the permeability of their skins to $NH_3$ despite the cell membranes being permeable to gaseous molecules like $O_2$ and $CO_2$, even though the permeability of $NH_3$ is less than those of $O_2$ and $CO_2$ (Marcaggi and Coles, 2001). $NH_3$ permeates the membrane by solvation and diffusion in the lipid bilayer and the limit is determined by the lipid properties of membrane (Ip *et al*., 2004a). The cholesterol and phospholipid fatty-acid contents of artificial membranes have been shown to affect the permeability of artificial membranes to ammonia, which decreases with decreasing membrane fluidity (Lande *et al*., 1994). However, there is no report of membrane fluidity manipulation in fishes during ammonia stress condition, except possibly in the mudskipper (*P. schlosseri*). The skin of the mudskipper has very high cholesterol content (4.5 µmol/g), which lowers the fluidity of biomembranes. The cholesterol content in the skin of (*P. schlosseri*) increased significantly to 5.5 µmol/g after 6 days of high environmental ammonia exposure (Ip *et al*., 2003a), suggesting a role for cholesterol as a defence mechanism against environmental ammonia toxicity. In the face of high environmental ammonia, a reduction in $NH_3$ permeability of the skin of the mudskipper would help to reduce the influx of $NH_3$. This is an important adaptation, which complements active $NH_4^+$ excretion through its gills because it would prevent back diffusion of ammonia through the cutaneous surfaces after buildup of high ammonia concentrations in the external medium (Randall *et al*., 1999; Ip *et al*., 2003a).

**4) Volatilization of $NH_3$:**

High temperatures and humidity increase the likelihood of the ammonia excreted into thin film of water covering the body surfaces of the tropical fishes to be eliminated by volatilization. In teleosts, ammonia volatilization was first reported in the temperate intertidal blenny (*Blennius pholis*), which account for 8% of the total ammonia excreted during emersion (Davenport and Sayer, 1986). The other tropical air-breathing fishes that are capable of volatizing ammonia during aerial exposure are the leaping blenny (*Alticus kirki*) (Rozemeijer and Plaut, 1993), the oriental weatherloach (*Misgurnus anguillicaudatus*) (Chew *et al*., 2001) and the mangrove killifish (*Rivulus marmoratus*) (Frick and Wright, 2002). However, only the oriental weatherloach has been reported to be capable of volatizing $NH_3$ during high environmental ammonia (Tsui *et al*., 2002). The amount of $NH_3$ volatized by the fish exposed to 20 mM $NH_4Cl$ during 24 h period was approximately 2 µmol/day/g. This was similar to the amount of ammonia excreted by the specimen to a thin film of water during aerial exposure at 25 °C for the same period (Chew *et al*., 2001). When the fish is prevented for gulping air, the volatilization of ammonia from the intestine which acts as the respiratory organ of this fish stopped. The pH of the mucosal surface of the anterior region of the intestine became significantly alkaline when exposed to high environmental ammonia (Tsui *et al*., 2002). Similarly, ammonia built up to very high levels in the muscle, liver and plasma of (*M. anguillicaudatus*) after 48 h of air exposure and leveled off thereafter. Some ammonia must be produced during prolonged exposure to air. To maintain the high levels at steady states without allowing it to build up further, weatherloach must be capable of sustaining a low rate of ammonia excretion as $NH_3$ gas in terrestrial conditions (Chew *et al*., 2001). With the building up of ammonia concentration to a very high level in plasma during aerial exposure results in the alkalinity of the blood pH. The progressive increase in the amount of internal ammonia and alkalinization are the essential prerequisites for volatilization to occur. These together would facilitate the $\Delta P_{NH3}$ gradient that favours efflux of ammonia from the non-branchial epithelial surfaces. In (*M. anguillicaudatus*), there appear to be at least two sites of $NH_3$ volatilization, the skin and the digestive tract, under terrestrial conditions; and surface of the skin was reported to become significantly more alkaline (Tsui *et al*., 2002).

### **5) Reduced ammonia production**:

One of the primary sources of energy in teleost fish is proteins and amino acids, rather than carbohydrates and lipids as in many animals (Moon and Johnston, 1981). The main storage site of utilizable proteins is white muscle. The steady state of concentration of FAAs in the tissues is maintained by the balance between their rates of degradation and production (through protein degradation or amino acid synthesis). During ammonia loading, the increase in the concentration of non-essential amino acids alone indicate an increase in amino acid synthesis from ammonia and α-keto acids catalysed by glutamate dehydrogenase (GDH) and various transaminases. Essential amino acids are released through the catabolism of proteins in fasted animals; therefore, the increase in these amino acids in the experimental animals without food supply would suggest reductions in their rates of catabolism. If the rate of proteolysis remains the same, but the rate of amino acid catabolism decreases, ammonia production will decrease but the concentration of free amino acids (FAAs) will increase. Hence the increase in the concentration of amino acids in which fishes have difficulty in excretion of ammonia cannot be the indication of ammonia detoxification as suggested by Iwata *et al*. (1981) for the mudskipper (*P. cantonensis*). It simply suggests a decrease in the rate of amino acid catabolism (Ip, *et al*., 2001a). However, when the mudskipper, (*P. schlosseri*) is exposed to terrestrial condition under constant darkness, there was an decrease in tissue total FAAs, under which it remains quiescent (Ip, unpublished observation); but when it is exposed to terrestrial condition under 12h: 12h dark and light regime, the fish can be very active and the levels of total FAAs increase significantly in the tissue and plasma after 24 h of the experiment. An analysis of the balance sheet between the reduction in ammonia excretion and accumulation of ammonia, urea, glutamine and glutamate in the 70 g mudskipper shows that the catabolism of amino acids is suppressed, but there is still an accumulation of ammonia in the tissues (Lim *et al*., 2001). Unlike (*P. schlosseri*) there is no increase in total FAAs and no accumulation of glutamate or glutamine in the tissues of (*B. boddaerti*) during aerial exposure (Ip *et al*., 1993), it also undergoes a reduction in the rate of amino acid catabolism under such condition (Lim *et al*., 2001). These data suggest that (*B. boddeati*) does not rely on protein as energy source during 24h aerial exposure. Comparing the glycogen content in the muscle of exercised submerged controls with that 24h air exposed followed by exercise, they found glycogen deceased significantly. Thus, this fish uses glycogen as metabolic fuel during aerial exposure. This strategy offers a limited amount of energy for a short period of time, as (*B. boddaerti*) does not stay away from water for long periods of time. The rainbow trout (*Oncorhynchus mykiss*) exposed to pH 10.0 water resulted in reduction of ammoniogenesis (Wilson *et al*., 1998). The Lohantan cutthroat trout (*Oncorhynchus clarki henshawi*), appears to permanently lower its rate of nitrogenous waste production immediately following transfer from its juvenile freshwater habitat (pH 8.4) to Pyramid Lake, Nevada (pH 9.4) (Wilkie *et al*., 1994). The actual reason is not clear. However, it can be suggested that the reduction in ammonia excretion can be due to reduced rate of proteolysis and/or amino acid catabolism (ammoniogenesis) as an effective strategy to slow the internal buildup of ammonia.

### 6) High ammonia tolerance at cellular and sub-cellular levels:

It would appear that high tolerance of air exposure and to high environmental ammonia in some tropical fishes is usually associated with high tolerance of ammonia at the cellular and sub-cellular levels, as observed in the Indian catfishes (*Hetereopneustes fossilis* and *C. batrachus*) (Saha and Ratha, 1990; 1994; 1998; Saha *et al*., 2002a), abehaze (*Mugilogobius abei*) (Iwata *et al*., 2000a), the oriental weatherloach *M. anguillicaudatus*) (Tsui et al., 2002), and the freshwater stringray (*Himantura signifer* (Ip *et al*., 2003b). The accumulated ammonia is not always evenly distributed within fish; some exhibit much higher levels of ammonia in the muscle than in other tissues. The plasma ammonia concentrations in various air-tolerant teleosts are usually around 1.6 µmol/ml after air exposure (Ip *et al*., 2001a). In contrast, the ammonia level in the plasma of (*M. anguillicaudatus*) raised upto 5.09 µmol/ml after 48 h of aerial exposure, the highest accumulation of ammonia in the plasma on land (Chew *et al*., 2001). During exposure to high environmental ammonia, the plasma ammonia concentration in (*M. anguillicaudatus*) increased from 0.9 to 4.2 µmol/ml after two days of ammonia exposure (Tsui *et al*., 2002) and in the plasma of (*C. batrachus*) which was reported to accumulate up to 4 µmol/ml under hyper ammonia stress without having any apparent deleterious effects (Saha and Ratha, 1998). It is well known that mammalian brains cannot tolerate ammonia levels of >1-2 µmol/g, beyond which encephalopathy would develop (Cooper and Plum, 1987; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002). How cells especially those of the brain and the heart, of these fishes tolerate these high ammonia levels is not clear at present. However, in view of toxic effects of ammonia on the brains of mammals, Ip *et al*. (2004) speculated that the weatherloach and probably other fishes which adopt a similar strategy to handle ammonia toxicity might have greater space in the skull to prevent the buildup of intracranial pressure, have special mechanisms to maintain the homeostasis of extra-cellular glutamate concentration, and/or possess NMDA receptors that are less sensitive to fluctuation in glutamate level. Moreover, these fishes may have $K^+$ channels and $Na^+$-$K^+$-ATPase with high substrate specificity for $K^+$ (i.e. to prevents its substitution by $NH_4^+$) in order to maintain the intracellular $K^+$ concentration and the resting membrane potential. This is especially important to the brain and cardiac muscle cells, although skeletal muscles and some non-excitable cells would also be affected. Also the accommodation of relatively high concentration of ammonia in the blood during exposure to high environmental ammonia at the cellular and sub-cellular levels in the tropical air-breathing fishes allows the favourable blood-water $\Delta P_{NH3}$ gradient. The sustained elevated plasma ammonia level reduces the influx of exogenous ammonia by preventing the inwardly directed $\Delta P_{NH3}$ pressure gradient, resulting in a smaller, if not zero, net influx of exogenous ammonia (Ip *et al*., 2004a).

**7) Urea synthesis**:

Urea forms an appreciable component of nitrogen output even when fish are in water (Campbell and Anderson, 1991; Wood, 1993; Saha and Ratha, 1998). The major pathways for urea synthesis in teleosts are arginolysis, uricolysis and the OUC (Mommsen and Walsh, 1989; 1992; Anderson and Walsh, 1995; Wright, 1995; Walsh, 1997; Saha and Ratha, 2007a) of these only OUC is the synthetic pathway call ureogenesis. Urea synthesis as the major mechanism of ammonia detoxification appears to occur in only a few species of teleosts, usually as a unique adaptation to unusual environmental circumstances. Such as Indian catfishes (*Heteropneustes fossilis* and *Clarias batrachus*) (Saha and Ratha, 1987) and (*Protopterus dolloi*) (Chew *et al*., 2003b) during aerial exposure, alkaline lake adapted tilapia (*Alcolapia graham*) (Randall, 1989), marine toadfishes (*Opsanus beta* and *O. tau*) (Mommsen and Walsh, 1989) and gobiid fish (*Mugilogobius abei*) (Iwata, 2000). The advantages of urea excretion are that subsequent excretion of urea is completely independent of the presence of ammonia in the external medium (Ip *et al*., 2004a) and water requirement is very less as it is excreted in the highly concentrated form which is the top priority during land excursion because water conservation is imperative. However, the formation of urea in fishes is highly energy dependent. A total of 5 mol of ATP are hydrolysed to ADP for each mole of urea assimilated (Ip *et al*., 2001a). It is probably because of this that ureogenesis is not universally adopted as single major strategy in handling ammonia toxicity in fishes (Ip *et al*., 2001a). The majority of tropical teleost fishes studied so far do not use ureogenesis as a major strategy to detoxify endogenous (during aerial exposure) or exogenous and endogenous ammonia (during ammonia loading). These include the mudskippers (*P. schlossseri*, *B. boddaerti* and *P. modestus*) (Iwata and Deguichi, 1995; Peng *et al*., 1998; Lim *et al*., 2001), the marble goby (*Oxyeleotris marmoratus*) (Jow *et al*., 1999), the four-eyed sleeper (*Botrichthys sinensis*) (Ip *et al*., 2001b; Anderson *et al*., 2002), the oriental weather loach (*M. agillicaudatus*) (Chew *et al*., 2001; Tsui *et al*., 2002), the mangrove killifish (*R. marmoratus*) (Frick and Wright, 2002), and the small snakehead (*Channa asiatica*) (Chew et al., 2003) when exposed to terrestrial conditions or ammonia loading for various periods.

### 8) Up-regulation of glutamine synthetase activity:

Ammonia toxicity in fishes can also be avoided by converting exogenous and/or endogenous ammonia to glutamine. Duda and Handler (1958) suggested that rapid incorporation of exogenous ammonia into glutamine acts as a general cellular mechanism for ammonia detoxification. Glutamine, a non-essential amino acid, is produced from glutamate and $NH_4^+$, the reaction catalysed by GS in the liver and/or muscle. Glutamate may in turn be produced from α-ketoglutarate (α-KG) and $NH_4^+$, catalysed by glutamate dehydrogenase (GDH), or α-KG and other amino acids catalysed by various transaminases. Thus the formation of one mole of glutamine allows the uptake of two moles of ammonia (Campbell, 1973). Thus, comparison with ureogenesis, glutamine formation is more energy efficient than ureogenesis. It is logical to utilize this pathway during critical situations, especially to protect the brain during ammonia loading. More importantly, glutamine is stored within the body and can be used for other anabolic processes (e.g., syntheses of purine, pyrimidine, mucopolysaccharides) when environmental conditions become more favourable. Moreover, in ureogenic fishes and ureo-osmotic elasmobranches glutamine serves as the nitrogen-donating substance in the synthesis of carbamyl phosphate by the mitochondrial CPS III the key enzyme, which serves as the precursor for the formation of urea through the ornithine urea cycle. Thus, GS acts as a feeder enzyme for ammonia detoxification via OUC.

In fishes the role of glutamine in detoxifying both endogenous and exogenous has been reported during exposure to high environmental ammonia and terrestrial conditions (Levi *et al*., 1974; Arillo *et al*., 1981; Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng *et al*., 1998). The marble goby (*O. marmoratus*) is a facultative air breather capable of surviving under terrestrial conditions for up to several days, but ammonia levels in its muscle do not increase during aerial exposure (Jow *et al*., 1999). It does not detoxify ammonia to urea, despite the fact that it has all the five enzymes of OUC in the liver. There was an increase in the hepatic GS activity in the marble goby exposed to air. A good correlation between the peak of hepatic GS activity and the peak of glutamine accumulation in the muscle of the fish exposed to terrestrial conditions was observed. The glutamine content in the muscle of the goby exposed to terrestrial condition increased 3-fold in 72 h and the hepatic glutamine content increased at 24 h. This indicates that the glutamine formed in the liver is perhaps later shuttled to muscle, which acts as a reservoir for glutamine accumulation. The sleeper, (*Bostrichthys sinensis*) inhabits brackish water near the mouths of rivers in Southeast Asia and is periodically subjected to air exposure for variable periods of time. This species also synthesizes and stores glutamine, primarily in the muscle, in the first few days of air exposure, which is accompanied by an increase in GS activity in the liver (Ip *et al*., 2001b). Exposure of sleeper to high concentrations of environmental ammonia results in significant increases in GS activity, GS protein and GS mRNA in all the tissues except the stomach (Anderson *et al*., 2002). Both the fishes (*O. marmoratus* and *B. sinesis*) are relatively inactive on land and the reduced energy-demand for muscular activity may provide them with the opportunity to exploit glutamine formation as a means to detoxify ammonia. Detoxification of accumulated ammonia to glutamine during high environmental ammonia exposure has also been reported during confinement/crowding stress in the ureogenic Gulf toadfish (*O. beta*) (Walsh *et al*., 1994; Walsh and Milligan, 1995; Julsrud *et al*., 1998; Kong *et al*., 2000). It has been suggested, however, that glutamine formation would contribute to toxicity because it leads to depletion of ATP and α-KG (Campbell, 1973: Cooper and Plum, 1987), and glutamine accumulation causes increased astrocyte cell volume, leading to cellular dysfunction, brain edema and death (Brusilow, 2002). In its natural brackish water habitat, (*B. sinensis*) may be passively exposed to air by a receding tide, when it remains in crevices above water level and thus is exposed to high environmental ammonia (HEA) conditions due to the accumulation of excreted ammonia in the external medium and to the evaporation of water. Infact, (*B. sinensis*) is the first non-ureosmotic teleost fish reported to respond to HEA exposure by increasing the expression of GS activity in the non-cerebral tissues (Anderson *et al*., 2002). Previously, it was believed that only cerebral GS was inducible by sub-lethal concentrations of environmental ammonia (Korsgaard *et al*., 1995). The cytosolic compartmentalization of the hepatic GS in this fish would eliminate the necessity for ammonia to penetrate the mitochondrial membranes before being detoxified.

**(9) <u>Partial amino acid catabolism</u>**:

Certain amino acids such as arginine, glutamine, histidine, and praline can be converted to glutamate which can undergoes deamination catalysed by GDH, producing $NH^{+}_{4}$ and α-KG (Campbell, 1991). Glutamate can also undergo transamination with pyruvate, caltalysed by alanine aminotransferase (ALT), producing α-KG without the release of ammonia (Ip *et al*., 2001c, Chew *et al*., 2003). This would facilitate the oxidation of the carbon chain of some amino acids without polluting the internal environment with ammonia. For fish that have difficulty in excreting endogenous ammonia, partial amino acid metabolism coupled with reduction in the rate of amino acid catabolism would be the most cost-effective strategy (in term of energy conservation) in reducing the rate of ammonia buildup in the body. It allows amino acids to be used as energy source during adverse conditions without polluting the internal environment with ammonia. However, it cannot be strictly speaking, regard as a strategy to detoxification of ammonia, because ammonia is not released and then converted back to alanine. Significant increase in the levels of alanine and total FAAs concentrations in the muscle, liver, plasma of (*P. schlosseri*) exposed to terrestrial conditions were reported (Ip et al., 1993; 2001). Since, (*P. schlosseri*) has difficulty in excreting ammonia on land; partial amino acid metabolism leading to the formation of alanine would provide energy in the form of ATP without ammonia release. This would allow the utilization of certain amino acids as energy source, at the same time, minimize the ammonia accumulation. The snake head (*Channa asiatica*) which is active during terrestrial exposure, also adopt partial amino acid catabolism as the strategy to avoid the toxic accumulation of ammonia as reported by Ip and Chew in unpublished report.

## Habit and Habitat of the mud eel:

The mud eel (*Amphipnous cuchia*) (Family: Synbranchidae; Order: Synbranchiformes; Class: Actinopterygii), one of the air-breathing fishes, is a bony fish that lack paired fins and have scale-less body, reaching a maximum length of 1.5 m and are voracious, nocturnal predators. They are primarily aquatic but obligatory air-breathing fish and would die of suffocation if access to air is denied. They are found widely distributed in tropical and sub-tropical freshwaters of India, South China, Malaysia and Indonesia (Graham, 1997a). Found inhabiting in a wide variety of freshwater habitats that are stagnant, slow-flowing and shallow water bodies such as swampy areas, muddy ponds, canals, rice fields, and even ditches and temporary pools where they burrow in moist earth during dry season, surviving for long periods without water during summer (Shih, 1940; Davidson, 1975). During prolonged drought and seasonal draining of paddy fields, they move in or hide in the mud to avoid total dehydration. Moreover, they can survive several days in the market bins without water. All these would lead to difficulties in excreting ammonia through their reduced gills and cutaneous surface. Further, they face the problem of high concentrations of environmental ammonia during agricultural fertilization in rice fields. Under the laboratory conditions, it was found that the mud eel was able to survive up to 200 mM $NH_4Cl$ for months without any deleterious effect to the fish. Thus, the immediate question arises is how the mud eels encounter the possible accumulation of endogenous ammonia to a toxic level, and how they tackle the problems of high external ammonia.

# OBJECTIVES:

To find out the possible strategies to ameliorate the ammonia toxicity by the mud eel during exposure to:

- High environmental ammonia, and
- Ammonia generated endogenously during exposure to air or semi-terrestrial conditions.

# PLAN OF WORK:

1. The mud eel was initially exposed to different concentrations of $NH_4Cl$ (50 mM, 100 mM, 200 mM, 250 mM and 300 mM) to find out the maximum tolerance limit by this fish.

Then the mud eel was exposed to 100 mM $NH_4Cl$ for 7 days and the following parameters were studied at different time intervals:

- changes in the levels of ammonia-N and urea-N in different tissues such as liver, kidney, muscle, brain, intestine, stomach and plasma,
- changes in the activity of all the five ornithine-urea cycle (OUC) enzymes such as carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG); and the amino acid metabolism related enzymes such as glutamine synthetase (GS), glutamate dehydrogenase (GDH, both oxidative and reductive amination), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in different tissues,
- changes in the levels of free amino acids (FAAs) in different tissues and in plasma,
- changes in the rate of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of both control and ammonia-treated fish and
- changes in the pattern of expression of GS enzyme protein and Hsp70.

2. The mud eel was exposed to air for 6 days and the following parameters were studied in different tissues at different time intervals:

- changes in the rates of excretion of ammonia-N and urea-N,
- changes in tissue levels of ammonia-N and urea-N in different tissues such as liver, kidney, muscle, brain, intestine, stomach and plasma,
- changes in the activity of all the five OUC enzymes and the amino acid metabolism related enzymes as mentioned above,
- changes in the levels of FAAs in different tissues and plasma,

- changes in the rates of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of both control and aerially exposed fish and
- changes in the pattern of expression of GS enzyme protein and Hsp70.

3. $NH_4HCO_3$ (10 mmoles/kg body weight) was injected intra-peritoneally to the mud eel and the following parameters were studied during 24 hours:
   - changes in the rates of excretion of ammonia-N and urea-N,
   - changes in the levels of ammonia-N and urea-N in different tissues such as liver, kidney, muscle, brain, intestine, stomach and in plasma,
   - changes in the activity of all the five OUC enzymes and the amino acid metabolism related enzymes as mentioned above,
   - changes in the levels of FAAs in different tissues and plasma,
   - changes in the rates of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of both control and $NH_4HCO_3$-treated fish after 24 h and
   - changes in the pattern of expression of GS enzyme protein after 24 h.
4. Cortisol (50 µmoles/kg body weight) was injected intra-peritoneally to the mud eel and the following parameters were studied at different time intervals:
   - changes in the rates of excretion of ammonia-N and urea-N,
   - changes in tissue levels of ammonia–N and urea-N in different tissues such as liver, kidney, muscle, brain, intestine, stomach and plasma,
   - changes in the activity of all the five OUC enzymes and the amino acid metabolism related enzymes as mentioned above,
   - changes in the levels of FAAs in different tissues and plasma,
   - changes in the rates of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of both control and cortisol-treated fish, and
   - changes in the pattern of expression of GS enzyme protein after 5 days.

# MATERIAL AND METHODS

## Experimental animal:

The freshwater air-breathing mud eel (*Amphipnous cuchia*) weighing 120–140 g were purchased from commercial sources and maintained in the fish room in cemented tanks containing natural stream water with a layer of mud at the bottom of each tank. The temperature of the room was maintained at 28±2 $^{o}$C with 12h:12h light and dark photoperiods. The fish were fed with dry fishes and earth worm on every alternate day. Fishes were acclimatized to the laboratory conditions for at least one month, when the mortality rate became zero and food consumption was normal, before performing any experiments. No sex differentiation was done while performing the experiments. Food was withdrawn 24 h prior to setting up of experiments and no food was provided to the fish during experimental periods.

## Experimental set up:

### High external ammonia exposure (100 mM $NH_4Cl$):

Five sets of fishes having five fishes in each set were kept in five different plastic tumblers containing 10 l of 100 mM $NH_4Cl$ in each tumbler. Another five sets of fishes having five fishes in each set were kept in five plastic tumblers containing 10 l of stream water in each which served as controls. The $NH_4Cl$ solution and also the stream water from each tumbler were changed every day with fresh media at a fixed time. On day 0, 1, 3, 5 and 7, five fishes from treated and five fishes from control tumblers were removed, killed immediately by decapitation after collecting blood from the caudal vein with heparinised syringe. Liver, kidney, muscle, stomach, intestine and brain were dissected out and plunged into liquid nitrogen before storing at –60°C. All enzyme assays and analyses were completed within two weeks of collecting tissues.

Another set of fishes (both from control and ammonia-treated for 7 days) was kept aside for performing liver perfusion experiment. The temperature of the experimental room was maintained at 28±2 °C with 12:12 h day and night photoperiods.

## Aerial exposure experiment:

Another set of fishes were isolated, weighed and kept individually in plastic tumblers containing 2 l of water. After 24 h, they were exposed to air in tumblers containing only 25 ml of water. The tumblers were covered with a finely meshed net and the fishes were exposed to dehydration stress for 144 h. Every 24 h, five fishes were removed, the fluid left in individual tumbler was collected and diluted to 100 ml with distilled water, and used for measurement of ammonia-N and urea-N excretion. Each fish was then kept in individual tumbler with 25 ml of $H_2O$ and repeated the same after every 24 h till 6 days. Another set of pre-weighed fishes were kept individually in plastic tumbler containing 2 l of stream water (pH 7.04±0.15) and water was replaced at every 24 h after collecting some water sample for the measurement of ammonia-N and urea-N excretion. Simultaneously, after every 24 h, five air-exposed fishes and five control fishes were killed by decapitation; tissues such as liver, kidney, muscle, brain, intestine and stomach were dissected out after collection of blood from the caudal vein with heparinised syringe. They were then immediately plunged in liquid nitrogen and stored at −60 °C until used for various analyses as mentioned above. At the end of six days of aerial exposure, five fishes were re-immersed in a known volume of water and water sample was collected at 12 and 24 h post immersion for estimation of ammonia-N and urea-N concentrations. The enzyme assays and various analyses were completed within 2 weeks of preserving tissues. One set of fishes (both from control and aerial exposed) is kept aside for performing the liver perfusion experiment.

## Ammonium bicarbonate treated experiment:

A set of five fishes were weighed and injected intraperitoneally with $NH_4HCO_3$ (10 mmoles/kg body wt, dissolved in 0.8% saline). Another set of five fishes was injected with the same volume of 0.8% saline, which served as controls. Fishes were then placed individually in plastic tumblers containing 2 l of stream water (pH 7.0 ± 0.14) and the tumblers were covered with finely meshed net. Water from each tumbler was replaced with fresh water at every 6 h interval after collecting some water from each tumbler for the measurement of ammonia-N and urea-N concentrations. After 24 h, both the control and treated fishes were killed by decapitation after collection of blood from caudal vein by heparinised syringe, and tissues such as liver, kidney, muscle, stomach, intestine and brain were dissected out. All the tissues were immediately plunged in liquid nitrogen and stored at –60 $^{o}$C until used for various analyses. All enzyme assays and other analyses were completed within two weeks after collecting the tissues. One set of fishes (both from control and $NH_4HCO_3$-treated) is kept aside for performing liver perfusion experiment.

## Cortisol treated experiment:

A set of five fishes was weighed and injected intraperitoneally with cortisol (50 mg/ml, dissolved in sham) at the rate of 50 mg/kg body weight. Another set of five fishes was injected with the same amount of sham, which served as controls. Fishes were then placed individually in plastic tumblers containing 2 l of stream water (pH 7.04 ± 0.10) and the tumblers were covered with a finely meshed net. Water from each tumbler was replaced at every 12 h interval after collection of some water from each tumbler for the measurement of ammonia-N and urea-N concentrations. After 5 days of treatment, fishes were removed, killed immediately by decapitation, and tissues such as liver, kidney, muscle, brain, intestine and stomach were dissected out after collection of blood from the caudal vein with the heparinised syringe. They were then immediately plunged in liquid nitrogen and stored at −60 °C until used for various analyses. The enzyme assays and various analyses were completed within 2 weeks of preserving the tissues. Another set of fishes (both from the control and cortisol-treated) after 5 days was kept aside for performing liver perfusion experiment.

**Blood collection and processing:**

From each of the above experiment blood was collected from each fish from the caudal vein by heparinised syringe and was centrifuged at 10000Xg for 10 min to separate out the blood cells, and the plasma was processed for the estimation of ammonia-N and urea-N, and for analysis of FAAs as described by Saha and Ratha (1989).

# Estimations:

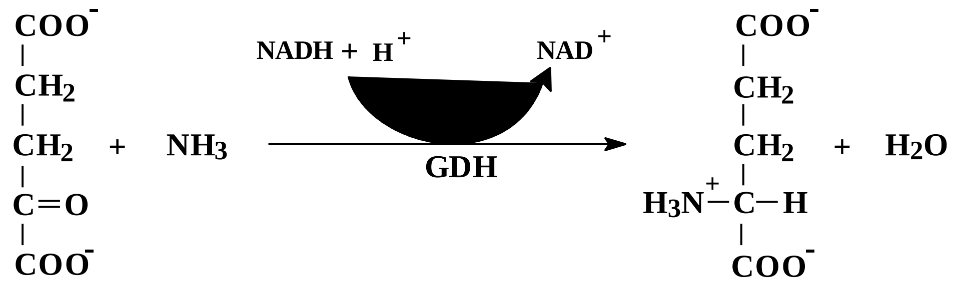
## Ammonia-N and urea-N:

The excretion of ammonia-N and urea-N by the whole fish, released into the effluent from the perfused liver, and also the tissue concentrations of ammonia-N and urea-N under different experimental conditions were measured enzymatically based on the procedure of Kun and Kearny (1974).

**Ammonia-N:**

For the measurement of ammonia-N in the water samples, different tissues and in the effluent, all the ammonia was converted to L-glutamate by the enzyme glutamate dehydrogenase (GDH) in presence of α-ketoglutarate and NADH. The amount of NADH oxidised was equivalent to the amount of ammonia present in the sample.

The reaction takes place as follows:



**α-Ketoglutarate** **L-Glutamate**

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

| | |
|---|---|
| Tris-HCl buffer (pH 8.0) | 100 µmoles |
| α-ketoglutarate | 2.5 µmoles |

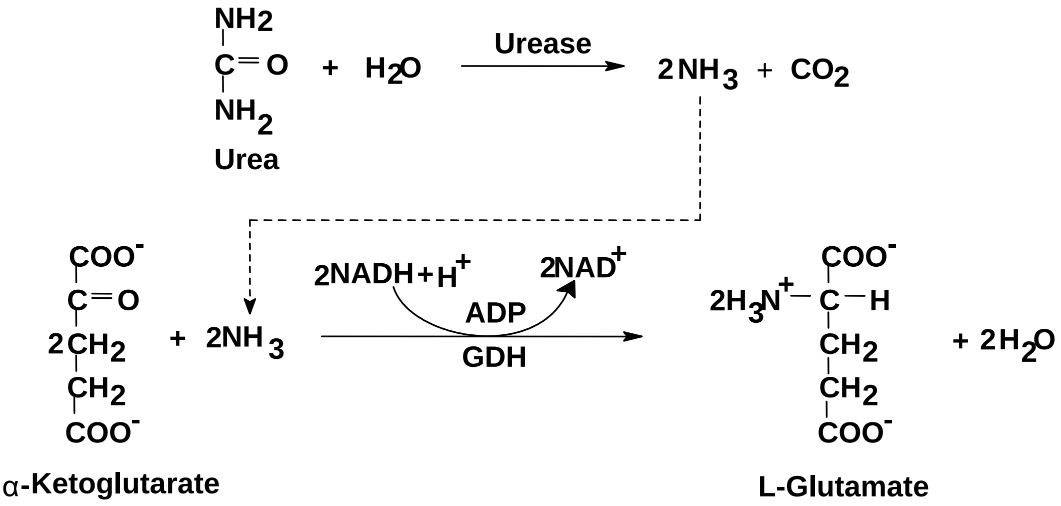
| | |
|---|---|
| EDTA | 0.2 µmole |
| ADP | 1.0 µmole |
| NADH | 0.4 µmole |
| GDH | 2 units |
| Sample | 0.2 ml |

The reaction mixture was incubated for 30 min at 37 ºC. A reagent blank was also prepared which contained everything in the reaction mixture as mentioned above except the sample, which was replaced by 0.2 ml of distilled water. The optical density (O.D.) was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer (Varian, Carry 50) both in the reagent blank and in the experimental samples. The differences in O.D. values obtained between these two were used to calculate the concentration of ammonia present in the effluent taking 6.22 x $10^6$ as molar extinction coefficient value for NADH.

**Urea-N:**

For the measurement of urea-N in different samples, urea was first converted to ammonia by the enzyme urease, and then to L-glutamate in presence of α-ketoglutarate and NADH by the enzyme GDH. The amount of NADH oxidised was equivalent to the amount of both ammonia-N and urea-N present in the sample. Finally, the amount of urea-N was calculated by substrating the value of ammonia for each sample.

The enzymatic reaction takes place as follows:



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

| | |
|---|---|
| Tris-HCl buffer (pH 8.O) | 100 µmoles |
| α-ketoglutarate | 2.5 µmoles |
| EDTA | 0.2 µmole |
| ADP | 1.0 µmole |
| NADH | 0.4 µmole |
| GDH | 2 units |
| Urease | 2 units |
| Sample | 0.2 ml |

The reaction mixture was incubated for 30 min at 37 °C. A reagent blank was prepared containing everything in the reaction mixture as mentioned above except the sample, which was replaced by 0.2 ml distilled water. The O.D. was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer both in the reagent blank and in the sample. The differences in O.D. values obtained between these two were used to calculate the concentration of urea-N present in the sample taking 6.22 x $10^6$ as molar extinction coefficient for NADH.

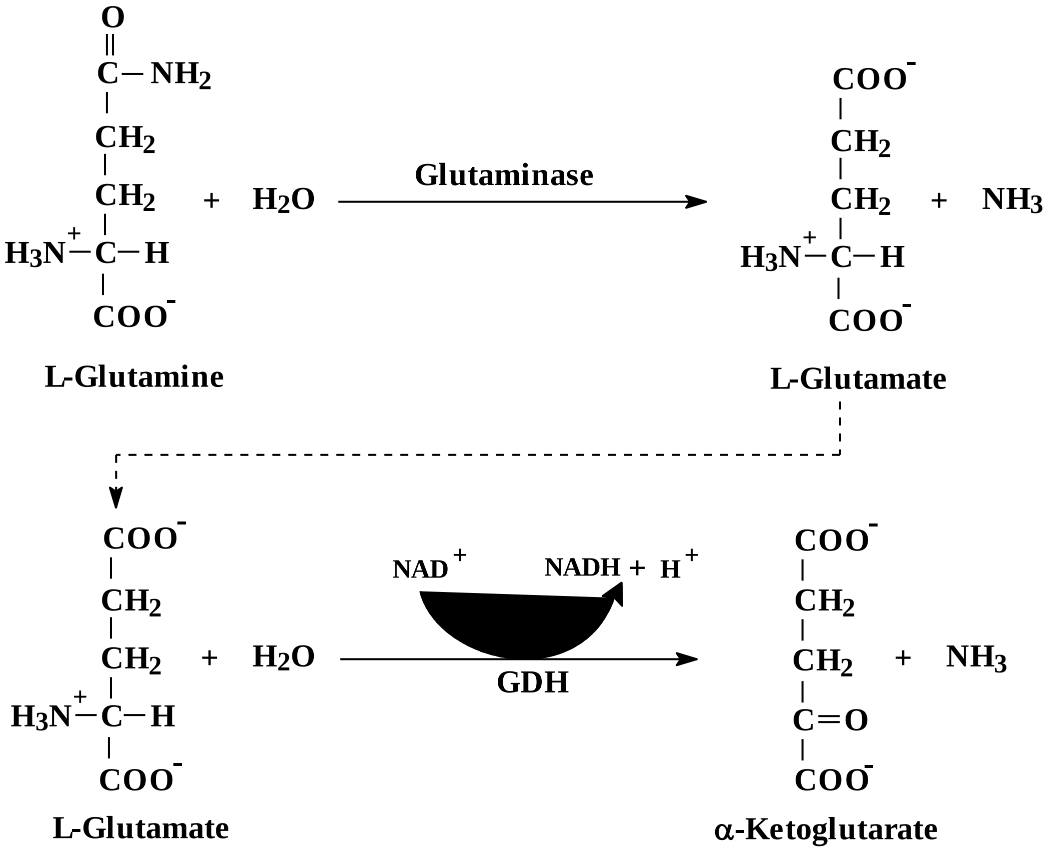
## Protein Estimation:

Protein was estimated following the dye-binding method of Bradford (1976) using bovine serum albumin as the standard.

## Glutamine estimation:

Glutamine level in the effluents from the perfused liver were measured enzymatically based on the procedure of Bergmeyer (1974). For the measurement of glutamine in the sample, the L-glutamine was first converted to L-glutamate by the enzyme glutaminase. Then L-glutamate was converted to α-ketoglutarate and ammonia by the enzyme GDH in the presence of NAD$^+$. The amount of NAD$^+$ reduced was equivalent to the amount of glutamine and glutamate present in the sample. Further, the efflux of glutamine was calculated by multiplying the glutamine concentration with 2 since each molecule of glutamine contains 2N.

The enzymatic reaction takes place as follows:



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

| | |
|---|---|
| Na-acetate buffer (pH 5.0) | 280 µmoles |
| Glycine | 500 µmoles |

| | |
|---|---|
| Hydrazine hydrate | 0.4 µmole |
| ADP | 1.0 µmole |
| $NAD^+$ | 0.6 µmole |
| GDH | 5 units |
| Glutaminase | 0.5 unit |
| Sample | 100 µl |

Initially, the sample and enzyme glutaminase was incubated for 2 h at 37 °C to convert all the glutamine to glutamate. Then the other components of the reaction mixture were [illegible] and incubated again for 30 min at room temperature. A reagent blank was also prepared containing everything in the reaction mixture as mentioned above except the sample, which was replaced by 100 µl of distilled water. The O.D. was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer (Varian, Carry 50), both in the reagent blank and in the experimental sample. The concentration of glutamate was also measured in each sample without adding glutaminase. The difference in O.D. values obtained between the reagent blank and sample was used to calculate the concentrations of glutamate present in the sample taking $6.22 \times 10^6$ as molar extinction coefficient value for NADH.

## Liver perfusion technique:

Fishes were anesthetized with neutralized 3-aminobenzoic acid ethyl ester (MS222, 0.2 g/l) for 2 min prior to the operation for liver perfusion. Livers of both control and treated fishes were perfused by way of a ventral incision made on the hepatic portal vein, in a non-circulating manner in the physiological antegrade direction (from the portal to the hepatic vein) with a haemoglobin-free medium as used by French *et al*. (1981) with certain modifications made by Saha *et al*. (1995). The osmolarity of the perfusing medium was 265 mOsmol/l, since the osmolarity of blood of mud eel was found to be 265 mOsmol/l (as determined by freezing point depression method with a Camlab Osmometer, Model 2000).

The composition of the isotonic medium as the basic solution for perfusion was as follows: -

| | |
|---|---|
| NaCl | 119 mM |
| $NaHCO_3$ | 5 mM |
| KCl | 5.4 mM |
| $Na_2HPO_4$ | 0.35 mM |
| $KH_2PO_4$ | 0.44 mM |
| $MgSO_4$ | 0.81 mM |
| $CaCl_2$ | 1.25 mM |

The livers were initially perfused with standard isotonic medium (265 mOsmol/l) containing 0.5 mM ornithine for 20 min, followed by infusion with 0.5 mM $NH_4Cl$ (corresponds to the infusion of ammonia-N at the rate of 2.15 μmoles/g liver/min). The medium also contained 1.0 mM lactate and 0.3 mM pyruvate. The perfusate was gassed with $O_2/CO_2$ (99:1, v/v) before infusing into the liver. The livers were perfused at a flow rate of 4-5 ml/g liver/min at a temperature of 30 °C for 20 minutes with standard medium; the pH of the medium was always maintained at 7.6, since the blood pH of this fish ranges from 7.5 to 7.7. Then 0.5 mM $NH_4Cl$ was infused into the perfused liver through a precision pump (Orion, M361) along with the standard perfusion medium under different experimental conditions. Effluents were collected at 2 min intervals through a cannula inserted at the superior vena cava for the measurement of ammonia, urea and glutamine released from the perfused liver. Immediately after perfusion, the mass of the liver was recorded.

## Free Amino Acid Analysis:

For analysis of free amino acids (FAAs) in different tissues, a 10% homogenate (w/v) was prepared with a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle in ice-cold HPLC grade water. Protein was immediately precipitated out from the homogenate by adding ice-cold 2 M PCA in 1:1 ratio, followed by centrifugation at 10,000 x g for 10 min. The plasma was also treated with 2 M PCA in a 1:1 ratio to precipitate out the protein, and further processed as above. All these steps were performed at 4 ºC. The supernatant was passed through a Millipore microfilter (0.45 µm pore size) before using for FAA analysis with HPLC.

## Sample preparation for HPLC:

After filtering the tissue and plasma samples with 0.45 µm membranes

↓

20 µl of the filtered sample was pipetted in a sample tube

↓

The sample was lyophilized in a cold vacuum dried lyophilizer

↓

10 µl of redrying solution was added to each sample

↓

Cold vacuum dried and then 20 µl of derivatizing reagent was added

↓

Kept for 20 min for reaction to occur and dried in a lyophilizer

↓

200 µl of sample diluent was added to re-dried sample just before injection.

The derivatized sample was then analyzed with a Waters HPLC using the pre-column derivatization method. FAAs was derivatized with phenyl isothiocyanide (PITC) with the standard protocol provided by the company and was separated out with a Pico Tag column (Waters, USA).

The eluted amino acids was detected in a UV-detector (Model 486) at 254 nm coupled with Millinium[32] data processor for quantification of the eluted peak areas. Two eluting mobile phases were used in a gradient for 72 min to separate out all the FAAs. The two eluent buffers were eluent A (70 mM Na-acetate buffer, pH-6.5) and eluent B (60% acetonitrile). Before starting the run, the column chamber temperature was set at 38 $^{o}$C and the column was purged with 90% eluent A and 10% eluent B and equilibrated for stabilization of the baseline. The run was started with 100% mobile phase A and the flow rate was set at 0.5 ml/min through out the run. In the first 13.50 min, the linear gradient progressed to 3% mobile phase B, followed by increase to 6% mobile phase B in 24 min, 9% increase of mobile phase B in 30 min, 34% linear increase of mobile phase B in 50 min and was held there till 62 min. The gradient was then increased to 100% mobile phase B from 62.01 min and continued till 72 min. After the gradient elution was complete, the column was washed thoroughly with 100% mobile phase B for 20 min, followed by washing with HPLC grade water for 30 min. The column was again re-equilibrated with 100% eluent A before subsequent njections. The mixture of standard physiological FAAs (Sigma) containing 38 amino acids and some amino compounds was also eluted under identical conditions as mentioned above for identification and quantification of amino acids.

## Western Blot Analysis:

Western blot analysis of GS enzyme protein and Hsp70 in different tissues such as liver, kidney, muscle, stomach, intestine, and brain of the mud eel was done by performing the following steps:

## 1. SDS-PAGE:

For separation of proteins, SDS-PAGE was carried out in the vertical slab gel electrophoresis apparatus (Hoefer SE 400 Series, Amersham Pharmacia Biotech, Sweden). Prior to electrophoresis, following preliminary steps were followed:

**a) <u>Sample preparation</u>:**

100 mg of each tissue was homogenized in 1 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 0.33 mM sucrose, 1 mM EDTA, 1% Triton-X 100 and also the protease inhibitor Complete (Roche, Germany) using a sonicator (Soniprep 150) at an amplitude of 20-22 μ for 30 x 4 = 120 sec. The tissue homogenates were then centrifuged at 10,000X g for 10 min to pellet out the cell debris and nuclei and the protein concentration of each sample was determined spectrophotometrically by the dye-binding method of Bradford (1976) and samples were diluted to get a final protein concentration of 5 μg/μl.

The loading buffer was added to the diluted protein sample in 1:2 ratio and incubated at 37 °C for 30 min. The samples were then stored at −40 °C until used for SDS-PAGE.

The loading buffer was prepared by mixing re-suspension buffer and 180 mM DTT (dissolved in distilled water) in the ratio of 2:1, followed by the addition of a few drops of tracking dye (Bromophenol Blue, 0.125 %).

The composition of the resuspension buffer was as follows:

| | |
|---|---|
| Tris-HCl buffer (pH 8.8) | 220 mM |
| EDTA | 22.5 mM |
| Sucrose | 1.8 mM |
| SDS | 9 % |

For separation of proteins, SDS-PAGE electrophoresis was carried out in the vertical slab gel electrophoresis apparatus (Hoefer SE 400 Series, Amersham Pharmacia Biotech, Sweden) using 14 x 16 cm gel of 1.5 mm thickness, the composition of which was as follows:

| **Solution components** | **Stacking Gel (5%) (ml)** | **Resolving Gel (10%) (ml)** |
|---|---|---|
| $H_2O$ | 6.8 | 11.9 |
| Acrylamide-bis-acrylamide (36.5:1) | 1.7 | 10.0 |
| 1.5 M Tris (pH 8.8) | - | 7.5 |
| 1.0 M Tris (pH 6.8) | 1.25 | - |
| 10% SDS | 0.10 | 0.3 |
| 10% Ammonium persulphate | 0.10 | 0.3 |
| TEMED | 0.01 | 0.012 |

### b) Gel casting:

A gel sandwich was prepared by using two glass plates of 14 x 16 cm. Two spacers of 2 x 16 cm, having thickness of 1.5 mm were placed along the edges and the three sides were sealed with a tape to avoid any leakage. First, the resolving gel (10%) was prepared and poured into the gel sandwich up to 4 cm below the top of the glass plate. Then a layer of stacking gel (5%) of 4 cm height was added after the resolving gel was completely polymerized. A comb was introduced into the sandwich to create wells for loading the samples. The resolving and stacking gels were prepared by mixing different reagents as mentioned earlier.

### c) Loading and running of sample:

The teflon comb was removed from the gel sandwich and each well was filled with electrophoresis buffer (composition of which was mentioned earlier). 30 µl each of the prepared samples was loaded into the wells using a micro syringe. 5 µl of full range rainbow recombinant protein molecular weight marker (RPN 800, Amersham Pharmacia Biotech, Sweden) with a molecular weight range of 10,000 – 2,50,000 Daltons was also loaded in one lane. Both the upper and lower buffer chambers were filled with electrophoresis buffer and the electrophoresis 49 s connected to the power supply unit. Initially, a low voltage of 80 V was applied till the sample crossed the separation or stacking gel. The voltage was then increased to 240 V and the run was continued till the tracking dye reached the bottom of the gel.

## 2. Blotting:

Following electrophoresis, the proteins were transferred from polyacrylamide gel onto a nitrocellulose paper (NCP) using the semi-dry electro-blotting apparatus (Hoefer TE 70 Series, Amersham Pharmacia Biotech, Sweden) as per the standard procedure given by the manufacturer. The composition of blotting buffer was as follows:

| | |
|---|---|
| Tris | 48 mM |
| Glycine | 39 mM |
| SDS | 0.03 % (w/v) |
| Methanol | 20 % |

After blotting of proteins to NCP, the different bands of Rainbow marker were marked. The blotter was then stained with Ponceau S (Sigma, USA) to check the status of blotting.

## 3. Membrane blocking:

Non-specific binding sites were blocked by immersing the NCP in 1% BSA, prepared in phosphate-buffered saline Tween (PBST), pH 7.6, and kept overnight at 4 °C in a shaker.

The composition of PBST (adjusted to pH 7.4) was as follows:

| | |
|---|---|
| NaCl | 1.37 M |
| KCl | 26.82 mM |
| $Na_2HPO_4$ | 1 M |
| $KH_2PO_4$ | 17.64 mM |
| Tween (Polysorbate-20) | 0.1 % |

## 4. Conjugation with primary and secondary antibodies:

The NCP was then incubated with GS primary antibody raised in rabbit and Hsp70 primary antibody raised in mouse (dilution of 1:2500 in PBST containing 1% BSA) for 2 h at room temperature in a shaker. The membrane was washed for 3 x 10 min with PBST using large volumes of buffer, to remove unbound or nonspecifically bound primary antibody. The membrane was then incubated with horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibody for GS and Hsp70, respectively (dilution of 1:10,000 in PBST containing 1% BSA) for 2 h at room temperature in a shaker. The membrane was again washed for 3 x 10 min with PBST using large volumes of buffer, to remove the unbound secondary antibody.

### 5. Immunodetection:

Immunodetection was performed with an enhanced chemiluminescence kit (Western Ligthning™ Chemiluminescence Plus, PerkinElmer Inc., U.S.A.) as per the manufacturer's instruction. The X-ray film was developed and then scanned with a computer assisted densitometer (Kodak, ID 3.6) and photographed.

## Tissue processing for:

### a) Assaying amino acid metabolism-related enzymes:

For assaying the activity of different amino acid-related enzymes, a portion of each frozen tissue was thawed on ice and a 10% homogenate (w/v) of each tissue was prepared with a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle in a homogenizing buffer containing 50 mM Tris-HCl (pH 7.4), 0.3 M sucrose and 1 mM EDTA. The homogenate was treated with 0.5% Triton X-100 for 30 min in 1:1 ratio, followed by mild sonication to facilitate the proper breakage of mitochondria. The homogenate was then centrifuged at 10,000 x g for 10 min and the resultant supernatant was used for the enzyme assay. All steps for the preparation of tissue extracts were carried out at 4 ºC.

### b) Assaying ornithine-urea cycle ezymes:

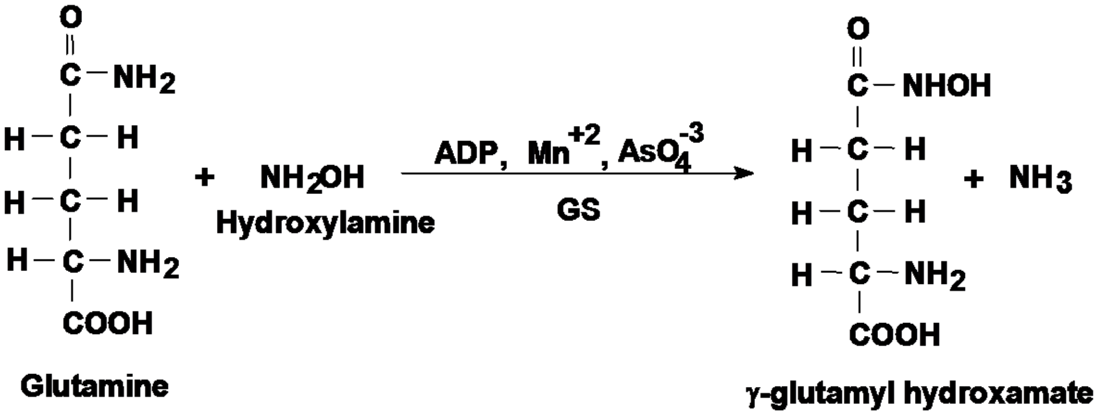
For assaying the activity of different OUC enzymes, a portion of each frozen tissue was thawed on ice and a 10% homogenate (w/v) of each tissue was prepared with a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle in a homogenizing buffer containing 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA and 1 mM dithiothreitol (DTT). The homogenate was treated with 0.5% Triton X-100 for 30 min in 1:1 ratio, followed by mild sonication to facilitate the proper breakage of mitochondria. The homogenate was then centrifuged at 10,000 x g for 10 min and the resultant supernatant was used for the enzyme assay. All steps for the preparation of tissue extracts were carried out at 4 ºC.

## Enzyme Assay:

### Glutamine synthetase (GS; E.C. 6.3.1.2)

GS was assayed by the γ-glutamyl transferase reaction following the method of Webb and Brown (1976).

The enzymatic reaction takes place as follows:



The reaction mixture in a final volume of 1.0 ml contained the following:

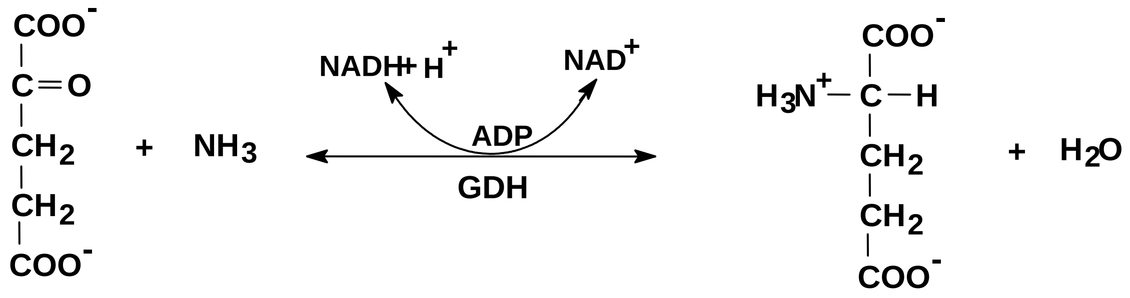
| | |
|---|---|
| Imidazole-HCl buffer (pH 7.5) | 60 μmoles |
| L-Glutamine | 10 μmoles |
| Sodium arsenate | 1 μmoles |
| Hydroxylamine hydrochloride | 12 μmoles |
| $MnCl_2$ | 0.02 μmole |
| ADP | 0.08 μmole |
| Tissue extract | 0.2 ml |

The assay mixture without the tissue extract was first preincubated for 5 min at 30 °C. The reaction was initiated by adding 0.2 ml of tissue extract and after 30 min of incubation; the reaction was stopped by the addition of 1 ml of 20% PCA. A tissue blank was also prepared simultaneously by adding 1 ml of 20% PCA to the reaction mixture prior to the addition of the tissue extract. The precipitated protein was separated out by centrifugation at 10,000 Xg for 15 min and the amount of γ-glutamyl hydroxamate formed during the reaction was measured in the supernatant by adding 1.5 ml of $FeCl_3$ reagent (containing 0.37 M $FeCl_3$, 0.67 M HCl and 0.2 M TCA) and measuring the O.D. at 500 nm in a UV-visible spectrophotometer. One unit of enzyme activity was defined as that amount of enzyme that catalysed the formation of 1 μmole of γ-glutamyl hydroxamate per h at 30 °C.

**Glutamate dehydrogenase** (GDH; E.C. 1.4.2.3)

GDH (both reductive amination and oxidative deamination) activity was assayed following the method of Olson and Anfinsen (1952) with modifications in the substrate concentration made by Das et al. (1991).

The enzymatic reaction takes place as follows:



α -Ketoglutarate

L- Glutamate

For the assay of GDH in the reductive amination (or ammonia utilizing) direction, the reaction mixture in a final volume of 1 ml contained the following:

| | |
|---|---|
| Tris-HCl buffer (pH: 7.5) | 70 μmoles |
| α- Ketoglutarate | 7 μmoles |

| | |
|---|---|
| $NH_4Cl$ | 225 µmoles |
| EDTA | 0.25 µmole |
| ADP | 3 µmoles |
| NADH | 0.3 µmole |
| Tissue extract (suitably diluted) | 25 µl |

For the assay of GDH in the oxidative deamination (or ammonia forming direction), the reaction mixture in a final volume of 1 ml contained the following:

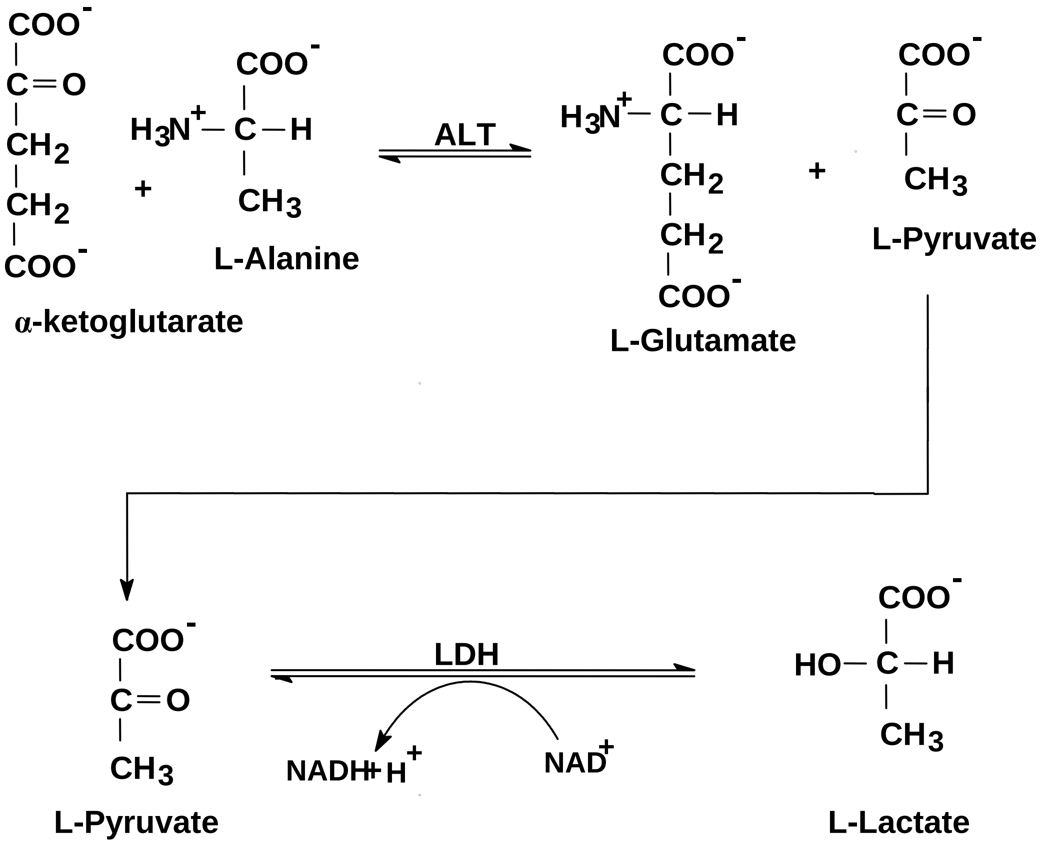
| | |
|---|---|
| Tris-HCl buffer (pH: 7.5) | 70 µmoles |
| L-Glutamate | 25 µmoles |
| NAD | 3 µmoles |
| ADP | 3 µmoles |
| EDTA | 0.25 µmole |
| Tissue extract (suitably diluted) | 20 µl |

The assay mixture without the tissue extract (in both reductive amination and oxidative deamination) was pre-incubated at 30 ºC for 5 min in a 1 ml quartz cuvette having 1 cm light path directly in a UV-visible spectrophotometer (Varian, Cary 50) having a peltier temperature control system fitted to it. The reaction was started by the addition of the tissue extract in the pre-incubated reaction mixture. The decrease in O.D. in case of reductive amination reaction and increase in O.D. in case of oxidative deamination reaction were recorded at 340 nm at 30 sec interval and the period of linear decrease/increase in O.D values were used for calculating the GDH activity. The amount of NADH oxidized in case of reductive amination reaction or the amount of $NAD^+$ reduced in case of oxidative deamination reaction was calculated taking $6.22 \times 10^6$ as the molar extinction co-efficient value for NADH for expressing the GDH activity. One unit of GDH activity was expressed as that amount of enzyme, which catalyzed the oxidation of 1 µmole of NADH to NAD$^+$ in case of GDH (reductive amination) or the reduction of 1 µmole of NAD$^+$ to NADH in case of GDH (oxidative deamination) per h at 30 ºC.

### **Alanine aminotransaminase** (ALT; E.C. 2.6.1.2)

ALT was assayed by the enzyme-coupled reaction following the method of Forster and Moon (1986) with modifications in the substrate (optimal) concentration. L-pyruvate so formed from L-alanine and α-ketoglutarate by the reaction of ALT was converted further to L-lactate by adding excess of lactate dehydrogenase (LDH) in the reaction mixture and the rate of oxidation of NADH was measured finally to find out the ALT activity.

The enzymatic reaction takes place as follows:



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

| | |
|---|---|
| K-phosphate buffer (pH: 7.5) | 125 µmoles |
| L-Alanine | 125 µmoles |

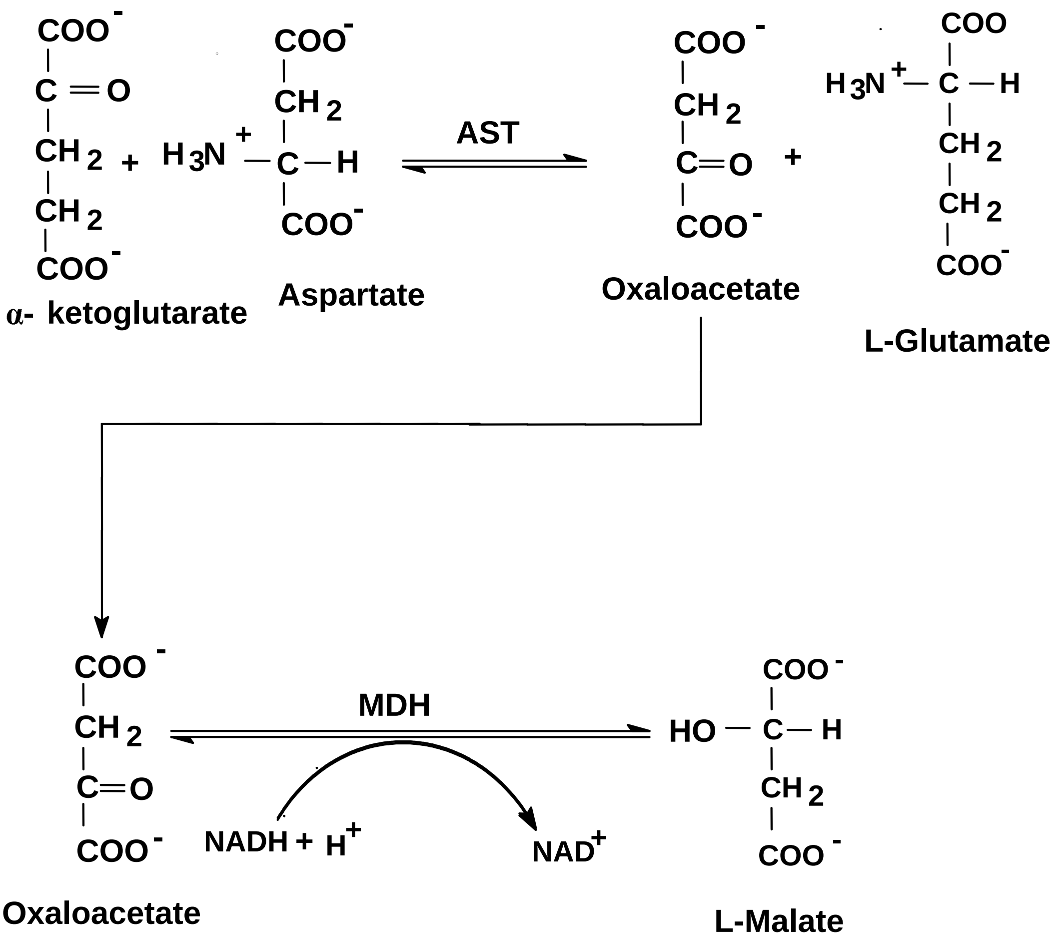
| | |
|---|---|
| α-Ketoglutarate | 15 μmoles |
| NADH | 0.3 μmole |
| LDH (ammonia free) | 10 units |
| Tissue extract (suitably diluted) | 20 μl |

The assay mixture was incubated at 30 $^{o}$C in a 1 ml quartz cuvette having 1 cm light path directly in a UV-visible spectrophotometer (Varian, Cary 50) having a peltier temperature controlled system fitted to it. The reaction mixture without L-alanine was pre-incubated at 30 $^{o}$C for 5 min. The reaction was started by addition of L-alanine into the reaction mixture. The decrease in O.D. was recorded at 340 nm at 30 sec interval. The period of linear decrease of O.D. was used for calculation of ALT activity. The amount of NADH oxidized per h was calculated taking $6.22 \times 10^{6}$ as molar extinction coefficient value for NADH. One unit of ALT activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to NAD$^{+}$ per h at 30 $^{o}$C.

### **Aspartate aminotransaminase** (AST; E.C. 2.6.1.1)

AST activity was assayed by the enzyme-coupled reaction following the method of Forster and Moon (1986) with certain modifications in the substrate (optimal) concentration. Oxaloacetate so formed from L-aspartate and α- ketoglutarate by the enzyme AST was converted further by adding excess of malate dehydrogenase (MDH) in the reaction mixture. The rate of oxidation of NADH was measured finally to find out the activity of AST.

The enzymatic reaction takes place as follows:



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

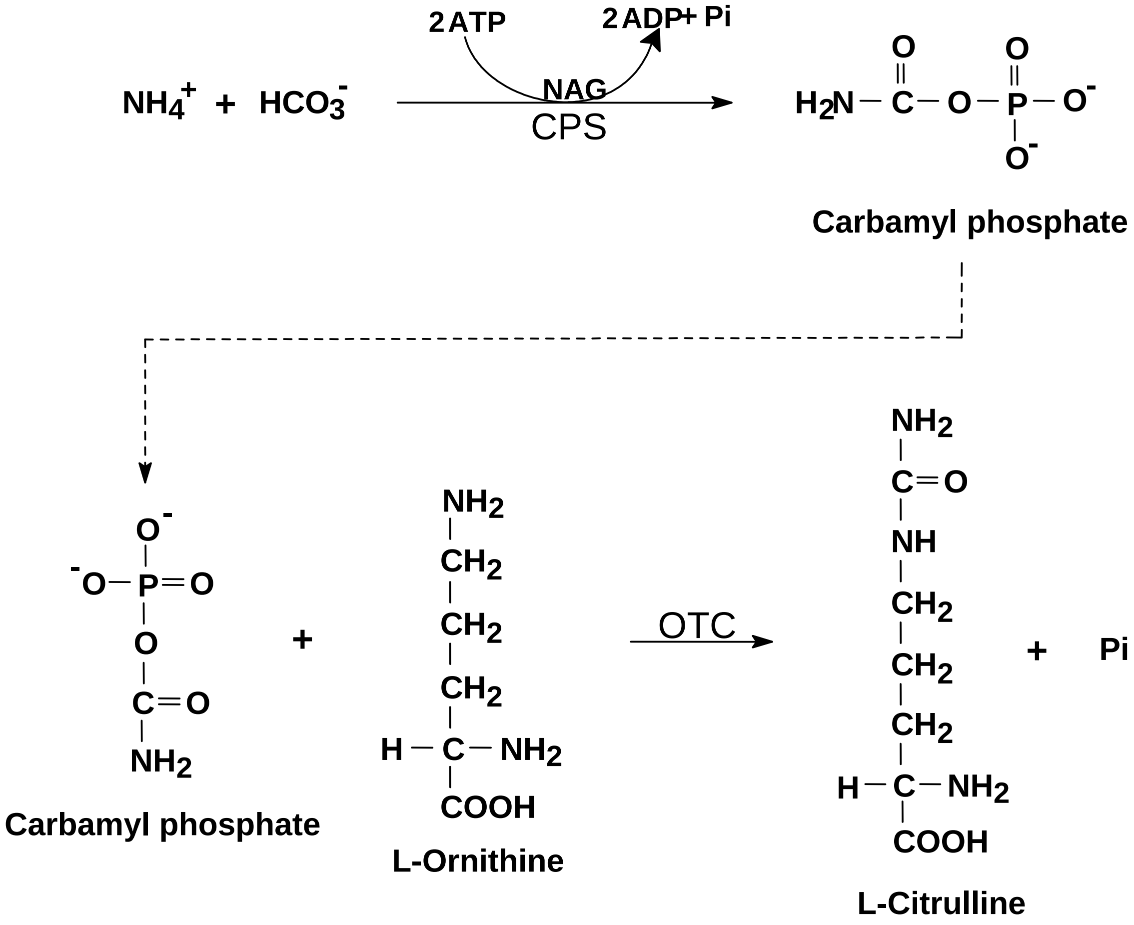
| | |
|---|---|
| K- phosphate buffer (pH: 7.5) | 125 µmoles |
| L-Aspartate | 125 µmoles |
| α-Ketoglutarate | 15 µmoles |
| *NADH* | *0.3 µmole* |
| MDH (ammonia free) | 10 units |
| *Tissue extract*<br>(suitably diluted) | *20 µl* |

The assay mixture was incubated at 30 $^{0}$C in a 1 ml quartz cuvette having 1 c 58 path directly in a UV-visible spectrophotometer (Varian, Cary 50) having a peltier temperature controlled system fitted to it. The reaction mixture without L-aspartate was pre-incubated at 30 $^{o}$C for 5 min. The reaction was started by addition of L-aspartate into the reaction mixture. The decrease in O.D. was recorded at 340 nm at 30 sec interval. The period of linear decrease was used for calculation of AST activity. The rate of NADH oxidized per h was calculated taking 6.22 × $10^6$ as molar extinction coefficient value for NADH. One unit of AST activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to $NAD^+$ per h at 30 °C.

### Carbamyl phosphate synthetase (CPS; E.C. 2.7.2.5)

The urea cycle-related CPS activity was assayed following the method of Brown and Cohen (1959) with modifications made by Saha *et al*. (1995).

The enzymatic reaction takes place as follows:



The assay mixture in a final volume of 1.0 ml contained the following:

| | |
|---|---|
| K-phosphate buffer (pH: 7.5) | 50 μmoles |
| Ammonium chloride | 50 μmoles |
| Na-bicarbonate | 50 μmoles |
| ATP | 20 μmoles |

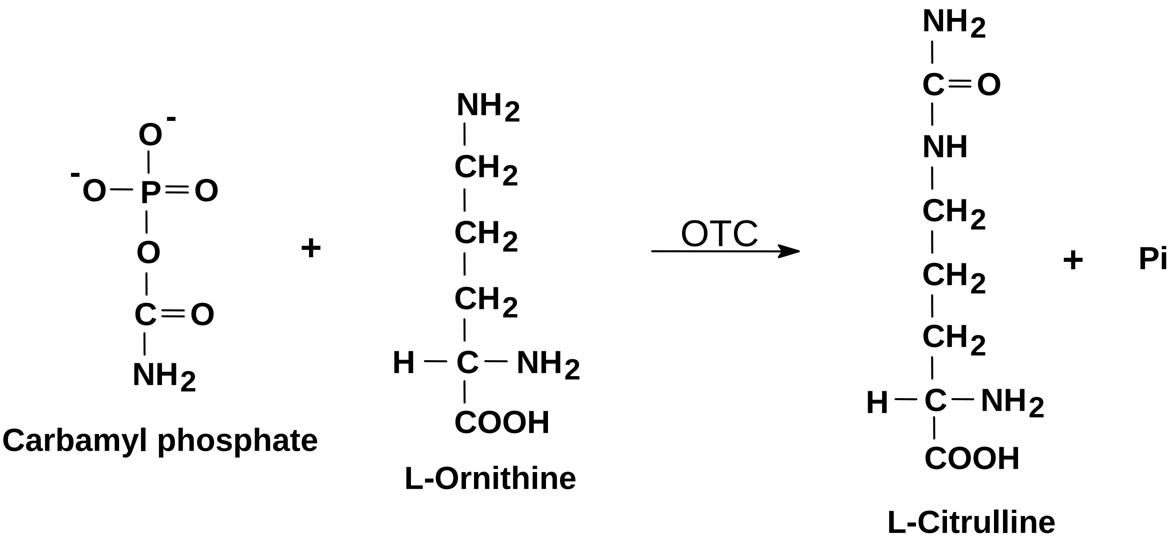
| | |
|---|---|
| L-Ornithine | 5 µmoles |
| N-Acetyl-L-glutamate | 5 µmoles |
| MgS0$_4$ | 10 µmoles |
| UTP | 1 µmole |
| OTC | 5 units |
| Tissue extract (suitably diluted) | 0.3 ml |

The assay mixture without the tissue extract was first preincubated for 5 min at 30 $^{o}$C. The reaction was initiated by adding 0.3 ml of the tissue extract and after 30 min of incubation; the reaction was stopped by the addition of 0.5 ml of 10% PCA. A tissue blank was prepared simultaneously by adding 0.5 ml of 10% PCA to the reaction mixture prior to the addition of the tissue extract. The precipated protein was separated out by centrifugation and the supernatant was used for the estimation of citrulline following the method of Moore and Kauffman (1970). To 0.5 ml of the supernatant, 0.5 ml of distilled water, 2.5 ml of acid mixture (prepared by mixing 150 ml conc. $H_3PO_4$, 50 ml $H_2SO_4$, and 0.118 g MnS0$_4$ and 0.015 g $FeCl_3$ in 500 ml of distilled water) and 0.25 ml of 3% (w/v) diacetyl monoxime were added. The mixture was kept for boiling in a hot water bath for 30 min, cooled and O.D. was measured at 490 nm in a UV-visible spectrophotometer (Varian, Carry 50) against the tissue blank. The amount of citrulline formed by the enzymatic reaction was calculated from the standard graph, prepared by using different concentrations of citrulline (0.01-0.1µmole), which was linear. One unit of CPS activity was expressed as that amount of enzyme which catalyzed the formation of 1 µmole of citrulline per h at 30 $^{o}$C.

## **Ornithine transcarbamylase (OTC; E.C. 2.1.3.3)**

The activity of OTC was assayed spectrophotometrically following the method of Brown and Cohen (1959) with modifications made by Saha *et al*. (1995).

The enzymatic reaction takes place as follows:



The assay mixture in a final volume of 2.0 ml contained the following:

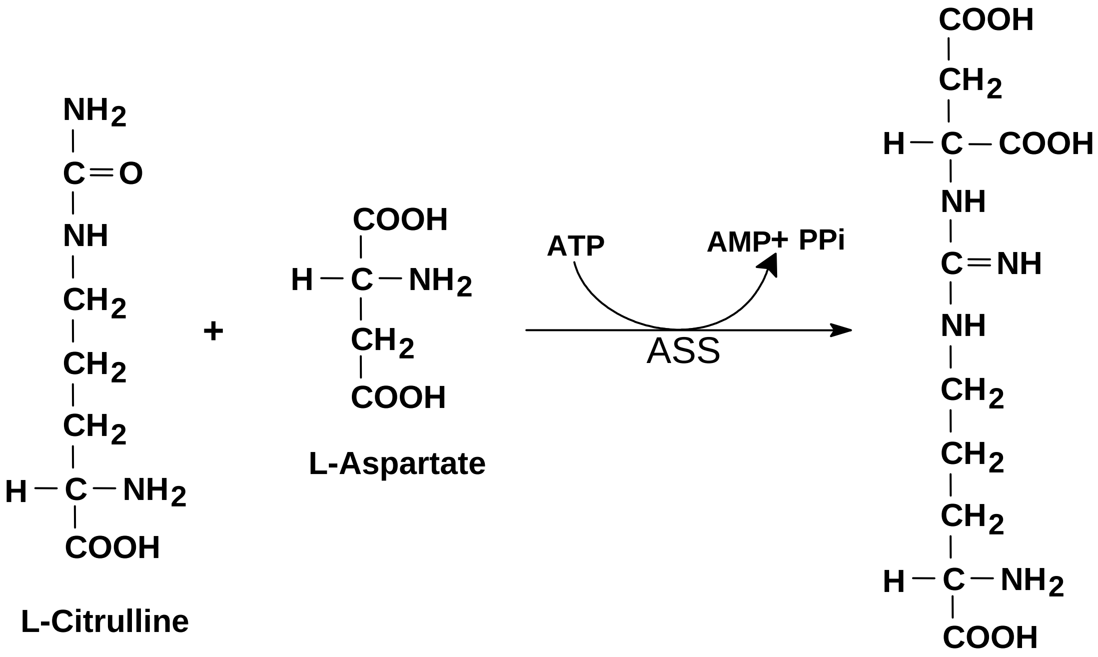
| | |
|---|---|
| Glycyl-glycine buffer (pH 8.3) | 50 µmoles |
| L-Ornithine | 20 µmoles |
| Dilithium carbamyl phosphate | 20 µmoles |
| Tissue extract<br>(suitably diluted) | 0.2 ml |

The assay mixture without the tissue extract was first preincubated for 5 min at 30 °C. The reaction was initiated by adding 0.2 ml of the tissue extract and after 20 min of incubation; the reaction was stopped by the addition of 1 ml of 10% PCA. A tissue blank was also prepared simultaneously by adding 1 ml of 10% PCA to the reaction mixture prior to the addition of the tissue extract. The precipated protein was separated out by centrifugation and citrulline so formed by the enzymatic reaction of OTC during the period of incubation was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above for CPS assay. One unit of OTC activity was expressed as that amount of enzyme which catalyzed the formation of 1 µmole of citrulline per h at 30 °C.

## **Argininosuccinate synthetase** (ASS; E.C. 6.3.4.5)

The ASS activity was assayed following the method of Ratner (1955) with modifications made by Saha *et al*. (1995).

The enzymatic reaction takes place as follows:



**L-Argininosuccinate**

The assay mixture in a final volume of 1.0 ml contained the following:

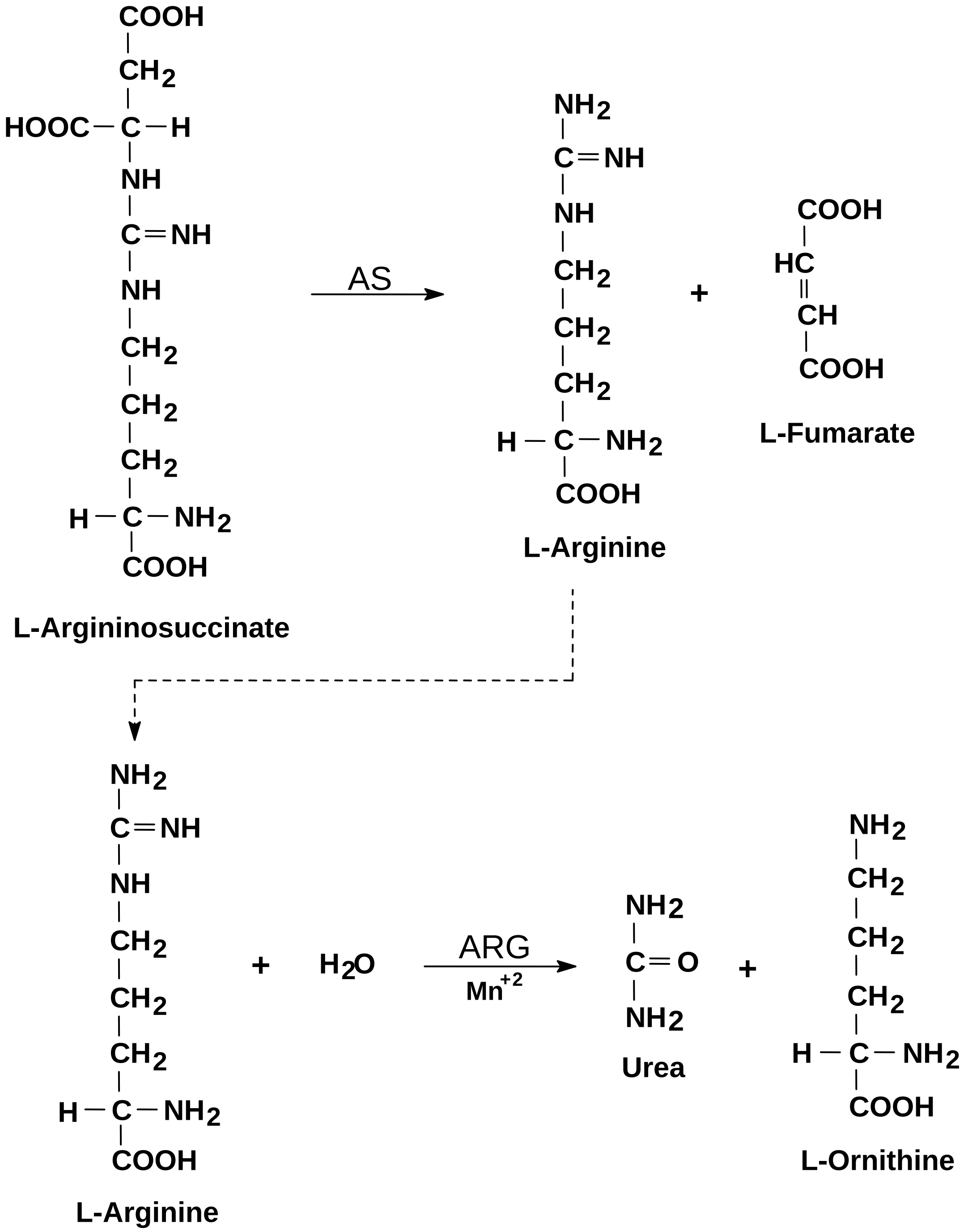
| | |
|---|---|
| K-phosphate buffer (pH: 7.0) | 60 µmoles |
| L- Citrulline | 3 µmoles |
| L- Aspartate | 5 µmoles |
| $MgSO_4$ | 10 µmoles |
| ATP | 20 µmoles |
| Urease | 20 units |
| Tissue extract (suitably diluted) | 0.2 ml |

The reaction mixture without citrulline was first preincubated for 5 min at 30 °C. The reaction was initiated with the addition of citrulline and after 30 min of incubation; the reaction was stopped by the addition of 0.5 ml of 10% PCA. In the tissue blank, PCA was added in the reaction mixture prior to the addition of citrulline. The precipitated protein was separated out by centrifugation and the amount of citrulline utilized during the incubation period was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above for CPS assay. One unit of ASS activity was expressed as that amount of enzyme which catalyzed the utilization of 1 μmole of citrulline per h at 30 $^{o}$C.

### Argininosuccinate lyase (ASL; E.C. 4.3.2.1)

ASL activity was assayed following the method of Brown and Cohen (1959) with modifications made by Saha *et al*. (1995). In this enzymatic assay, the arginine so formed by ASL was further converted to urea by taking excess of arginase in the reaction mixture and the amount of urea formed was expressed as ASL activity.

The enzymatic reaction takes place as follows:



The assay mixture in a final volume of 1.0 ml contained the following:

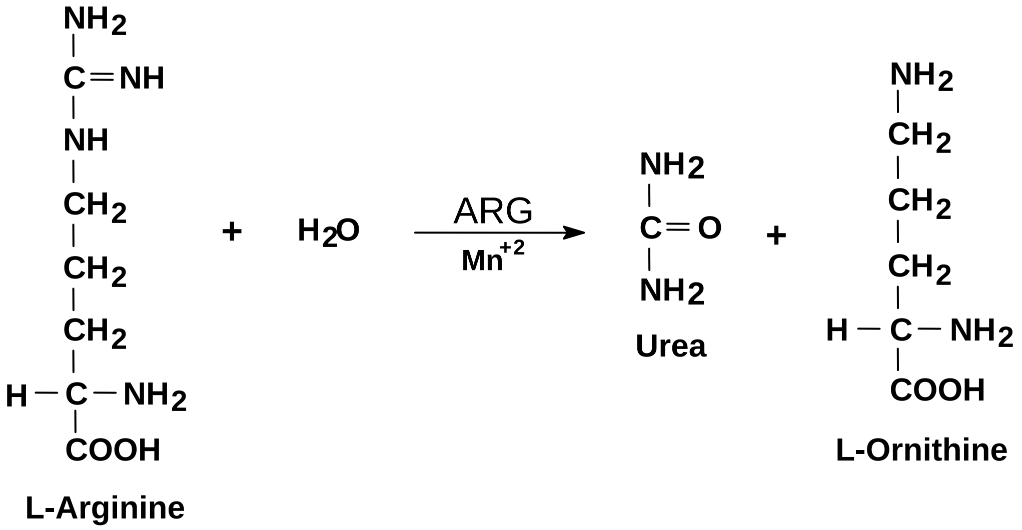
| | |
|---|---|
| K-phosphate buffer (pH: 7.3) | 60 µmoles |
| L-Argininosuccinate | 4 µmoles |
| Arginase | 10 units |
| Tissue extract<br>(suitably diluted) | 0.2 ml |

The assay mixture without the tissue extract was first preincubated for 5 min at 30 °C. The reaction was initiated by the addition of 0.2 ml of the tissue extract and after 30 min of incubation; the reaction was stopped by the addition of 0.5 ml of 10% PCA. A tissue blank was also prepared simultaneously by adding 0.5 ml of 10% PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation and urea so formed by the enzymatic reaction was estimated in the supernatant following the method of Moore and Kauffman (1970). The method was same as described for citrulline estimation, except the O.D. was measured at 478 nm. The concentration of urea was calculated from a linear standard graph prepared with different concentrations (0.01 to 0.1 μmole) of urea. One unit of ASL activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of urea per h at 30 °C.

## Arginase (ARG; E.C. 3.5.3.1)

ARG activity was assayed following the method of Brown and Cohen (1959) with modifications made by Saha *et al*. (1995).

The enzymatic reaction takes place as follows:



The assay mixture in a final volume of 2.0 ml contained the following:

| | |
|---|---|
| Na- glycinate buffer (9.5) | 100 μmoles |
| L-Arginine | 50 μmoles |
| $MnCl_2$ | 0.5 μmole |
| Tissue extract<br>(suitably diluted) | 0.1 μmole |

The reaction mixture without L-arginine was first preincubated for 5 min at 30 °C. The reaction was initiated by the addition of L-arginine, and after 15 min of incubation, the reaction was stopped by adding 1.0 ml of 10% PCA. A tissue blank was also prepared simultaneously by adding 1.0 ml of 10% PCA to the reaction mixture prior to the addition of L-arginine. The precipitated protein was separated out by centrifugation and the supernatant was used for urea estimation. The amount of urea formed during the incubation period was estimated following the method of Moore and Kauffman (1970) as described above for ASL assay. One unit of arginase activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of urea per h at 30 °C.

## Measurement of osmolarity:

The osmolarity of blood plasma of the mud eel under different experimental conditions was determined by the freezing point depression method with a Camlab Osmometer (Model 2000).

## Chemicals:

All the enzymes, co-enzymes, substrates, mixtures of physiological amino acids and bovine serum albumin were obtained either from Sigma Chemical Co., St. Louis, U.S.A. or from Roche, Germany. The primary antibody for GS raised in rabbit was from Santa Cruz Biotech., Inc., U.S.A. Secondary antibodies (anti-rabbit) conjugated with HRP were obtained from Sigma-Aldrich Co., USA. Primary antibody for Hsp70 raised in mouse was from Sigma Chemical Co., St. Louis, U.S.A. Secondary antibody (anti-mouse) from Santa Cruz Biotech., Inc., U.S.A. Enhanced chemiluminescence kits (Western Lightning™ Chemiluminescence Reagent Plus) were from PerkinElmer LAS, Inc., Boston, U.S.A. All the other chemicals used were of analytical grades were obtained from local sources, developer and fixer from Kodak India Ltd., Mumbai. Deionized double glass distilled water was used in all preparations.

## Statistical analysis and presentation of data:

The data was calculated from at least three to six observations at each point and presented as mean ± standard error of the mean (SEM). One-way ANOVA test was performed when the control value was compared with more than one experimental data. P values at <0.05, <0.01 and <0.001 were regarded as statistically significant. Besides the presentation of data in tabulated forms, histograms, and line diagrams were prepared to highlight the results.

# RESULTS

## 1. 100 mM $NH_4Cl$ Exposure Experiment:

*1.1 Changes in tissue levels of ammonia-N and urea-N*:

There was a significant increase in the concentration of ammonia-N in all the tissues studied and in the plasma of the mud eel within the first day of exposure to 100 mM $NH_4Cl$, followed by further increase of approximately 1.5-2.0 fold in most of the tissues compared to 0 day and 7 days controls. Percentage wise the concentration of ammonia increased maximally in plasma (91%), followed by brain (71%), liver and stomach (69%), muscle (65%), intestine (64%), and kidney (63%) after 7 days of treatment (Table 1, Fig. 1). Whereas, there was no significant changes in the concentration of urea-N in different tissues of $NH_4Cl$-treated fish, except for a significant increase in liver (24%) after 7 days, in stomach (29%) after 3 days, in brain (32%) after 5 days and in plasma (25%) after 3 days of exposure (Table 1, Fig. 2).

*1.2 Fluxes of ammonia-N, urea-N and glutamine-N by the perfused liver*

The changes of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of the mud eel kept in 100 mM $NH_4Cl$ for 7 days with relation to controls are shown in Table 2, Fig. 3. The rates of efflux of ammonia-N, urea-N and glutamine-N (calculated by taking 2N for each molecule of glutamine) by the perfused liver, following infusion of 0.5 mM $NH_4Cl$ both in the control as well as in the fish exposed to 100 mM $NH_4Cl$ for 7 days, were determined to assess the changes of efflux of these compounds as a consequence of upregulation of GS, the key enzyme responsible for detoxification of ammonia-N to glutamine-N. In the control fish, the rates of efflux of ammonia-N, urea-N and glutamine-N by the perfused liver were recorded to be 1.32 ± 0.14, 0.22 ± 0.05 and 0.37 ± 0.07 µmol/g liver/min, accounting for 69%, 12% and 19% of the total-N efflux, respectively. Whereas in the $NH_4Cl$-treated fish, the rates of efflux of ammonia-N, urea-N and glutamine-N were recorded to be 0.62 ± 0.1, 0.28 ± 0.04 and 0.99 ± 0.07 µmol/g liver/min, accounting for 33%, 15% and 52% of the total-N efflux, respectively. However, while comparing with the control fish with that of $NH_4Cl$–treated fish, there was significant decrease of ammonia-N efflux by 53%, and increase of glutamine-N and urea-N efflux by 167% and 27%, respectively.

### *1.3 Changes in the activity of some key amino acid metabolism-related enzymes*:

Tables 3-7 and figs. 4-13 represent the changes of tissue and specific activities of certain key amino acid metabolism-related enzymes such as GS, GDH (reductive amination and oxidative deamination), ALT and AST in the $NH_4Cl$-treated fish compared to respective controls. The physiological level of activity of GS was maximal in brain, followed by stomach, intestine, kidney, muscle and liver (Table 3, Fig. 4). The activity of GS increased significantly in most of the tissues after first day of exposure to 100 mM $NH_4Cl$, followed by further increase at later stages of exposure with maximum increase in liver (220%), followed by kidney (124%), muscle (122%), stomach (103%), intestine (84%) and brain (33%) after 7 days. In general, the maximum increase of GS activity was seen after the fifth day of exposure in liver, kidney and muscle. The changes of specific activity of $NH_4Cl$-treated fishes followed almost the similar pattern to that of tissue activity, with maximum percentage increase in liver (233%), followed by kidney (130%) on the fifth day, muscle (124%), stomach (81%) and intestine (65%) on the seventh day of exposure (Table 3, Fig. 5).

The physiological levels of activity of GDH (both reductive and oxidative directions) were found to be quite high in all tissues. For reductive amination (R.A.), the maximum tissue activity was seen in liver, followed by brain, kidney, intestine, muscle, and least in stomach. The increase in tissue activity of GDH (R.A.) in $NH_4Cl$-treated fish was maximum in liver (70%), followed by kidney (58%), stomach (57%), muscle (48%) with no changes of activity in brain and intestine (Table 4, Fig. 6). For oxidative deamination (O.D.), liver showed the maximum physiological activity, followed by kidney, brain, intestine, stomach, and muscle. The increase in tissue activity of GDH (O.D.) in $NH_4Cl$-treated fish was maximum in stomach (79%), followed by intestine (41%) and liver (26%) after 7 days of exposure, while in kidney, muscle and brain there was no significant changes of activity. The changes of specific activity of GDH (both R.A. and O.D.) showed almost similar pattern to that of tissue activity (Table 5, Fig. 7). The percentage increase of the specific activity of GDH in reductive amination direction was maximum in kidney (104%), followed by liver (68%), stomach (53%) on the fifth day, muscle (53%) on the seventh day with no significant changes in intestine and brain. The percentage increase of specific activity of GDH in oxidative deamination direction was maximum in stomach (74%) on the seventh day, followed by liver (32%) on the fifth day, kidney and liver (28%) on the seventh day of exposure.

The physiological level of tissue activity of AST was maximum in stomach, followed by liver, intestine, muscle, kidney and brain (Table 6, Fig. 10). The AST activity in $NH_4Cl$-treated fish increased significantly in liver (176%) on the fifth day of exposure, followed by kidney (99%), muscle (72%), intestine (44%) and brain (25%), with no changes of activity in stomach. However, the maximum increase was recorded in muscle (138%), followed by liver (108%), intestine (61%) and brain (42%) after 7 days. The changes of specific activity of AST in different tissues of $NH_4Cl$-treated fish showed almost similar pattern to that of tissue activity (Table 6, Fig. 11). The percentage increase of specific activity for AST was maximum in liver (179%), followed by kidney (99%) after the fifth day, muscle (78%) after the seventh day, intestine (44%) after the fifth day and brain (45%) after the seventh day of treatment.

For ALT, maximum physiological level of activity was seen in liver, followed by kidney, brain, muscle, intestine and stomach (Table 7, Fig. 12). The tissue activity of ALT in $NH_4Cl$-treated fish increased significantly within the first day of exposure especially in stomach, intestine and muscle, followed by further increase after 7 days. The percentage increase of ALT activity was recorded to be maximum in muscle (122%), followed by liver (119%), intestine (109%), kidney (88%), stomach (47%) and brain (42%). The changes of specific activity of ALT in different tissues of $NH_4Cl$-treated fish showed almost similar pattern to that of tissue activity (Table 7, Fig. 13). The percentage increase of specific activity for ALT was maximum in liver

(174%) after the fifth day, followed by muscle (104%), intestine (92%) after the seventh day, kidney (83%), stomach (53%) after the fifth day and brain (41%) after the seventh day of exposure.

### *1.4 Changes in the activity of OUC enzymes*:

The physiological levels of activity of five OUC enzymes such as carbamyl phosphate synthase (CPS), ornithine transcarbamylase (OTC), argininosucinate synthase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) were found to be quite low in both liver and kidney tissues of the mud eel (Table 8, Fig. 14). Exposure to high environmental ammonia (100 mM $NH_4Cl$) also did not cause any significant changes of activities of any of the OUC enzymes in both the tissues.

### *1.5 Changes in concentrations of different FAAs*:

There was significant increase in concentrations of different non-essential FAAs in most of the tissues of the mud eel following exposure to 100 mM $NH_4Cl$ compared to respective controls. In liver, the total concentrations of non-essential FAAs increased significantly within the first day of exposure to $NH_4Cl$, followed by further increase by 159% after 7 days of exposure (Table 9, Fig. 15). This increase was mainly attributable due to the increase in concentrations of Asp (64%), Gly (68%), Ala (67%), Asn (73%), Glu (51%), Gln (385%) and Tau (118%) after 5 days of exposure. Significant increase in the concentrations of few essential FAAs such as Met (27%), Ile (100%), Leu (45%) and Arg (33%) were observed mainly after 5 days of exposure.

In kidney, the pattern of changes of total concentration of non-essential FAAs was almost the same as that of liver. The total non-essential FAAs increased significantly by 121% after 5 days of exposure, which was mainly attributable due to the increase in concentrations of Asp (97%), Gly (366%), Ala (212%), Glu (116%), Gln (268%), Pro (109%), and Tau (89%) after 5 days of exposure (Table 10, Fig. 16).

In muscle, the total concentrations of non-essential FAAs also increased significantly within the first day of exposure to $NH_4Cl$, followed by further increase by to a maximum level by 126% after 7 days of exposure (Table 11, Fig. 17), which was mainly attributable due to the increase in the concentrations of Asp (138%), Ala (202%), Asn (204%), Glu (142%) and Gln (409%) after 5 days of exposure. Significant increase in the concentrations of few essential FAAs such as Met (27%), Ile (100%), Leu (45%) and Arg (33%) were also observed after 5 days of exposure.

The total concentrations of non-essential FAAs also increased significantly in brain within the first day of exposure to $NH_4Cl$, followed by further increase with a maximum increase by 100% after 5 days of exposure (Table 12, Fig. 18), which was mainly attributable due to the increase of Ala (95%), Gln (182%) and Pro (228%) after 5 days of exposure. The concentrations of total FAAs did not show any significant changes following exposure to 100 mM $NH_4Cl$ for 7 days.

In plasma, the concentrations of total non-essential FAAs also increased significantly within the first day of exposure to $NH_4Cl$, followed by further increase with a maximum increase by 179% after 5 days of exposure (Table 13, Fig. 19) which was mainly attributable to the increase of Asp (366%), Glu (408%), Gln (389%), Pro (200%) after 5 days of exposure. The concentrations of the total essential FAAs did not show any significant change even after 7 days of $NH_4Cl$ exposure.

### *1.6 Changes of GS enzyme protein and hsp70 concentrations*:

Western blot analyses indicated that there was significant increase of GS enzyme protein concentrations in different tissues of the mud eel following exposure to 100 mM $NH_4Cl$ for 7 days (Fig. 21). The fold of increase of GS enzyme protein was maximum for stomach (3.2 fold), followed by intestine (2.9 fold), muscle (2.7 fold), kidney (2.6 fold), liver (2.5 fold) and brain (1.4 fold). The hsp70 was seen to be expressed in all the tissues studied in the control fish, whose concentration was found to increase significantly in most of the tissues of the mud eel following exposure to 100 mM $NH_4Cl$ for 7 days. The maximum increase was seen in stomach and least in kidney ranging the fold of increase by 1.5 to 4.2 fold in different tissues (Fig. 22).

## 2. Aerial exposure Experiment:

### *2.1 Rates of excretion of ammonia-N and urea-N*:

The changes in the rates of excretion of ammonia-N and urea-N by the mud eel during aerial exposure and upon re-immersion are presented in table 14 and fig. 23.The rate of excretion of ammonia-N by the control fish was relatively stable over time period and averaged to be about 45 μmoles/kg body wt/h throughout the experimental periods. However, the excretion of ammonia-N excretion by the fish exposed to air decreased by 37% after 24 h, followed by further decrease to almost 54% after 96 h of aerial exposure. But the rate of ammonia-N excretion recovered almost to the basal level after 24 h of re-immersion.

The rate of excretion of urea-N by the control fish was stable and averaged to be about 6.0 μmoles/kg body wt/h throughout the experimental periods, with the percentage of N excreted as urea-N accounting for 14% of the total-N excretion (ammonia-N + urea-N). However, the rate of urea-N excretion by the fish exposed to air decreased significantly by 35% after 48 h, followed by returning almost to the basal level after 72 h of aerial exposure, which maintained even during re-immersion.

### *2.2 Changes of tissue levels of ammonia-N and urea-N*:

The physiological concentrations of ammonia-N and urea-N in different tissues both in control fish and as well as in the fish exposed to air for 144 h are presented in table 15 and figs. 24 & 25. Ammonia-N accumulated significantly in all the tissues of the mud eel following exposure to air. In most of the tissues, ammonia-N accumulated significantly within 24 h of aerial exposure, followed by further increase after 72 and 144 h of aerial exposure. The ammonia-N concentration increased maximally from 27.11 to 41.34 µmoles/g wet wt (46%) in kidney, from 26.58 to 46.72 µmoles/g wet wt (76%) in liver, from 25.56 to 41.12 µmoles/g wet wt (61%) in intestine, from 25.38 to 45.41 µmoles/g wet wt (79%) in muscle, from 25.12 to 39.75 µmoles/g wet wt (58%) in stomach from 20.02 to 26.35 µmoles/g wet wt (32%) in brain, and from 2.78 to 5.84 µmoles/ml (110%) in plasma after 72 h of aerial exposure.

However, urea-N concentration did not show any significant change in any of the tissues of the mud eel following exposure to air compared to respective controls (Table 15, Fig. 25).

### *2.3 Fluxes of ammonia-N, urea-N and glutamine-N by the perfused liver*

Table 16 and fig. 26 show the fluxes of ammonia-N, urea-N and glutamine-N from the perfused liver of both control and air-exposed mud eel. The rates of efflux of ammonia-N, urea-N and glutamine-N (calculated by taking 2N for each molecule of glutamine) by the perfused liver following infusion of 0.5 mM $NH_4Cl$ both in the control as well as in the air-exposed fish for 6 days were determined to assess the changes of efflux of these compounds mainly due to upregulation of GS, the key enzyme responsible for detoxification of ammonia-N to glutamine-N. In the control fish, the rates of efflux of ammonia-N, urea-N and glutamine-N by the perfused liver were recorded to be 1.35 ± 0.12, 0.23 ± 0.05 and 0.36 ± 0.06 µmoles/g liver/min, accounting for 70%, 12% and 19% of the total-N efflux, respectively. Whereas in the air-exposed fish, the rates of efflux of ammonia-N, urea-N and glutamine-N were recorded to be 0.59 ± 0.05, 0.27 ± 0.06 and 1.06 ± 0.08 µmoles/g liver/min, accounting for 28%, 14% and 55% of the total-N efflux, respectively. Thus, in the air-exposed fish with respect to control fish, there was a significant decrease of ammonia-N efflux by 56% and significant increase of glutamine-N efflux by 194%, with no significant change of urea-N efflux.

### *2.4 Changes in the activity of some key amino acid metabolism-related enzymes*:

Tables 17-21 and figs. 27-36 represent the changes of tissue and specific activities of certain key amino acid metabolism-related enzymes such as GS, GDH (both reductive amination and oxidative deamination), AST and ALT in the mud eel exposed to air along with the physiological levels of activity of these enzymes in different tissues. The activity of GS increased maximally in liver of air-exposed fish by 171%, followed by kidney (96%), muscle (68%), stomach (60%) and intestine (41%) on the third day of exposure and continued till the sixth day, while in brain there was no significant change of GS activity (Table 17, Fig. 27). The specific activity of GS was found to increase maximally in liver (164%), followed by muscle (104%), kidney (59%), intestine (55%) and stomach (47%) following 6 days of air exposure, and in brain (26%) on the third day (Table 17, Fig. 28).

The GDH activity in the reductive amination direction was found to increase maximally in liver (80%), followed by kidney (67%), muscle (55%) and stomach (45%) after three days of aerial exposure, which maintained till the sixth day (Table 18, Fig. 29). Whereas, the GDH activity in oxidative deamination direction no significant changes were noticed in any of the tissues of air-exposed fish except for a significant increase in muscle (33%) and stomach (48%) only after 6 days of aerial exposure (Table 19, Fig. 31). The changes of specific activity of GDH (both R.A. and O.D.) in the air-exposed fish followed almost the similar pattern to that of tissue activity. The percentage increase of the specific activity of GDH in reductive amination direction was maximum in kidney (76%), followed by liver (69%), muscle (46%) on the sixth day, with no significant changes in stomach, intestine and brain (Table 18, Fig. 30). However, there was no significant change in the specific activity of GDH in oxidative deamination direction during 6 days of aerial exposure in any of the tissues (Table 19, Fig. 32).

The activity of AST (both tissue and specific) also changed significantly in the mud eel exposed to air for 6 days compared to control fish (Table 20, Figs. 33 & 34). The tissue activity of AST increased maximally in liver (119%), followed by kidney (77%), muscle (65%), brain (47%) and intestine (39%) after 6 days of aerial exposure. The specific activity of AST increased maximally in liver (256%), followed by kidney (70%), muscle (57%), brain (53%) and intestine (39%) after 6 days of aerial exposure.

The changes of both tissue and specific activity of ALT in the mud eel exposed to air for 6 days are shown in table 21 and figs. 35 & 36. The tissue activity of ALT increased maximally in liver (137%), followed by kidney (106%), muscle (56%), stomach (53%), intestine (46%) and brain (43%) either after 3 or 6 days of aerial exposure. Likewise, the specific activity of ALT also increased maximally in liver (153%), followed by kidney (134%), stomach (66%), intestine (54%), muscle (51%) and brain (32%) either after 3 or 6 days of aerial exposure.

### *2.5 Changes in the activity of OUC enzymes*:

The activity of the five OUC enzymes did not show any significant changes in both in liver and kidney tissues following 6 days of aerial exposure (Table 22, Fig. 37).

### *2.6 Changes in concentrations of different FAAs*:

The concentrations of different non-essential FAAs increased significantly in most of the tissues of the mud eel following exposure to air compared to respective controls. In liver, the total concentrations of various non-essential FAAs increased significantly within the first day of exposure to air, followed by further increase by 125% after 3 days exposure (Table 23, Fig. 38). This was mainly attributable due to the increase in concentrations of Asp (98%), Gly (55%), Ala (180%), Asn (92%), Glu (139%), Gln (292%) and Cit (61%) after 3 days of exposure. Significant increase of few essential FAAs such as Val (78%), Met (29%), Ile (100%), Phe (46%) and Orn (44%) were observed mainly after 3 days and further increase after 6 day of exposure.

In kidney, the pattern of changes in the concentrations of different non-essential FAAs was almost the same as that of liver. The total non-essential FAAs increased maximally by 134% after 5 days of exposure, which was mainly attributable due to the increase in the concentrations of Asp (169%), Gly (188%), Ala (125%), Ser (78%), Glu (352%) and Gln (174%) after 3 days aerial exposure which maintained till the sixth day of exposure (Table 24, Fig. 39).

In muscle, the concentrations of total non-essential FAAs increased significantly after the first day of air exposure (36%), followed by further increase up to 74% after 3 days of exposure, which was mainly attributable due to the increase in the concentrations of Asp (137%), Ala (110%), Asn (104%), Glu (125%) and Gln (172%) (Table 25, Fig. 40). There was no significant change in the concentration of essential FAAs in the muscle of mud eel following 6 days of aerial exposure.

In brain, significant changes in the concentrations of total non-essential FAAs were observed on the third day of exposure to air (60%), which was further increased on the sixth day (87%) compared to control, which was mainly attributable due to the increase in the concentrations of Asp (49%), Ala (92%), Asn (350%), Glu (184%), Gln (73%) and Tau (68%) (Table 26, Fig. 41). The concentrations of essential FAAs remained unchanged throughout the experimental periods.

The concentrations of total non-essential FAAs in plasma increased significantly on the first day of exposure by 52%, which further increased up to 85% on the sixth day. This was mainly attributable due to the increase in the concentrations of Gln (162%), Glu (78%), Asn (150%), Asp (63%), Ala (60%) (Table 27, Fig. 42). There were also significant changes in the total concentration of essential FAAs by 26% on the first day of exposure, followed by further increase by 31% after the sixth day.

*2.7 Changes of GS enzyme protein and hsp70 concentrations*:

Western blot analyses indicated that there was significant increase of GS enzyme protein concentrations in different tissues of the mud eel following 6 days of aerial exposure (Fig. 44). The fold of increase of GS enzyme protein was maximum in intestine (2.9 fold), followed by stomach (2.7 fold), muscle (2.6 fold), liver (2.3 fold), kidney (2.1 fold) and brain (1.6 fold). The hsp70 was seen to be expressed in all the tissues studied in the control fish, whose concentration increased further in most of the tissues of mud eel following 6 days of aerial exposure. The maximum increase was seen in stomach and least in kidney ranging the fold of increase by 2 to 3.1 fold in different tissues (Fig. 45).

## 3. $NH_4HCO_3$ Injection Experiment:

*3.1 Rates of excretion of ammonia-N and urea-N*:

The rates of excretion of total ammonia-N and urea-N by the control fish remained relatively stable over a period of 24 h and averaged to be about 47.3 and 5.9 µmoles/kg body wt/h, respectively (Table 28, Fig. 46). The rate of excretion of ammonia-N increased significantly within 6 h of $NH_4HCO_3$ injection, which further increased to 39% during 12-24 h of injection. However, the rate of excretion of urea-N by the fish injected with $NH_4HCO_3$ did not change significantly following $NH_4HCO_3$ injection except during 12-24 h.

*3.2 Fluxes of ammonia-N, urea-N and glutamine-N by the perfused liver*:

The changes of efflux of ammonia-N, urea-N and glutamine-N from the perfused liver of the mud eel after 24 h of injection of $NH_4HCO_3$ are shown in table 29 and fig. 47. The rates of efflux of ammonia-N, urea-N and glutamine-N (calculated by taking 2N for each molecule of glutamine) by the perfused liver, following infusion of 0.5 mM $NH_4Cl$ both in the control as well as in the $NH_4HCO_3$ injected fish were determined to assess the changes of efflux of these compounds as a consequence of upregulation of GS. In the control fish, the rates of efflux of ammonia-N, urea-N and glutamine-N by the perfused liver were recorded to be 1.32 ± 0.14, 0.22 ± 0.05 and 0.37 ± 0.07 µmoles/ g liver/min, accounting for 69%, 11% and 20% of the total-N efflux, respectively. Whereas in the $NH_4HCO_3$ injected fish, the rates of efflux of ammonia-N, urea-N and glutamine-N were recorded to be 0.85 ± 0.11, 0.27 ± 0.04 and 0.74 ± 0.07 µmoles/ g liver/min, accounting for 45%, 14% and 40% of the total-N efflux, respectively. However, comparing with the control fish with that of the treated fish, there was a significant decrease of ammonia-N efflux by 36%, increase of glutamine-N and urea-N by 100% and 23%, respectively after infusion of ammonia to the liver of treated fish.

### *3.3 Changes in the activity of some key amino acid metabolism-related enzymes*:

Tables 30-32 and figs. 48-52 represent the changes of tissue and specific activities of certain key amino acid metabolism-related enzymes such as GS, GDH (both R. A. and O. D.), AST and ALT in the $NH_4HCO_3$-injected fish compared to respective controls. Significant increase of tissue activity of GS was observed $NH_4HCO_3$-injected fish with maximum increase in the stomach (148%), followed by kidney (94%), muscle (75%), liver (73%) and intestine (20%) after 24 h of injection, while in brain no significant change of GS activity was observed (Table 30, Fig. 48). The changes of specific activity of $NH_4HCO_3$-injected fish followed almost the similar pattern to that of tissue activity, with maximum percentage increase in stomach (177%), followed by liver (105%), kidney (71%), intestine (69%) and muscle (60%) and no change in brain after 24 h of $NH_4HCO_3$ injection (Table 30, Fig. 48).

The activity of GDH in reductive amination direction was found to increase significantly in the $NH_4HCO_3$-injected fish with maximum in kidney (86%), followed by liver (60%), brain (34%), stomach (29%), muscle (27%), and intestine (20%) (Table 31, Fig. 49). However, for GDH in oxidative deamination direction no significant changes of activity was observed in any of the tissue except for liver (56%) (Table 31, Fig. 50). The changes of specific activity of GDH (both R.A. and

O.D.) in the $NH_4HCO_3$-injected fish followed almost the similar pattern to that of tissue activity with liver showing the highest activity. The percentage increase of the specific activity of GHD (R. A.) was maximum in liver (91%), followed by brain (73%) and stomach (50%); and that of GDH (O. D.) was significant only in liver (105%) (Table 31, Figs. 49 & 50).

The tissue activity of AST increased significantly in different tissues of the mud eel injected with $NH_4HCO_3$ with a maximum increase in muscle (155%), followed by kidney (79%), liver (43%) and brain (23%) (Table 32, Fig. 51). Similarly, the $NH_4HCO_3$ injection led to significant increase of tissue activity of ALT also, with maximum increase in muscle (77%), followed by brain (43%) and liver (34%) and kidney, stomach and intestine did not show any significant increase (Table 32, Fig. 52).

### *3.4 Changes in the activity of OUC enzymes*:

The physiological activities of the five OUC enzymes such as activity CPS, OTC, ASS, ASL and ARG were quite low in both liver and kidney of the mud eel, with no changes of activity in both the tissues after 24 h of $NH_4HCO_3$ injection (Table 33, Fig. 53).

### *3.5 Changes in concentrations of different FAAs*:

Tables 34a & 34b, figs. 54-59 represent the changes in the concentrations of different FAAs in various tissues of the mud eel after 24 h of $NH_4HCO_3$ injection. The total concentrations of non-essential FAAs show significant changes in liver (96%), followed by kidney (60%), plasma (59%), stomach (38%), and brain (32%) after 24 h of $NH_4HCO_3$ injection, while there was no change in muscle. The increase of non-essential FAAs in the liver was mainly attributable due to the increase in the concentrations of Gln (190%), Glu (183%), Ala (91%) and Asp (33%) (Table 34, Fig. 54). In kidney, the increase was mainly attributable due to the increase in the concentrations of Asp (174%), Gln (91%), Ser (85%), Cit (81%) (Table 34, Fig. 55). In stomach, the increase was mainly attributable due to the increase in the concentrations of Gln (94%), Glu (76%), Asp (63%), Ala (85%) and Pro (54%) (Table 34b, Fig. 57). In brain, the increase was mainly attributable due to the increase in the concentrations of Asp (146%), Gln (138%), Glu (65%) and Tyr (125%) (Table 35, Fig. 58). Whereas, the total concentrations of essential FAAs in all the tissues of the mud eel did not show any significant changes after 24 h of $NH_4HCO_3$ injection.

### *3.6 Changes of GS enzyme protein concentration*:

The increase of the GS enzyme activity in the $NH_4HCO_3$-injected fish was accompanied with significant increase in the GS enzyme protein concentrations in different tissues of the mud eel as evidenced by the western blot analysis after 24 h of $NH_4HCO_3$ injection (Fig. 61). The fold of increase was maximum in intestine (2.3 fold), followed by kidney (1.8 fold), muscle (1.6) liver stomach (1.5 fold) and brain (1.2 fold).

## **4. Cortisol Injection Experiment**:

### *4.1 Rates of changes in excretion of ammonia-N and urea-N*:

The pattern of changes in rates of excretion of ammonia-N and urea-N by the mud eel following intra-peritoneal injection of cortisol (50 mg/kg body wt) are presented in table 36 and fig. 62. The total-N excreted as ammonia-N and urea-N increased significantly during 6-12 h of injection of cortisol up to 24%, followed by further increase up to 51% during 24-48 h and remained more or less constant till 5 days of injection. The rate of excretion of ammonia-N increased significantly during 6-12 h by 27%, followed by further increase up to 61% during 24-48 h post injection and thereafter maintained more or less the same level till 120 h (5 days) of cortisol treatment. However, the rate of urea excretion did not show any significant change except during 12-24 h (29%) and 72-96 h (23%).

### *4.2 Changes of tissue levels of ammonia-N and urea-N*:

The tissue levels of ammonia-N and urea-N in different tissues of the mud eel after 5 days of intra-peritoneal injection of cortisol were presented in the table 37 and fig. 63. The ammonia-N concentration increase significantly in all the tissues of cortisol-injected mud eel except in brain. The accumulation of ammonia-N was maximum in liver (85%), followed by kidney (83%) and muscle (45%) as compared with control fish. The urea-N level also significantly in liver (32%), kidney (50%), muscle (55%) and plasma (20%) in cortisol treated fish (Table 37, Fig. 63).

### *4.3 Fluxes of ammonia-N, urea-N and glutamine-N by the perfused liver*:

The changes of efflux of ammonia-N, urea-N and glutamine-N by the perfused liver of the mud eel after 5 days of cortisol injection with relation to controls are presented in table 38 and fig. 64. The rates of efflux of ammonia-N, urea-N and glutamine-N (calculated by taking 2N for each molecule of glutamine) by the perfused liver following infusion of 0.5 mM $NH_4Cl$ both of the control as well as of the treated fish were determined to assess the changes of efflux of these compounds due to injection of cortisol. In the control fish, the rates of efflux of ammonia-N, urea-N and glutamine-N by the perfused liver were recorded to be 1.32 ± 0.14, 0.22 ± 0.05 and 0.37 ± 0.07 µmoles/g liver/min, accounting for 69%, 12% and 19% of the total-N efflux, respectively. Whereas, the rates of efflux of ammonia-N, urea-N and glutamine-N in the cortisol-injected fish were recorded to be 0.89, 0.35 and 0.69 µmoles/g liver/min, accounting for 46%, 18% and 36% of the total-N efflux, respectively. However, while comparing with the control fish, there was a significant decrease of ammonia-N efflux by 33%, and increase of glutamine-N and urea-N efflux by 86% and 59%, respectively.

### *4.4 Changes in the activity of some key amino acid metabolism-related enzymes*:

The activities of different amino acid metabolism related enzymes were measured after 5 days of intra-peritoneal injection of cortisol and compared with respective controls (Tables 39-41 and figs. 65-69). The activity of GS increased significantly in most of the tissues after 5 days of cortisol injection with maximum increase in muscle (103%), followed by liver (90%), kidney (77%) and brain (53%) (Table 39, Fig. 65). The changes of specific activity of cortisol-injected fishes followed almost similar pattern to that of tissue activity, with maximum percentage increase in kidney (63%), followed by liver (56%), brain (49%) and muscle (36%) on the seventh day of exposure(Table 39, Fig. 66).

The GDH (R.A.) also increased significantly in different tissues of cortisol-treated fish compared to respective controls with maximum increase in kidney (84%), followed by liver (52%), intestine (30%), brain (24%), stomach (22%) and muscle (21%) (Table 40, Fig. 67). Percentage increase of specific activity of GDH (R. A.) was maximum in kidney (84%), followed by liver (52%), intestine (30%), brain (24%) and muscle (21%) after 120 h (5 days) of cortisol injection (Table 40, Fig. 67). However, the tissue and specific activities of GDH (O. D.) did not show any significant change in any of the tissues of the mud eel following cortisol injection (Table 40, Fig. 68).

The activity of AST and ALT increase significantly in the treated fishes compared to their respective controls. Maximum increase of AST activity was observed in muscle (165%), followed by stomach (105%), kidney (68%) and liver (39%) with no significant change in brain. Percentage change in the specific activity was maximum in stomach (83%), followed by muscle (74%), kidney (70%) and liver (35%) after 5 days of cortisol injection (Table 41, Fig. 69). The activity of ALT increased maximally in muscle (45%) and stomach (44%), followed by kidney (34%) and liver (33%). In brain and intestine no significant change of ALT activity was observed after 5 days cortisol injection. The specific activity of ALT increased maximally in kidney (54%), followed by stomach (38%), muscle (37%) and liver (25%) after 5 days of injection with cortisol (Table 41, Fig. 69).

### *4.5 Changes in the activity of OUC enzymes*:

Out of the five OUC enzymes, the CPS, the rate limiting enzyme of OUC, was found to increase significantly by 37% and 38% in liver and kidney of the mud eel after 5 days of intra-peritoneal injection of cortisol (Table 42, Fig. 70).

### *4.6 Changes in concentrations of different FAAs*:

The concentrations of different FAAs increased significantly in various tissues after 5 days of cortisol injection. In liver, the total concentrations of non-essential FAAs increase significantly by 95% after 5 days of cortisol injection, which was mainly attributable due to the increase in the concentrations of Gln (292%), Tau (122%), Glu (77%) and Ala (54). Significant increase in the concentrations of few essential FAAs was also observed in liver such as Trp (145%), His (119%), Arg (79%), Leu (77%) and Iso (61%), which consequently led to a significant increase in the concentration of total essential FAAs (39%) in liver of injected fish (Table 43, Fig. 71).

In kidney, the concentrations of total non-essential FAAs increased significantly by 73% in the cortisol-injected fish, which was mainly attributable due to increase in the concentrations of Gln (164%), Glu (123%), Asn (76%), Ala (75%), Asp (73%), Gly (52%) and Pro (32%) compared with respective controls. There was no significant change in the concentrations of total essential FAAs, except for change in Val (81%), Iso (82%) and Arg (55%) (Table 43, Fig. 72).

In muscle, the concentrations of total non-essential FAAs increased significantly by 86%, which was mainly due to the increase in the concentrations of Ala (319%), Gln (148%), Gly (140%), Glu (95%), Cit (63%) and Asp (28%). There was also a significant increase in the total concentration of essential FAAs by 39%, which was mainly attributable due to the increase in the concentrations of Ile (211%), His (154%), Thr (125%), Met (70%), Arg (44%), Phe (34%) and Lys (33%) (Table 43, Fig. 73).

In stomach there was significant increase in the concentration of the total non-essential FAAs by 41% due to cortisol injection, which was mainly attributable due to the increase in the concentrations of Gln (177%), Pro (300%), Ala (125%), Tyr (103%), Gly (100%) and Ser (100%), with no significant changes in the concentrations of essential FAAs (Table 44, Fig. 74).

In brain of cortisol-injected fish the total non-essential FAAs increased by 75%, which was mainly attributable due to the increase in the concentrations of Gln (129%), Ala (154%), Asp (146%), Glu (54%) and Tyr (94%); while the concentrations of total essential FAAs did not change in the cortisol-injected fish (Table 44, Fig. 75).

In plasma, the concentrations of total non-essential FAAs increased significantly by 132% after 5 days of cortisol injection, which was mainly attributable due to the increase in the concentrations of Gln and Glu (500% each), Ala (460%), Gly (417%) and Ser (100%). There was also significant increase in the concentrations of total essential FAAs by 39%, which was mainly attributable due to the increase in the concentrations of Thr (125%), Ile (211%), His (154%), Arg (44%), Met (70%) and Lys (33%) after 5 days of cortisol-injection (Table 44, Fig. 76).

### *4.7 Changes of GS enzyme protein*:

As evidenced by the western blot analysis, treatment with cortisol (50mg/ kg body wt) led to a significant increase of GS enzyme protein concentrations in different tissues of the mud eel compared with respective control (Fig. 78). The fold of increase of GS enzyme protein was maximum in liver (1.5 fold), followed by brain (1.3 fold) with no significant changes in other tissues.

# DISCUSSION

## Exposure to high environmental ammonia (100 mM $NH_4Cl$) for 7 days

The mud eel normally lives in muddy ponds, swamps, canals and rice fields (Rainboth, 1996), where it burrows in moist earth in dry season and survives for long periods without water during summer (Davidson, 1975). It would encounter high environmental ammonia concentrations (HEA), which may increase to about 90 mM (Freney *et al*., 1981) during agricultural fertilization in rice fields. Moreover, during drought, water dries up in ponds and rice fields, and it is likely that ammonia would be concentrated in the external medium. Therefore, in the present investigation the mud eel was exposed to 100 mM $NH_4Cl$ for 7 days, and studied the possible strategies to avoid the ammonia toxicity in this mud eel.

One of the strategies adopted by this mud eel to avoid the ammonia toxicity is the capacity to tolerate a very high ambient ammonia. Under laboratory conditions, it has been observed that this mud eel could survive up to 200 mM $NH_4Cl$ without any mortality for at least 2 weeks. In contrast, typical water-breathing fishes have comparatively very low tolerance to external ammonia (for review, see Saha and Ratha, 1998, 2007a). Iwata (1988) reported that when five water-breathing teleosts were exposed to 10 mM $NH_4Cl$, all died within 24 h. However, certain air-breathing catfishes such singhi catfish (*H. fossilis*) and walking catfish (*C. batrachus*) have been reported to tolerate up to 75 mM $NH_4Cl$ concentration in the water for months without any mortality (Saha and Ratha, 1990, 1994, 1998). The high tolerance of the mud eel to environmental ammonia correlates well with conditions of the natural habitats, where it might encounter HEA during application of fertilizer and/or drying up to the external media in dry seasons.

Another possible strategy adopted by this fish to adapt in HEA is to allow high accumulation of ammonia *in vivo* in various tissues while exposing to HEA. Accumulation of ammonia was tissue specific with a maximum accumulation in liver and kidney, followed by muscle, intestine, stomach and plasma, and least in brain (Table 1 and fig.1). This was possibly due to total inhibition of ammonia excretion as a result of decrease or reversal in blood-to-water diffusion gradient of ammonia through gills and skin as reported in walking catfish (*C. batrachus*) (Saha *et al*., 2003), accompanied with the net uptake of ammonia by the mud eel from the HEA. The capacity for ammonia accumulation in this mud eel was found to be much higher than many teleosts, gobiid fishes (Dabrowska and Wlasow, 1986; Iwata, 1988; Jow *et al*., 1999; Iwata *et al*., 2000) and even higher than the two air-breathing catfishes (*H. fossilis* and *C. batrachus*) (Saha and Ratha, 1994; Saha et al., 2003). Although ammonia accumulation was not maximum in muscle under exposure to high environmental ammonia, it appears to be a main storage tissue for ammonia since it constitutes >50% of the body mass. This probably would serve in lowering down the level of circulating ammonia in the blood that resulted from ammonia loading. In this mud eel, about 1.8 fold increase of ammonia concentration was observed from day 3 and continued till 7 days of exposure. Significant accumulation of ammonia in various tissues including plasma, which appears to be much higher in many other teleosts (Dabrowska and Wlasow, 1986; Iwata, 1988; Jow *et al*., 1999; Iwata *et al*., 2000; Ip *et al*., 2004a) during exposure to HEA, is possibly due to net influx of ammonia through the gills as well as through the scale less skin of the mud eel from the external medium. Thus the increase accumulation of ammonia *in vivo* appears to be another possible strategy adopted by the mud eel to tolerate HEA, as observed in the gobiid fish (Iwata *et al*., 2000), swamp eel (Ip *et al*., 2004c), and the oriental weather loach (Tsui *et al*., 2002).

We also looked at one of the most potential mechanisms to avoid ammonia toxicity, such as active conversion of accumulated ammonia to various non-essential FAAs enabling the mud eel to survive in such high ambient ammonia. Interestingly, it was found that there was a significant accumulation of various non-essential FAAs in different tissues and also in plasma of the mud eel exposed to 100 mM $NH_4Cl$. Liver is the most important organ with relation to nitrogen metabolism and also in governing the amino acid homeostasis in fish (Campbell, 1991). Significant increase in the concentration of total non-essential FAAs was observed in the $NH_4Cl$-exposed fish liver (121%

on the fifth and seventh day), 92% of which were mainly attributable to the increase of Asp, Ala, Glu, Gln and Tau on the first day of exposure, and increased further after seven days of exposure. Similar observation was also made in kidney of the mud eel while exposing to HEA for seven days. The total non-essential FAAs increased to approximately 100%, which were attributable mainly to the increase of Asp, Gly, Ala, Glu, Gln, Pro and Tau concentrations, taken together accounted for 69% on day 1 of exposure, which was maximum on day 5 of exposure. It is known that the synthesis of Glu by assimilating ammonia with α-ketoglutarate by GDH (reductive amination) plays a crucial role in preventing ammonia reaching to a toxic level that is generated endogenously from the catabolism of amino acids and tissue proteins in various tissues including the brain (Cooper and Plum, 1987; Jürss and Bastrop, 1995). In this fish, the activity of GDH enzyme in the ammonia utilizing direction was found to be approximately 1.5-2 fold higher in liver, kidney and stomach of treated fish comparable to other ammoneotelic teleosts (Wilson, 1973; Chew and Ip, 1987; Iwata, 1988; Walton and Cowey, 1977; Peng *et al.*, 1998). Non-essential FAAs were also reported to increase significantly in different non-ureogenic ammoniotelic teleosts such as the carp (Dabrowska and Wlasow, 1986), mudskippers (Iwata *et al.*, 1981; Iwata and Deguchi, 1995; Ip *et al.*, 1993; Kok *et al.*, 1998; Peng *et al.*, 1998), goldfish (Levi *et al.*, 1974), Indian air-breathing catfish (Saha *et al.*, 2002b) under hyper-ammonia stress.

The activities of all the amino acid metabolism related enzymes in muscle were comparatively lower than other tissues. However, considering its size, muscle is the main organ for ammonia detoxification as suggested in another teleost fish such as walking catfish (Saha *et al.*, 2002a). The mechanism(s) by which these enzymes are induced in this fish under hyper-ammonia stress cannot be explained at this moment with the available data. The increase in ammonia concentration in muscle accompanied by approximately 2 fold increase in the concentrations of total non-essential FAAs as seen on 7 days of $NH_4Cl$ exposure, was mainly due to increase of Asp, Gly, Ala, Glu and Gln concentrations, which together accounted for more than 84% increase.

Significant increase in concentrations of non-essential FAAs was also recorded in plasma of the $NH_4Cl$-exposed fish. It was probably due to the inter-organ transportation of amino acids synthetised in other tissues during high ammonia loading.

The increase in concentrations of various non-essential FAAs in different tissues was probably due to upregulation of the amino acid metabolism related enzymes such as GS, GDH (reductive amination), AST and ALT. The basal levels of activity of these key amino acid metabolism-related enzymes were also quite high in this fish as compared to many ammoneotelic teleosts including the gobiid fishes (Wilson, 1973; Waarde and Kesbeke, 1982; Chew and Ip, 1987; Iwata, 1988; Walton and Cowey, 1977; Peng *et al*., 1998; Anderson, 2001; Lim *et al*., 2001), with further simulation in their activities in different tissues of $NH_4Cl$-treated fish. It is believed that the higher accumulation of ammonia in different tissues in HEA is the possible cause of induction of activities of these enzymes in the mud eel as suggested in the walking catfish (Saha *et al*., 2002b).

Further, parallel to the increase of Gln concentration, GS activity also increased significantly in ammonia-treated fish. The western blot analysis in the present study certainly indicated that the increase in GS activity was mainly due to upregulation of GS enzyme protein concentration in the mud eel while exposing to HEA, since the fold of increase of enzyme activity was almost equal to increase in enzyme protein concentration. Therefore, it is believed that the induction of GS activity in this fish is primarily by translational activation, and possibly also by transcriptional activation during exposure to HEA. This is in contrast to the situation in some non-ureogenic teleosts (Levi *et al*., 1974; Arillo *et al*., 1981; Peng *et al*., 1998) and ureogenic marine toad fishes (Wang and Walsh, 2000), where GS activity did not increase in HEA, although there was an increase of Gln level. However, similar to the present observation in the mud eel, significant increase of GS activity, GS protein and/or GS mRNA levels in different tissues has been reported during exposure to HEA in the walking catfish (Saha *et al*., 2007b), in the sleeper

(Anderson et al., 2002), and in the marble goby (Iwata *et al*., 2000). Whether the GS of this mud eel could additionally be regulated by allosteric modification of pre-existing enzyme needs to be investigated, although there was a suggestion that the fish GS is normally not regulated by this process (for a review, see Mommsen *et al*., 1999). Recently, possession of multiple genes for GS and their variable levels of tissue expression in adults (Murray *et al*., 2003) and in early developmental stages (Essex-Fraser *et. al*., 2005) of rainbow trout (*O. mykiss*), in the adults of Gulf toadfish (Walsh *et al*., 2003) have been reported. It is more likely that the mud eel also possesses multiple GS genes with the possibility of different levels of tissue expression under HEA, since the increase of GS activity varied among different tissues. However, a detailed study must be considered to know the possible occurrence of multiple GS genes and their possible transcriptional activation under hyper-ammonia stress.

The liver perfusion experiment provided evidences of enhancing the capacity of incorporation of more exogenous ammonia-N to Gln-N and to a lesser extend to urea-N by the ammonia-treated fish liver as supported by the fact that the efflux of ammonia-N, which was 69% of the total-N infused in the control fish liver, was reduced to 33% and the fluxes of Gln-N and urea-N increased from 19 to 167% and 12 to 15% respectively, in ammonia treated fish without causing any change of efflux of total-N (Table 2 and fig. 3). It was believed that this was mainly because of upregulation of GS enzyme activity.

The brain is the most sensitive organ to ammonia toxicity (Cooper and Plum, 1987). The most efficient way of ammonia detoxification in vertebrate brain is to convert ammonia to Gln by the enzyme GS (for review, see Anderson, 2001), and like most other fishes, brain tissue of mud eel was found to have an exceptionally high level of GS activity. Unlike that of other fishes, brain of the mud eel accumulated quite a bit of ammonia in HEA. The mode of ammonia detoxification in vertebrate brain was suggested to be through Glu and subsequent Gln synthesis through the coupled steps of GDH and GS (Cooper and Plum, 1987; Mommsen and Walsh, 1991; Wright and

Wright, 1996). The activities of these two enzymes in brain of the mud eel were very high, and ammonia did not induce further in their activities, which suggest that their activities are always at the saturation level. Due to these two reasons, it is believed that this fish has a higher tolerance limit to ammonia loading than other fishes. The high levels of activity of these two enzymes led to fixing the loaded ammonia into non-essential FAAs such as Glu and Gln and some other non-essential FAAs, which were seen to increase linearly during the 7 days of treatment. Increase in Gln concentration in brain due to exposure to HEA has also been reported in some non-ureogenic teleosts like rainbow trout (*Oncorhynchus gairdneri*) (Arillo *et al*., 1981), goldfish (*Carassius auratus*) (Levi et al., 1974) and common carp (*Cyprinus carpio*) (Dabrowska and Wlasow, 1986). Although the Gln concentration in the muscle of ammonia-treated mud eel increased by 5.5 fold, it remained lower than in brain, liver and kidney. However, the overall capacity for ammonia storage as Gln in muscle would be much higher than in other tissues considering that muscle comprises >50% of the total body mass.

Thus, it is evident from this experiment that active synthesis and accumulation of various non-essential FAAs in the mud eel play a significant role for the detoxification of accumulated ammonia under hyper-ammonia stress. Further, it has the capacity to accumulate ammonia in different tissues and also in plasma as another strategy to tolerate such a high ambient ammonia. Although a full compliment of OUC enzymes were detected in liver and kidney of the mud eel (Table 8 and fig. 14), the levels of activity of all the five OUC enzymes were found to be quite low as compared to other ureogenic teleosts (Saha and Ratha, 1989; Mommsen and Walsh, 1989; Randall *et al*., 1989; Iwata *et al*., 2000). Further, there was no significant increase of activities of any of the OUC enzymes in HEA as reported in two air-breathing catfishes (Saha and Ratha, 1994; Saha *et al*., 1995, 2003, 2007b), with no significant change in the levels of urea-N in any of the tissues of the mud eel (Table 1 and fig. 2). Thus, it appears that the conversion of ammonia-N to urea-N via the OUC is not the major strategy adopted by this mud eel to tackle the problem of ammonia toxicity in HEA.

Exposure to HEA also led to upregulation of hsp70 expression (Fig. 22), the major molecular chaperone, in the mud eel probably to defend against the ammonia-mediated stressors. As has been discussed earlier, ammonia gets diffused inside body of the fish during exposure to HEA and has various toxic effects particularly causing ionic and acid-base imbalances in different cell types. All these would result in the mis-folding, unfolding or denaturation of protein molecules. The hsps are self protective proteins that maintain cell homeostasis against various forms of stress as an adaptive response (Lindquist and Craig, 1988), and play some vital roles inside the cell including the maintenance of protein integrity, preventing premature folding and aggregation of proteins, protein translocation, and mediating steroid and receptor binding (Iwama *et al*., 1999). Therefore, the increase in the concentration of hsp70 in different tissues of the ammonia-treated fish observed in the present study is believed to be primarily for preventing the ammonia-mediated cytotoxic effects during exposure to HEA.

Thus, it can be concluded that the mud eel is able to tolerate very high ambient ammonia that it faces in its natural habitat by adopting the strategies of (i) allowing the *in vivo* accumulation of ammonia in different tissues, and (ii) active conversion of accumulated ammonia to various non-essential FAAs and to store as such in different tissues. The conversion of ammonia to urea via the active ureogenesis, as has been reported in some Indian air-breathing catfishes, is not possible in this mud eel to neutralize the ammonia toxicity. Further, the higher expression of hsp70, the major molecular chaperone, probably helps this mud eel to defend the ammonia toxicity and to adapt in a better way in HEA.

## Aerial exposure experiment

Air breathing is one of several adaptive responses utilized by fishes that dwell in habitats where $O_2$ supplies may be severely depleted (Sayer and Davenport, 1991). The tropical aquatic environment is warm and often hypoxic; hence, air breathing is a common occurrence in tropical fishes (Graham, 1997b). Many tropical fishes have evolved mechanisms to deal with ammonia toxicity during aerial exposure (Ip *et al*., 2001b; Chew *et al*., 2005). These mechanisms include the active excretion of ammonia ($NH_4^+$) (Randall *et al.,* 1999*;* Ip *et al.,* 2004d), prevention of ammonia production in general through a reduction in amino acid catabolism and/or partial amino acid catabolism leading to the formation of Ala (Ip *et al*., 2001c, 2005c; Loong, 2005), the conversion of ammonia to less toxic compound such as Gln (Ip *et al*., 2004d) or urea (Jenssens and Cohen, 1968; Saha and Ratha, 1994; Saha *et al*., 1995, 2001, 2003), and excretion of ammonia into air by volatilization (Frick and Wright, 2002; Tsui *et al*., 2002). All these mechanisms can be found in various species of tropical air-breathing fishes, which can survive on land for various periods without water. The responses of tropical fishes to impediment of ammonia excretion on land are many and varied, determined by the behavior of the fish and the nature of environment in which they live (Ip *et al*., 2004a).

In the tropics, the capacity for air breathing facilitates the exploitation of terrestrial environment by many fish species in certain extreme cases- e.g. the weather loach (Chew *et al*., 2001) and the mud eel (Graham, 1997b). They are capable not only of locomotion on land (in normoxia) but also of burrowing into the mud and surviving for a long period during drought. As mentioned in the introduction, mud eel lives in muddy ponds, swamps, canals and rice fields, where it burrows in the moist earth in dry season, surviving for a long period without water during summer (Shih, 1940; Davidson, 1975). During prolong drought, it burrows deeper into the mud to remain in contact with the water table (Liem, 1987). The mud eel, while living in the air or inside the mud during drought, will face the problem of ammonia toxicity since ammonia excretion by the gills and scale less cutaneous surface is not possible without the availability of sufficient water in the surrounding habitat. Furthermore, most teleost fishes depend mainly on catabolism of tissue proteins and amino acids for energy generation. Therefore, ammonia is being continuously generated inside their body through the process of transdeamination mainly in liver (Wilkie, 1997). Therefore, the present investigation was aimed at determining the pattern of nitrogen excretion and also the handling of toxic ammonia in the mud eel while living in air for 6 days.

The mud eel is ammoniotelic in water excreting ammonia as the major nitrogenous end product (Table 14). The total nitrogen excretion (ammonia-N and urea-N) by the fish under control conditions averaged to be about 58 µmoles/kg/h, out of which 88% was excreted as ammonia-N and 12% as urea-N. Whereas, the ammonia-N excretion by the mud eel, while keeping outside the water, found to reduce significantly with 30% reduction in the first day itself, followed by further decrease to about 62% on the sixth day. Aerial exposure also affected the rate of urea-N excretion, which decreased to about 34% on the first day of exposure and continued till the third day, after which it is more or less the same as control. The decrease of ammonia-N excretion was accompanied by significant accumulation of ammonia-N in different tissues of the mud eel during exposure to air, with maximum accumulation in liver, kidney and muscle, followed by intestine, stomach, brain and plasma (Table 15). This is a unique phenomenon, because in mammals, the brain ammonia level of >1 µmole/g wet wt leads to encephalopathy (Brusilow, 2002; Felipo and Butterwoth, 2002; Rose, 2002). By contrast, ammonia in different tissues of the mud eel built up to very high levels upon aerial exposure reaching about 42 µmoles/g wet wt in liver, kidney and muscle, 25 µmoles/g wet wt in brain, 5 µmoles/ml in plasma. It would appear that resistance to aerial exposure for longer duration by the mud eel is associated with its high tolerance of ammonia at the cellular and sub-cellular levels. To our knowledge, this is the only fish which can accumulate ammonia in plasma to such a high level while on land without any feasible damage of ammonia toxicity. The mud eel muscle attributes by > 50% of the total body weight and the level of ammonia increased significantly by 76% in muscle after 6 days of aerial exposure, thereby suggesting that major ammonia accumulated in muscle tissue. However, how the cells and tissues of the mud eel tolerate such a high ammonia levels is not clear at present. Whereas, when we looked at the accumulation of urea-N level in different tissues of the mud eel during aerial exposure, no significant changes were observed in any of the tissues and plasma (Table 15). This indicated that conversion of ammonia to urea, as reported in Indian air-breathing catfishes (Saha *et al*., 2001, Datta, 2006), is not the major strategy adopted by the mud eel to avoid the accumulation of ammonia to a toxic level during aerial exposure.

The possible conversion of the accumulated ammonia to some non-essential FAAs was also looked into as another major strategy adopted by the mud eel during aerial exposure. The pattern of changes in the levels of different FAAs in different tissues of the air-exposed fish was almost similar to the $NH_4Cl$-exposed fish along with the changing pattern of all the amino acid metabolism-related enzymes. There was significant accumulation of various non-essential FAAs in different tissues and plasma of the mud eel while exposing to air for 6 days. The cause of enhancement of synthesis and accumulation of non-essential FAAs during aerial exposure would also probably be the same with $NH_4Cl$-exposed fish, i.e., to avoid the *in vivo* accumulation of ammonia to a lethal concentration. In liver, the concentration of the total non-essential FAAs increased significantly by 80% on the first day of exposure, which was mainly attributable due to the increase of Asp, Gly, Ala, Glu, Gln, Pro concentrations, followed by further increase by 125% on the fifth day. Similarly, in kidney of mud eel, the concentrations of total non-essential FAAs increased approximately to by 100%, 90% of which was mainly attributable due to the increase in concentrations of Asp, Gly, Ala, Glu, Gln and Pro.

The increase in the concentrations of FAAs was accompanied with increase in activity of certain key amino acid metabolism-related enzymes such as GS, GDH, AST and ALT in different tissues of the mud eel (Tables 17-21 and Figs. 27-36). The activity of GS, which assimilate ammonia with Glu to form Gln, increased significantly in all tissues of this fish during aerial exposure for 6 days. Ammonia toxicity can be avoided by converting ammonia to Gln, since formation of one mole of Gln molecule assimilates two ammonia molecules (Campbell, 1973). One mole of ATP is required for the production of every amide group of Gln via GS, whereas 2.5 moles of ATP is needed for every mole of nitrogen detoxified via the OUC (Ip, *et al*., 2001a). Hence, Gln synthesis would be more efficient than ureogenesis in term of energy expenditure. Moreover, excretion of urea requires water to flush out from the branchial and cutaneous surfaces, whereas Gln can be retained inside the body and the requirement of water does not arise. In addition, Gln, stored within the body after its synthesis, can be used for the anabolism of purine, pyrimidine etc. when favourable environmental condition prevails (Tay *et al*., 2003). Gln has been found to play a significant role in the detoxification of endogenously generated ammonia in different fish species exposed to air for different time periods (Chew *et al*., 2001; 2003a). There was a significant accumulation of Gln in all tissues including plasma of the mud eel exposed to air with maximum accumulation in liver, followed by brain, plasma, stomach, and muscle. Even though, Gln concentration in muscle is lower than that in other tissues, but the capacity of total storage of Gln in muscle would be much higher than in other tissues, as it constitutes >50% of the total body mass. Therefore, muscle acts as a good reservoir for Gln accumulation in the mud eel, as suggested in the walking catfish (Saha *et al*., 2002b), marble goby (*O. marmoratus*) and sleeper (*B. sinensis*) (Jow *et al*., 1999; Ip *et al*., 2001b; Anderson, *et al*., 2002). The brain of the mud eel has the highest activity of GS as compared to other tissues studied, and this would probably contribute to the extreme tolerance to high ammonia concentration in the body. The Gln content in brain of the mud eel reached up to 8.55 µmoles/g wet wt after 6 days of aerial exposure. Ironically the high Gln synthesis and storage in the brain contribute to toxicity, causing brain ATP and α-KG depletion (Campbell, 1973; Cooper and Plum, 1987), and astrocyte cell volume increase, leading ultimately to brain edema and death (Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002).

How the brain of fish tackles this problem of high Gln accumulation is unknown, but it opens up for future studies. Elevated tissue ammonia levels are associated with increased Gln levels in brains of many vertebrates (Ip, *et al*., 2001b; Brusilow, 2002). A similar phenomenon of ammonia detoxification to Gln on aerial exposure has also been reported in loach (*M. anguillicaudatus*) (Chew *et al*., 2001), sleeper (*B. sinensis*) (Ip, *et al*., 2001b), swamp eel (*M. albus*) (Tay *et al*., 2003), lungfish (*P. dolloi*) (Chew *et al*., 2003), African sharptooth catfish (*C. gariepinus*) (Ip, *et al*., 2005), and walking catfish (*C. batrachus*) (Saha *et al*., 2007b). Thus, it appears that Gln formation is adopted as one of the important strategies to defend against endogenously generated ammonia in the mud eel.

The liver perfusion experiment provided further evidences of enhancing the capacity of incorporation of more endogenously built ammonia-N to Gln-N and to a lesser extent to urea-N by the air-exposed fish liver as supported by the fact the efflux of ammonia-N, which was 70% of the total-N infused in the control fish liver, was reduced to 28%, and the fluxes of Gln-N and urea-N increased from 19% to 55% and 12% to 14%, respectively, in air-exposed fish without causing any change of total-N (Table 16 and Fig. 26). It is believed that this was mainly because of upregulation of GS enzyme activity.

The activity of GDH in ammonia utilizing direction was found to be very high in this fish, which further enhanced by 88% and 80% in liver and kidney, respectively. Glu formed by GDH can also undergo transdeamination with pyruvate, catalyzed by ALT producing α-KG without the release of ammonia (Ip *et al*., 2001c). In the mud eel, the concentration of Ala increased significantly in liver, kidney and muscle (94%, 104%, and 132%, respectively) on the first day of exposure, accompanied by the increase in activity of the enzyme ALT in these tissues. Thus, the formation of Ala or partial amino acid metabolism without releasing of ammonia could be one possible strategy to lower down the concentration of endogenously build ammonia concentration in this mud eel during aerial exposure as suggested in *C. asiatica* during 48 h of aerial exposure

(Chew et al., 2003b). This would allow the utilization of certain amino acids as energy sources, and at the same time minimize the ammonia accumulation in this fish. Further, the AST activity was also upregulated by aerial exposure. The increase in the activity of all these enzymes helps in fixing the accumulated ammonia to Glu, Gln, Ala and others non-essential FAAs under the ammonia-stress. In order to slow down the buildup of tissue ammonia concentrations during decrease ammonia excretion on land, fish can decrease the rate of ammonia production by reducing the rate of amino acid catabolism. The steady state concentrations of FAAs in tissues are maintained by the balance between the rate of anabolism and catabolism of tissue proteins and amino acids. Any alteration of these two rates would lead to changes in the concentrations of FAAs, and the ability to alter these rates is important for a fish that is often exposed to terrestrial condition, because it slows down the buildup of endogenous ammonia (Ip *et al*., 2004a). It was earlier suggested that the mudskippers reduce the rate of nitrogen metabolism in response to aerial exposure (Gordon *et al*., 1969; 1970; 1978), although they did not provide any data to support this proposition. In contrast, Ip *et al*. (1993) observed an increase in total FAA levels in different tissues of *P. schlosseri* after 24 h of aerial exposure but not in those of *B. boddaerti*. They attributed this difference between the two species of the mudskippers to the greater activity of *P. schlosseri* on land. In addition, they speculated that *P. schlosseri* increased the rate of amino acid catabolism to sustain its activity on land. However, Lim *et al*. (2001) suggested a reduction in the rates of protein and amino acid catabolism to slow down the accumulation of endogenous ammonia in two mudskippers rather than enhanced synthesis during aerial exposure. However, with the present data it is difficult to say whether the protein and amino acid catabolism went down in the mud eel during aerial exposure.

The western blot analysis certainly indicated that the GS enzyme protein concentration in different tissues of mud eel increased significantly by 1.6-3.0 fold after 6 days of aerial exposure, which was parallel to the increase of GS enzyme activity. Therefore, it is believed that the induction of GS activity in this fish is primarily by translational activation, and possibly also by transcriptional activation during aerial exposure as suggested during HEA exposure. The cause of induction is probably the same, i.e. higher accumulation of ammonia *in situ*, which was generated endogenously during aerial exposure. Further, it is suggestive that the mud eel possesses more than one mRNA transcripts for GS and that probably leads to synthesis of more than one isoforms of GS enzyme as evidenced from the western blot analysis. In muscle, there were two bands for GS both in control and air-exposed fish (Fig. 44), which was accompanied with different levels of tissue expression during aerial exposure since the increase of GS activity varied among different tissues.

Exposure to air for 6 days also led to upregulation of hsp70 expression (Fig. 45), the major molecular chaperone, in the mud eel probably to defend against the ammonia-mediated stressors that resulted due to accumulation of endogenously built ammonia and also due to dehydration stress. All these would result in the mis-folding, unfolding or denaturation of various stress-sensitive protein molecules. The hsps are self protective proteins that maintain cell homeostasis against various forms of stress as an adaptive response (Linquist and Craig, 1988), and play some vital roles inside the cells including the maintenance of protein integrity, preventing premature folding and aggregation of proteins, protein translocation, and mediating steroid and receptor binding (Iwama *et al*., 1999). Therefore, the increase in the concentration of hsp70 in different tissues of the air-exposed fish, observed in the present study, is believed to be primarily for preventing endogenously built ammonia-mediated cytotoxic effects and also to prevent the dehydration mediated stressors during aerial exposure.

Thus, it can be concluded that the mud eel is able to survive for longer duration either in the air or inside the mud in semidry conditions by adopting the strategies of (i) allowing the *in vivo* accumulation of ammonia in different tissues that is generated endogenously, (ii) the formation of Ala or partial amino acid metabolism without releasing ammonia, and (iii) active conversion of accumulated ammonia to various non- essential FAAs and to store as such in different tissues. The conversion of ammonia to urea via the OUC is not adopted in this fish during aerial exposure. Further, the higher expression of hsp70, the major molecular chaperone, probably helps this mud eel for better adaption to desiccation stress.

## Ammonium bicarbonate injection experiment

In the first experiment, the external ammonia got loaded inside the body of fish by reverse partial pressure of external ammonia, and in the second experiment the excretion of endogenously generated ammonia was impeded due to lack of water in the surrounding medium to flush the branchial and the scale-less cutaneous surfaces. In both cases, ammonia concentration built up inside the body of stressed fish. In the former case, the fish encountered retention of endogenously generated ammonia and loading from the external source, while in the latter case, they encountered only with retention of endogenously generated ammonia, which accumulated inside the body due to impediment of its excretion. In contrast, in this experiment, ammonia in the form of $NH_4HCO_3$ was injected directly in the peritoneal cavity of the mud eel. The idea was to increase the level of ammonia concentration artificially inside the body of fish and aimed at investigating the role of endogenous ammonia in controlling the nitrogen metabolism in this fish. Further, bicarbonate of ammonia was used instead of $NH_4Cl$, because it can be utilized in the OUC pathway. Since the injected fishes were again kept back in water, ammonia excretion would not be impeded, as the partial pressure was greater in the outwardly direction.

The total-N (ammonia-N and urea-N) excreted by the mud eel under normal physiological condition averaged to be 53.24 µmoles/kg body wt over a period of 24 h, out of which ammonia-N excretion accounted for 89% (47.35 µmoles/kg body wt) and urea-N 11% (5.89 µmoles/kg body wt), suggesting again that the mud eel is ammoniotelic while living in the normal aquatic habitat. However, significant increase in excretion of ammonia-N (24%) was observed after 6 h post injection and increased further up to 37% after 24 h, and urea-N excretion during these periods was not significantly increased. The conversion of loaded ammonia to urea was not possible via the existing OUC in liver and kidney to avoid the toxic effects of the accumulated ammonia since the activities of OUC enzymes are as such low with no further induction of activities of any of the enzymes by ammonia injection (Table 33, fig. 53). Thus, the nitrogen excretion pattern did not change from ammoniotelism to ureotelism as reported in the Indian air-breathing catfishes which have relatively high activity of the OUC enzymes in the hepatic and non-hepatic tissues (Saha and Ratha, 1989; Saha *et al*., 1999; 2003). Thus, this experiment again proves that the conversion of ammonia-N to urea-N via the OUC is not the major strategy adopted by this mud eel to tackle the problem of ammonia toxicity.

Several recent studies, however, have suggested that ureotelism is not the universal response of fishes to survive under various adverse ecological conditions that interfere with ammonia excretion (Ip *et al*., 2004a). Few more strategies to ameliorate the ammonia toxicity, either generated endogenously or coming from the external sources, have been suggested by various workers in certain fishes and mentioned in earlier chapters. The mud eel, when confronted with intra-peritoneal injection of $NH_4HCO_3$, did not detoxify accumulated ammonia to urea, instead ammonia was excreted as such in the surrounding medium by simple diffusion from their branchial and scale-less cutaneous surfaces. Further, since not all ammonia injected was excreted out by the fish during 24 h, the majority of ammonia was retained inside the body of the fish. As has been discussed in the previous experiments, this fish has a very high tolerance to accumulated ammonia at cellular and sub-cellular levels. Hence, the injected ammonia retained by the fish was apparently assimilated with other defense mechanisms for detoxification such as active conversion to non-essential FAAs by upregulating various amino acid metabolism-related enzymes to prevent the accumulation of ammonia to a lethal level.

$NH_4HCO_3$ injection significantly increased the activity of various amino acid metabolism related enzymes such as GS, GDH, AST and ALT. The GS activity was upregulated in all tissues of the mud eel except in brain, where its activity was already at a very high level. The maximum induction was recorded in stomach of the treated fish after 24 h of injection, increasing from 88.76 µmoles/g wet wt to 219.97 µmoles/g wet wt (148%), followed by kidney from 49.59 µmoles/g wet wt to 96.00 µmoles/g wet wt (94%), muscle from 26.16 µmoles/g wet wt to 45.88 µmoles/g wet wt (73%), and liver from 28.3 µmoles/g wet wt to 48.88 µmoles/g wet wt (73%). As evidenced by the western blot analysis (Fig. 61), the upregulation of GS activity in different tissues of the treated fish was accompanied by 1.5 to 2.0 fold increase of the GS enzyme protein concentration in liver, kidney, stomach and muscle as compared to respective controls. Corresponding to the increase in the GS enzyme activity, the concentration of Gln also increased significantly in all tissues including plasma (324%) of the injected fish. The accumulation of Gln in plasma was maximum, followed by liver (190%), brain (138%), stomach (94%), kidney (91%) and muscle (88%). From the result, it was observed that Gln concentration in muscle was the least, but again as discussed earlier, considering its mass, much of Gln was safely stored in this tissue. Thus, the upregulation of GS activity helped to convert much of the injected ammonia to Gln for safe storage in muscle and other tissues, which appears to be one of the major strategies adopted by the mud eel to avoid the accumulation of toxic ammonia to a lethal level. In addition, the perfusion experiment provided evidence of the enhanced capacity of GS to incorporate more exogenous ammonia-N to Gln-N in liver from the treated fishes (Table 29). This was mainly due to upregulation of GS activity induced by the injected ammonia in the liver of the fish.

Significant increase in tissue concentrations of various non-essential FAAs was also observed in different tissues of treated fish. The total concentrations of non-essential FAAs increased significantly in liver (96%) after 24 h, followed by kidney (60%), plasma (59%), stomach (38%) and brain (32%). The increase was mainly attributable to the increase in concentrations of Asp, Ala, Ser, Glu, and Gln, which together accounted for about 63% of the total FAAs in liver of the treated fish. Similarly in kidney, plasma, stomach and brain the increase in the total concentrations were due to the increase of these few non-essential FAAs. These results indicated that a decrease in the rate of FAA catabolism, as suggested by Ip *et al*. (2003) in the swamp eel accompanied with the increasing rate of their synthesis by the increase in the activity of the amino acid metabolism related enzymes apart from GS such as GDH (in ammonia utilizing direction), AST and ALT almost in all tissues studied. The GDH activity in the reductive amination direction (forming Glu from α-KG and $NH_4^+$) increased significantly in kidney and liver of the treated fish after 24 h (Table 31). Iwata *et al*., (1981) reported a significant increase in GHD activity (assayed in the Glu forming direction) in the mud-skipper (*P. schlosseri*) under increase ammonia content. Peng *et al*., (1998) also reported significant increase of GDH (reductive amination) and ALT activities in liver and brain of *P. schlosseri* under hyper-ammonia stress. Accompanied with the increase of activity of GDH (ammonia utilizing direction), the ALT activity was also upregulated by the injected ammonia in this fish.

Thus, it is clear from this experiment that the extra loading of ammonia in different tissues of the mud eel is the major cause of upregulation of GS enzyme at the translational level and possibly also at the transcriptional level along with induction of activities of other amino acid metabolism related enzymes, which may involve in detoxification of endogenously built ammonia. This probably results in detoxification of ammonia to Gln and other non-essential FAAs as one of the major strategies to handle the ammonia toxicity in this fish. Further, it is clear from this experiment that ureogenesis via the OUC does not play any role in detoxification of ammonia to urea in the mud eel.

## **Cortisol injection experiment**

Cortisol is the metabolic hormone in fish (Vijayan *et al*., 1991; 1994) and limited investigations have reported that it increases the metabolic rate of fish (Chan and Woo, 1978). The circulating cortisol level in teleost fishes rises drastically during exposure to stress and is the major regulator of intermediary metabolism and normal physiological parameter (Mommsen *et al*., 1999). Therefore, it is one of the most commonly used indicators of stress in fish. Certain studies, have also demonstrated that cortisol might contribute to the regulation of ureogenesis in fish (for review, see Mommsen *et al*., 1999), and suggested a link between the hormone and the OUC (Vijayan *et al*., 1996). Increase in plasma cortisol level during confinement/crowding has been suggested to be the primary cause of induction of GS enzyme in the gulf toadfish (Hopkins *et al*., 1995). Similarly, the intraperitoneal injection of cortisol stimulated the *in vivo* GS activity in sea-raven (*Hemitripterus americanus*) (Vijayan *et al*., 1996), tilapia (Mommsen *et al*., 2003a) and rainbow trout (McDonald and Wood, 2004). A sharp rise in the plasma cortisol level was also observed in the walking catfish within 24 h of HEA exposure, which could be as a result of high ammonia stress as a consequence of ammonia accumulation in body tissues and plasma (Saha *et al*., 2007b). Moreover, amino acids are known to be the main catabolic substrates for the energy generation in teleost fish (van Waarde, 1983), and in the process of amino acid catabolism ammonia is generated by transdeamination. Hence, the main objective in this study was to investigate the effect or the involvement of cortisol on the pattern of nitrogen metabolism and induction of various enzymes related to nitrogen metabolism in the mud eel.

The total-N excretion (ammonia-N and urea-N) by the mud eel in control condition averaged to be about 58.64 µmoles/kg body wt/day over a period of 5 days, out of which 82% (48.35 µmoles/kg body wt/day) was excreted as ammonia-N and 18% (10.3 µmoles/kg body wt/day) as urea-N, thus suggesting again that this fish is ammoniotelic in nature while living in normal aquatic habitat as suggested in other teleosts (for review, see Wilkie, 1997). However, ammonia-N excretion by the cortisol-injected fish increased significantly up to 27% after 12 h and further increased after 48 h and 72 h of injection accounting for about 61% and 59%, respectively. The urea-N excretion increased to some extend following injection of cortisol. As a result, the total-N excretion increased significantly following 120 h of cortisol injection. This indicated that nitrogen catabolism increased by the cortisol treatment as suggested by Mommsen *et al*., (2003) leading to an increase in nitrogen turn-over. Elevation of endogenous cortisol in fishes was reported to enhance hepatic gluconeogenesis from amino acids (Freeman and Idler, 1973; Whiting and Wiggs, 1977; Chan and Woo, 1978; Leach and Taylor, 1982; Davis *et al*., 1985; Vijayan *et al*., 1996; 1997). Cortisol also elevates plasma amino acids rapidly and is likely to act through elevated proteolysis in liver being the primary target for cortisol-mediated release of amino acids (Milligan, 1997). Consequently, in many teleosts, a rise in endogenous cortisol is reported to elevate in circulating glucose and an increase in ammonia production and excretion (Chan and Woo, 1978).

Accompanied with enhanced rate of excretion of ammonia-N, there was significant accumulation of ammonia-N almost in all tissues of the cortisol-treated fish. The ammonia-N concentration increased maximally in liver (85%), followed by kidney (83%), muscle (45%) and stomach (24%). Urea-N was also found to increase significantly in muscle (55%), kidney (50%) and liver (47%) with slight increase of urea-N excretion rate. All these indicated that the N turn-over rate increased significantly in the mud eel due to cortisol injection. Recent evidences indicated that cortisol may contribute to the regulation of urea production in fish (Hopkins *et al*., 1995; Vijayan e*t al*., 1996; for review see Mommsen *et al*., 1999). Vijayan *et al*., (1996) showed that cortisol enhances liver arginase activity in ammoniotelic marine sea raven (*H. americanus*) with a 3 fold increase in plasma urea concentration, and suggested a link between the hormone and the OUC. Rainbow trout (*O. mykiss*), treated with cortisol, had a 3 fold elevation in plasma and urine urea concentrations, and a 2-3 fold elevation in branchial and urinary urea excretion rates. Similarly, Cortisol injection also stimulated the ureogenesis in the walking catfish (Dutta, 2006).

However, in the mud eel, where very low level of activities of different OUC enzymes was reported, cortisol injection could not cause any significant induction in any of the OUC enzymes. In rats (mammal), treatment with cortisol caused an upregulation of three out of five OUC enzymes, viz. CPS, ASS, and ARG (Christowitz *et al*., 1981). Hence, it can be suggested that cortisol treatment affected the nitrogen metabolism in general and amino acid metabolism in particular that in turn would result in increased availability of amino acid-derived carbon for oxidation or anabolic pathways in this fish.

Cortisol exerts a proteolytic role, especially on the fish white muscle and possibly in the liver as part of its wide spread catabolic activities (Barton *et al*., 1987; Vijayan *et al*., 1997). The plasma amino acid concentration tends to increase in fish implanted with cortisol (Andersen *et al*., 1991; Vijayan *et al*., 1997). In the present investigation, it was noticed that intra-peritoneal injection of cortisol caused significant increase of different non-essential FAAs in all the tissues including plasma of the mud eel with the maximum percentage increase in plasma (132%), followed by liver (95%), muscle (86%), kidney (76%)  and brain (75%,). In liver, the increase of non-essential FAAs was mainly due to the increase of Gln, Glu, Ala and Tau, which together contributed about 84% of the total increase. The amino acid concentrations are influenced by the two processes, viz., their catabolism and synthesis. The levels of FAAs concentration can be increased by the enhanced rate of proteolytic process of tissue proteins by cortisol as suggested in fish muscle (Barton *et al*., 1987). The catabolic pathways lead to the production of ammonia and flux of amino acid carbons into glycogen and gluconeogenesis under the influence of the hormone resulting in the reduction of their concentrations. Conversely, as observed in this fish, the plasma and other tissue concentrations of non-essential FAAs increased significantly, thereby suggesting that probably the rate of synthesis out balanced their rate of catabolism under the influence of exogenous cortisol. However, the rate of proteolysis might had also increased as supported by the fact that the concentrations of total essential FAAs also increased significantly in liver, muscle and plasma. Liver has been suggested to be the primary target of cortisol-mediated release of amino acids (Milligan 1997). Same might be true in the case of the mud eel. Though muscle proteolytic activity is also affected by cortisol (Barton *et al*., 1987), but it is characterized by slow metabolic rate (Mommsen *et al*., 1999) as compared to other tissues such as liver. But the role of cortisol on proteolysis is based on numerous indirect observations, mainly plasma amino acid concentrations (Vijayan *et al*., 1997), and very little piscine evidences have been proposed in direct support of its role (Mommsen *et al*., 1999). Moreover, it is rather difficult to establish its proteolytic role experimentally, unless protein and amino acid turnover are investigated, which till date only one study has been done in this direction (Andersen *et al*., 1991).

Increase in the levels of FAAs concentrations, following cortisol injection, was accompanied with significant increase in the activity of some key amino acid metabolic enzymes especially involving the processing of Glu such as GS, AST, ALT and GDH (ammonia utilizing direction). Significant increase in GS enzyme activity was observed in various tissues such as liver (90%), kidney (77%), muscle (103%) and brain (53%). Cortisol has been shown to stimulate the hepatic GS enzyme activity in the ureogenic toadfish (*O. beta*) (Hopkins *et al*., 1995), ammoniotelic sea raven (*H. americanus*) (Vijayan *et al*., 1996), tilapia (*Oreochromis niloticus*) (Mommsen et al., 2003b), rainbow trout (*O. mykiss*) (Anderson, *et al*., 1991; McDonald and Wood, 2004) and Indian walking catfish (*C. batrachus*) (Saha *et al*., 2007b). GS is the feeder enzyme controlling the entry of nitrogen into the OUC in ureogenic teleosts (Mommsen and Walsh, 1991), and is considered as the key regulatory or rate limiting step for the ureogenic toadfish (Walsh *et al*., 1994). However, the OUC enzymes in the mud eel are not induced significantly following cortisol injection, more so as discussed earlier that ureogenesis is not adopted as the strategy to avoid ammonia toxicity in this fish. Hence, it can be said that cortisol in the mud eel induced GS activity only to assimilate the ammonia generated from the enhanced rate of amino acids catabolism by cortisol, suggesting its main role for ammonia detoxification.

Increase in GS enzyme activity was accompanied with the increase in the concentration of GS enzyme protein as evidenced by western blot analysis (Fig. 78), which probably might be due to increase in ammonia level by cortisol injection, as suggested in the walking catfish (Saha *et al*., 2007b). In addition, the perfusion experiment provided evidences of enhanced capacity of GS to incorporate more of ammonia-N to glutamine-N by the cortisol-injected fish liver. All these clearly indicated that cortisol injection resulted in the upregulation of GS enzyme protein at the translation level and/or probably also at the transcriptional level. Since fish hepatic GS does not seem to be activated allosterically (Shankar and Anderson, 1985), cortisol must be regulating transcription of GS gene in fish (Mommsen *et al.,* 1999). Preliminary studies with isolated Gulf toadfish hepatocytes indicated that treatment with cortisol induces GS mRNA expression (Ip *et al*., 2001a). As suggested in the case of mammalian and avian GS genes (Zhang and Young, 1991; Fahrner *et al*., 1993), the GS gene(s) of the mud eel also might contain upstream glucorticoid-response elements. Therefore, it opens a future field of investigation on the possible involvement of cortisol in the transcriptional regulation of GS with the possibility of multiple GS transcripts and their regulation in the mud eel. Now a day, the investigation on GS activity is not only restricted to liver but extended to other fish extra-hepatic tissues such as intestine, stomach and muscle (Anderson *et al*., 2002; Mommsen *et al*., 2003a). Like in most other fishes (Anderson and Walsh, 1995; Chakravorty et al., 1989; Felkie et al., 1998; Wang and Walsh, 2000), presence of exceptionally high level of activity of GS in brain tissue of the mud eel, reported here, must be playing an important role in detoxifying endogenous ammonia to Gln, resulting in significant increase in the concentration of Gln by about 129% (Table 44), following cortisol injection.

The effects of cortisol on the activity of amino acid metabolism related enzymes are tissue and species specific. Coticosteroids have been shown to increase aminotransferase activities in some fish species such as goldfish (*Carassius auratus*) (Storer, 1967), eels (*Anguilla japonica* and *A. rostrata* ) (Chan and Woo, 1978; Foster and Moon, 1986), brook charr (*Salvelinus fontinalis*)

(Freeman and Idler, 1973; Whiting and Wiggs, 1977; Vijayan *et al*., 1991), channel catfish (*Ictalurus punctatus*) (Davis *et al*., 1985), rainbow trout (*O. mykiss*) (Freeman and Idler, 1973; Barton *et al*., 1987; Morales *et al*., 1990), toadfish (*O. beta*) (Mommsen *et al*., 1992) and sea raven (*H. americanus*) (Vijayan *et al*., 1996). In the mud eel, the activity of GDH (ammonia utilizing direction), AST and ALT increased significantly following cortisol-treatment. The GDH activity in the ammonia assimilation direction increased maximally in kidney and liver (84% and 52%, respectively), followed by brain (24%), intestine (30%), stomach (22%) and muscle (21%) (Table 40). The increase in hepatic GDH activity in sea raven following cortisol treatment suggests a role for cortisol in the enhanced hepatic capacity for ammonia production (Vijayan *et al*., 1996) as proposed also in Japanese eel (*A. japonica*) (Chan and Woo, 1978). Also, toadfish showed higher GDH activity in isolated hepatocytes with dexamethasone treatment (Mommsen *et al*., 1992). The activity of ubiquitous enzymes such AST and ALT also are upregulated by the cortisol treatment, suggesting the higher rate of amino acids metabolism. The fact that higher activity of ALT and GDH could potentially increase the flux of Ala through the transdeamination pathway, as evidenced from the isolated sea raven and trout hepatocytes (Mommsen *et al*., 1992; 1999) and enhances Ala oxidation and gluconeogenesis in rainbow trout hepatocytes (Vijayan *et al*., 1993; 1994), as a consequence resulting in higher production of ammonia. Thus, our results supported that the increase in tissue ammonia concentration finally stimulated the amino acids metabolic enzymes for clearing the excess ammonia generated endogenously. However, there are inconsistencies and confusion in the literature regarding the action of cortisol during stress in fish. Much of the confusion probably arises due to differences in the method of administration of the hormone (Mommsen *et al*., 1999), species differences (Vijayan and Moon, 1994) among the many factors that influence cortisol levels in fishes. Nevertheless, it would be interesting to look at the compartmentalized induction of cortisol especially AST and ALT, which are locating both in the cytosolic and mitochondrial compartments in fish hepatocytes. Further, it would be interesting to study the possible induction of different mRNA transcripts of GS in the mud eel.

# CONCLUSION

The mud eel, *Amphipnous cuchia* is an obligatory air-breather, and lives in muddy ponds, swamps, canals and rice fields, where it burrows in moist earth during dry seasons and survives for long periods without water during summer. Thus, it faces the problem of ammonia toxicity in its natural habitats due to build up of high ammonia during exposure to high external ammonia (HEA) and also during desiccation stress. It possesses various adaptational strategies to adapt to these unique environmental conditions. Therefore, the main aim in the present study was to find out the possible biochemical strategies related to nitrogen metabolism adopted by this mud eel to survive under various environmental stresses that it faces in its natural habitats with special emphasis on amino acid metabolism under high environmental ammonia and desiccation stress.

The mud eel is primarily ammoniotelic while living in water excreting ammonia as the major nitrogenous excretory end product. It tolerates a very high ambient ammonia (upto 200 mM $NH_4Cl$), which is much higher than any other teleosts reported till date. It has the capacity to allow accumulation of ammonia at high level in different body tissues including plasma, while encountering the problem of HEA and also to terrestrial conditions for longer duration in its natural habitats, as one of the strategies to tackle the problem of ammonia toxicity.

Though the mud eel possesses a full complement of OUC enzymes in liver and kidney, but the levels of activity of all the five OUC enzymes are quite low as compared to other ureogenic teleosts. Further, exposure to HEA and to terrestrial conditions does not cause the induction of activity of the OUC enzymes as a consequent of higher accumulation of ammonia. Therefore, the conversion of accumulated ammonia to urea is not possible in this mud eel, as has been reported in some Indian air-breathing catfishes. Thus, ureogenecity is ruled out in this fish, as a strategy to neutralize and ameliorate ammonia toxicity. However, active conversion of accumulated ammonia to various non-essential FAAs and to store as such in different tissues are seen as the major strategy adopted by the mud eel in HEA and also in terrestrial habitats. The activities of GDH and

GS in brain and other tissues of the mud eel are as such very high, which further get induced in HEA and terrestrial habitat along with the induction of two important transaminase enzymes, AST and ALT. All these enzymes due to induction help in fixing the accumulated ammonia into Gln, Glu, Asp, Ala and some other non-essential FAAs in different tissues. Further, the higher accumulation of Ala in various tissues might be due to partial amino acid metabolism, which could be another important strategy to avoid ammonia toxicity, whereby, no ammonia is released in the process to cause ammonia-pollution in internal environment of the animal. Furthermore, the higher expression of hsp70, the major molecular chaperone, in all the tissues studied, probably helps the mud eel to defend against ammonia-mediated stressors and aid to adapt in a better way of dealing with high accumulation of ammonia encountered during exposure to various environmental constraints, and can be seen as another important strategy to avoid the toxic effects of ammonia.

The role of GS, a very important enzyme for detoxification of toxic ammonia and the key enzyme in nitrogen metabolism, has been explored in the present study in this fish. GS enzyme was found to be widely distributed at relatively high levels in different tissues of the mud eel, with maximum activity in brain, followed by stomach, intestine, kidney, liver and muscle. Interestingly, a high level of GS enzyme activity was found in stomach and intestine of this fish, where the enzyme may have a role unrelated to that in the liver and kidney. Exposure to HEA and terrestrial habitat causes upregulation of GS enzyme activity and also the GS enzyme protein concentrations in different tissues of the mud eel. It is suggested that high ammonia load causes upregulation of GS enzymes at translational, and possibly also at transcriptional level in this mud eel. Upregulation of GS activity leads to the detoxification of accumulated ammonia to Gln. With Gln serving as the primary nitrogen donating substrate for urea synthesis via the OUC in ureogenic teleosts, it is interesting to note that the activity of GS in this non-ureogenic fish is mainly for detoxification of ammonia accumulated inside tissues of stressed fishes. Upregulation of GS activity on exposure to HEA and terrestrial habitat is further evidenced by the fact that there is an increase in the efflux of glutamine-N by the perfused liver of the fish kept in HEA and terrestrial habitat, compared to the control fish. High accumulation of ammonia *in vivo* due to hyper-ammonia stress, caused during exposure to HEA and terrestrial habitat, believed to be the primary cause of induction of GS enzyme activity, is further evidenced by the fact that the intra-peritoneal injection of $NH_4HCO_3$ causes upregulation of GS enzyme activity, increase of GS enzyme protein concentrations and high Gln accumulation, along with increased efflux of glutamine-N by the perfused liver.

Further, the injection of cortisol, which is one of the most important stress hormones in fish and also is known to stimulate the proteolytic activities, is reported to enhance the rate of N excretion mainly in the form of ammonia-N with no changes of urea-N excretion, more accumulation of ammonia and non-essential FAAs in different tissues with concomitant increase of activities of GS and other amino acid metabolism-related enzymes in the mud eel. This again emphasizes the fact that whatever may be the cause of accumulation of ammonia *in vivo* in this mud eel, but its accumulation is the primary cause of induction in the synthesis of various non-essential FAAs in this mud eel as an adaptation to avoid the accumulation of ammonia to a toxic level.

Thus, it can be concluded that the mud eel (*A. cuchia*) is uniquely adapted to nitrogen metabolism using multiple strategies to tackle the problem of ammonia toxicity, thus enabling them to survive in extreme habitats that are totally unsuitable for any typical teleost.

# REFERENCES

Alabaster, J. S. and Herbert, D. W. H., (1954) Influence of carbon dioxide on the toxicity of ammonia. ***Nature,*** **174**: 404.

Alabaster, J. S. and Lloyd, R., (1982) Ammonia. In : *Water Quality Criteria for Freshwater Fish*, Butterworths, London, pp.85-102.

Alabaster, J. S., Shurben, D. G. and Knowles, G. (1979) The effect of dissolved oxygen and salinity on the toxicity of ammonia smolts of *Salmo salar* L. ***J. Fish Biol***., **15**: 705-712.

Alderson, R. (1979) The effect of ammonia on the growth of juvenile Dover sole, *Solea solea* L. and turbot, *Seophthalmus maximus* L. ***Aquaculture***, **17**: 291-309.

Andersen, D. E., Reid, S. D., Moon, T. W. and Perry, S. F. (1991) Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). ***Can. J. Fish. Aquat. Sci***., **48**: 1811-1817.

Anderson P.M., Broderius, M.A., Fong, K. C., Tsui, K. N.T., Chew, S. F., and Ip, Y. K., (2002) Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichyths sinensis* in response to exposure to high exogenous ammonia concentration. ***J. Exp. Biol***. **205**: 2053-2065.

Anderson, P. M. and Walsh, P. J. (1995) Sub-cellular localization and biochemical properties of the enzymes of carbamyl phosphate and urea synthesis in the batrachoidid fishes *Opsanus beta*, *Opsanus tau* and *Porichthys notatus*. ***J. Exp. Biol***., **198**: 755-766.

Anderson, P. M., (1995) Urea cycle in fish: Molecular and mitochondrial studies. In: *Fish Physiology*, Vol. 14, (Edited by Wood, C. M. and Shuttleworth, T. J.), Academic Press, New York, pp. 57-83.

Anderson, P.M., (2001) Urea and glutamine synthesis: environmental influence on nitrogen excretion. In: *Fish Physiology Vol. 20. Nitrogen Excretion*, (Edited by Wright, P.A. and Anderson, P. M.), Academic Press, New York, pp 239-277.

Anderson, P. M., Broderius, M. A., Fong, K.C., Tsui, T. K. N., Chew, S. F., Ip, Y. K. (2002) Glutamine synthetase expression in liver, muscle, stomach, and intestine of *Bostrichyths sinensis* in response to exposure to a high exogenous ammonia concentration. ***J. Exp. Biol***., **205**: 2053-2065.

Arillo, A., Margioco, C., Melodia, F., Mensi, P., and Schenon, G., (1981) Ammonia toxicity mechanisms in fish: studies on rainbow trout (*Salmo gairdneri* Rich). ***Ecotox. Environ. Saf***. **5**: 316-325.

Atkinson, D. E. (1992) Functional role of urea synthesis in vertebrates. ***Physiol. Zool***., **65**: 243-267.

Barton, B.A., Schreck, C. B. and Barton, L. D. (1987) Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. ***Dis. Aquat. Org***., **2**: 173-185.

Basu, N., Nakano, T., Grau, E. G. and Iwama, G. K. (2001) The effects of cortisol on heat shock protein 70 levels in two fish species. ***Gen. Comp. Endo***., **124**: 97-105.

Bever, K., Chenoweth, M. and Dunn, A., (1981) Amino acid gluconeogenesis and glucose turnover in kelp bass (*Paralabrax* sp.). ***Am. J. Physiol***., **240R**: 246-152.

Blei, A. T., Olafsson, S. M., Therrien, G., and Butterworth, R.F., (1994) Ammonia-induced cerebral edema and intracranial hypertension in rats after portacaval anastomosis. ***Hepatology***, **19**:1431-1444.

Bradford, H. F., Ward, H. K., and Foley, P., (1989) Glutaminase inhibition and release of neurotransmitter glutamate from synaptosomes. ***Brain Res***. **476**: 29-34.

Brett, J. R. and Zala, C. A. (1975) Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. ***J. Fish Res. Bd. Can***., **32**: 2479-2486.

Brown Jr, G.W. and Cohen, P.P. (1959) Comparative biochemistry of urea synthesis-I. Methods for the quantitative assay of urea cycle in liver. ***J. Biol. Chem***., **234**: 1769-1774.

Brusilow, S. W., (2002) Reviews in molecular medicine- Hyperammoniemic encephalopathy. ***Med***. **81**: 240-249.

Burrows, R. E., (1964) Effects of accumulated excretory products on hatchery-reared salmonids. ***U. S. Fish Wildl. Ser. Bur. Sport Fish. Wildl. Res. Rep***., **66**:12.

Cameron, J. N. and Heisler, N., (1983) Studies of ammonia in rainbow trout: Physico-chemical parameters, acid-base behavior and respiratory clearance. ***J. Exp. Biol***. **105**:107-125.

Campbell, J.W. (1973) Nitrogen excretion. In: *Comparative Animal Physiology* 3rd edn. (Edited by Prosser, C.L.), Saunders College Publishing, Philadelphia, pp. 279-316.

Campbell, J. W., Aster, P. L. and Vorhaben, J. E. (1983) Mitochondrial ammoniagenesis in liver of the channel catfish *Ictalurus punctatus*. ***Am. J. Physiol***., **244**: R709-R717.

Campbell, J. W. and Anderson, P. M., (1991) Evolution of mitochondrial enzyme systems in fish: The mitochondrial synthesis of glutamine and citruline. In: *Biochemistry and Molecular Biology of Fishes, Vol. 1*, (Edited by Hochachka, P.W. and Mommsen, T. P.), Elsevier Science Publishers, Amsterdam, pp. 43-76.

Campbell, J.B., (1991) Excretory nitrogen. In: *Experimental and Metabolic Animal Physiology: Comparative Animal Physiology* (edited by Prosser, C. L.) 4th edn. New York: Wiley-Interscience, pp 277-324.

Carter, G. S. (1957) Air breathing. In: *Physiology of Fishes* (Edited by Brown, M). Academic Press, New York, pp. 65-79.

Chakravorty, J., Saha, N. and Ratha, B.K. (1989) A unique pattern of tissue distribution and sub-cellular localization of glutamine synthetase in a freshwater air-breathing teleost, *Heteropneustes fossilis* (Bloch). ***Biochem. Int***., **19**: 519-527.

Chan, D. K. O. and Woo, N. Y. S. (1978) Effect of cortisol on the metabolism of the eel, *Anguilla japonica*. ***Gen. Comp. Endocr***., **35**: 205-215.

Chen, J.C. and Nan, F.H. (1993) Effects of ammonia on oxygen consumption and ammonia-N excretion of *Penaeus chinensis* after prolonged exposure to ammonia. ***Bull. Environ. Contam. Toxicol***., **51**: 122-129.

Chew, S.F. and Ip, Y.K. (1987) Ammoniogenesis in mudskippers *Boleophthalmus boddaerti* and *Periophthalmodon schlosseri*. ***Comp. Biochem. Physiol***., **87B**: 941-948.

Chew, S. F., Jin, Y., and Ip, Y. K., (2001) The loach *Misgurnus anguillicaudatus* reduces amino acid catabolism and accumulates alanine and glutamine during aerial exposure. ***Physiol. Biochem. Zool***., **74**: 226-237.

Chew, S. F., Ong, T. F. Ho, L., Tam, W. L., long, A. M. Hiong, K. C., Wong, W. P., Ip, Y. K., (2003a) Urea synthesis in the African lungfish *Protopterus dolloi* hepatic carbamoyl phosphate synthetase III and glutamine synthetase are up-regulated by 6 days of aerial exposure. ***J. Exp. Boil***. **206**: 3615-3624.

Chew, S. F., Wong, M. Y., Tam, W. I., and Ip, Y. K. (2003b) The snakehead *Channa asiatica* accumulates alanine during aerial exposure, but is incapable of sustaining locomotory activities on land through partial amino acid catabolism. ***J. Exp. Biol***., **206**: 693-704.

Chew, S. F., Chan, N. K. Y., Loong, A. M., Hiong, K.C., Tam, W. L. and Ip, Y. K. (2005) Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. ***J. Exp. Biol***., **207**: 77-786.

Christowitz, D., Matteyse, F.J. and Balinsky, J.B. (1981) Dietary and hormonal regulation of urea cycle enzymes in rat liver. ***Enzyme***, **26**:113–121.

Cooper, A.J.L., Plum, F. (1987) Biochemistry and physiology of brain ammonia. ***Physiol. Rev***., **67**: 440–519.

Claiborne, J. B., Evans, D. H. and Goldstein, L. (1982) Fish branchial $Na^+/H^+$ exchange is via vasolatral $Na^+$, $K^+$-activated ATPase. ***J. Exp. Biol***., **96**: 431-434.

Clayton, D.A. (1993). Mudskippers. ***Oceanogr. Mar. Biol. Annu. Rev***. **31**: 507–577.

Cohen, P.P. and Brown, G.W. Jr. (1960) Ammonia metabolism and urea biosynthesis. In: *Comparative Biochemistry, Vol. 2* (Edited by Florkin, M. and Mason, H.S.), Academic Press, New York, London, pp. 161-244.

Cohen, P.P. (1976) Evolutionary and comparative aspects of urea biosynthesis. In: *The Urea Cycle* (Edited by Grisolia, S., Baguena, R. and Mayor, F.), John Wiley and Sons, New York/London/Sydney/Toronto, pp. 21-28.

Cooper, A. J. L. and Plum, F. (1987) Biochemistry and physiology of brain ammonia. ***Physiol. Rev.,* 67:** 440-519.Cowey et al., 1977.

Cowey, C.B., De La Higuera, M. and Andron, J.W. (1977) The effect of dietary composition and insulin on gluconeogenesis in rainbow trout (*Salmo gairdneri*). ***Brit. J. Nutr***., **38**: 385-395.

Craig, E.A. and Gross, C. A. (1991) Is hsp70 the cellular thermometer? ***Trends Biochem. Sci***., **16 (4)**: 135-140.

Dabrowska, H. and Wlasow, T. (1986) Sublethal effect of ammonia on certain biochemical and haemotological indicators in common carp (*Cyprinus carpio*). ***Comp. Biochem. Physiol.,* 80C:** 179-184.

Davenport, J. and Sayer, M.D.J. (1986) Ammonia and urea excretion in the amphibians teleost *Blennius pholis* (L) in sea-water and in air. ***Comp. Biochem. Physiol.,* 84A:** 189-194.

Davidson, A. (1975) Fish and Fish dishes of Laos. In: "*Imprimerie Nationale, Vientiane"*. pp-202.

Davis, K.B., Torrance, P., Parker, N.C. and Suttle, M.A. (1985) Growth, body composition and hepatic tyrosine aminotransferase activity in cortisol-fed channel catfish, *Ictalurus punctatus* Rafinesque. ***J. Fish Biol***., **27**: 177-184.

DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982) The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. ***Cell***, **31(3 Pt. 2)**: 593-603.

Driedzic, W. R. and Hochachka, P.W. (1976) Control of energy metabolism in fish white muscle. ***Am. J. Physiol***., **230**: 579-582.

Driedzic, W.R. and Hochachka, P.W. (1978) Metabolism of fish during exercise. In: *Fish Physiology*, Vol. 7 (Edited by Hoar, W.S. and Randall, D.J.), Academic Press, New York, pp. 503-543.

Duda, G.D. and Handler, P. (1958) Kinetics of ammonia metabolism *in vivo*. ***J. Biol. Chem.,*** **232:** 303-314.

Dunn, J. (1983) Air-breathing fishes. Waters, J. Vancouver Aquarium, 6: 20-24.

Dutta, S. (2006) Purification and characterization of glutamine synthetase and its regulation under various environmental stresses in an air-breathing catfish (*Clarias batrachus*). (*Ph. D. Thesis*).

Eddy, F. B., (2005) Ammonia in estuaries and effects on fish. ***J. Fish Biol***., **67**: 1495 – 1513.

Essex-Fraser, P. A., Steele, S. L., Bernier, N. J., Murray, B. W., Stevens, E. D., Wright, P.A. ( 2005) Expression of four glutamine synthetase genes in early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relation to nitrogen metabolism. ***J. Biol. Chem***., **280**: 20268-20273.

Evans, D. H. and Cameron, J. N. (1986) Gill ammonia transport. ***J. Exp. Zool***., **239**: 17-23.

Fahrner, J., Labruyere, W. T., Gaunitz, C., Moorman, A. F. M., Gebhardt, R., Lamers, W. H. (1993) Identification and functional characterization of regulatory elements of the glutamine synthetase gene from rat liver. ***Euro. J. Biochem***., **213**: 1067-1073.

Fan, P. and Szerb, J. C., (1993) Effects of ammonium ion on synaptic transmission and responses to quisqualate and N-mythyl-D-aspartate in hippocampal CA1 pyramidal neurons in vitro. ***Brain Res***., **632**: 225-231.

Felipo, V. and Butterworth, R. F. (2002) Neurobiology of ammonia. ***Prog. Neurobiol***., **67**: 259-279.

Felskie, A.K., Anderson, P.M. and Wright, P.A. (1998) Expression and activity of carbamoyl phosphate synthetase III and ornithine urea cycle enzymes in various tissues of four fish species. ***Comp. Biochem. Physiol***., **119B**: 355-364.

Forster, R.P. and Goldstein, L. (1969) Formation of excretory products. In: *Fish Physiology***,** Vol. 1 (Edited by Hoar, W.S. and Randall, D.J.), Academic Press, New York, London, pp. 313-350.

Foster, G. D. and Moon, T. W. (1986) Cortisol and liver metabolism of immature American eels, *Anguilla rostrata* (LeSueur). ***Fish Physiol. Biochem***., **1**:113-124.

Freeman, H. C. and Idler, D. R. (1973) Effects of corticosteroids on liver transaminase in two salmonids, the rainbow trout (*Salmo gairdneri*) and the brook trout (*Salvelinus fontinalis*). ***Gen. Comp. Endocr***., **20**: 69-70.

Freney, J. R., Denmead, O. T., Watanabe, I. and Craswell, E. T. (1981) Ammonia and nitrous oxide losses following applications of ammonium sulfate to flooded rice. ***Aust. J. Agric. Res***., **32**: 37-45.

Frick, N.T. and Wright, P.A. (2002) Nitrogen metabolism and excretion in the mangrove killifish, *Rivulus marmoratus*. II. Significant ammonia volatilization in a teleost during air exposure. ***J. Exp. Biol.,*** **205:** 91-100.

Fromm, P.O. and Gillette, J.R. (1968) Effects of ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout (*Salmo gairdneri*). ***Comp. Biochem. Physiol.,*** **12:** 489-499.

Gardiner, B. (1980) tetrapod ancestry: A reappraisal. In: *The Terrestrial Environment*. (Edited by Panchen, A. L.). Academic Press, London, pp. 177-185.

Gibson, R.N. (1969) The biology and behavior of littoral fish. ***Oceanogr. Mar. Biol. Annu. Rev***. **7**: 367–410.

Gibson, R.N. (1982) Recent studies on the biology of intertidal fishes. ***Oceanogr. Mar. Biol. Annu. Rev***. **20**: 363–414.

Goldstein, L. and Forster, R. P. (1970) Nitrogen metabolism in fishes. In: *Comparative Biochemistry*, (Edited by Campbell, J. W.), Academic Press, New York, London, pp. 496-518.

Goldstein, L. and Forster, R.P. (1971) Urea biosynthesis and excretion in freshwater and marine elasmobranchs. ***Comp. Biochem. Physiol.,*** **39B:** 415-421.

Gordon, M. S. and Olson, E. C. (1994) The Invasions of the Land. Columbia University Press, New York, pp. 312.

Graham, J.B. (1997a) Respiratory Organs. In: *Air-breathing fishes: Evolution, Diversity and Adaptation*, Academic Press, San Diego. pp. 65-133.

Graham, J.B. (1997b) Metabolic adaptation. In: *Air-breathing fishes: Evolution, Diversity and Adaptation*, Academic Press, San Diego, pp. 223-240.

Griffith, R. W. (1991) Guppies, toadfish, lungfish, coelacanths and frogs: a scenario for the evolution of urea retention in fishes. ***Env. Biol. Fish***., **32**:199-218.

Hampson, B.L. (1976) Ammonia concentration in relation to ammonia toxicity during a rainbow trout rearing experiment in a closed freshwater-seawater system. ***Aquaculture,*** **9:** 61-70.

Hansson, E., Blomstrand, F., Khabiti, S., Olsson, T. and Ronnback, L., 1997. Glutamate induced astroglial swelling- methods and mechanisms. ***Acta. Neurochir***. **70**: 148-151.

Haywood, G.P. (1983) Ammonia toxicity in teleost fishes: a review. ***Can. Tech. Rep. Fish. Aquat. Sci.,*** **1177:** 35.

Heiler, N. (1990) Mechanisms of ammonia elimination in fishes. In: *Animal nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects*. (Edited by Truchot, J. P. and Lahlou, B.). Karger, Basel. pp. 137-151.

Hillaby, B. A. and Randall, D. J. (1979) Acute ammonia toxicity and ammonia excretion in rainbow trout (*Salmo gairdneri*). ***J. Fish. Res. Bd. Can.,*** **36:** 621-629.

Hindfelt, B., Plum, F. and Duffy, T.E. (1977) Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. ***J. Clin. Invest.,*** **59:** 386-396.

Hoar, W.S. (1983) Excretion. In: *General and Comparative Physiology***,** 3rd edn., Prentice Hall, Inc., U.S.A, pp. 586-626.

Hochachka, P. W. and Somero, G. N.(1984) In: *Biochemical Adaptation*, Princeton University Press, Priceton, NJ. p.525.

Holeton, G. F., Neumann, P. and Heisler, N. (1983) Branchial ion exchange and acid-base regulation after strenous exercise in rainbow trout. ***Resp. Physiol***., **51**: 303-318.

Hong, L. N. (1997) A comparative study of the effects of environmental pH on two mudskippers. Honours thesis, Department of Biological Sciences, National University of Singapore. P. 87.

Hong, J., Salo, W. L., Lusty, C. J., Anderson, P. M. (1994) Carbomyl phosphate synthetase III, an evolutionary intermediate in the transition between glutamine-dependent and ammonia-dependent carbomyl phosphate synthetase. ***J. Mol. Biol***., **243**: 131-140.

Hopkins, T.E., Wood, C.M. and Walsh P.J. (1995) Interactions of cortisol and nitrogen metabolism in the ureogenic Gulf toadfish *Opsanus beta*. ***J. Exp. Biol***., **198**: 2229-2235.

Ip, Y. K., Lee, C. Y., Chew, S. F. Low, W. P. and Peng, K. W. (1993) Differences in the responses of two mud skippers to terrestrial exposure. ***Zool. Sci***., **10**: 511-519.

Ip, Y.K., Chew, S.F. and Randall, D.J. (2001a) Ammonia toxicity, tolerance and excretion. In: "*Nitrogen excretion Vol. 20*," (Edited by Wright, P.A. and Anderson, P.M.), Academic Press, New York, pp. 109-148.

Ip, Y.K., Chew, S.F., Leong, I.A.W., Jin, Y., Lim, C.B. and Wu, R.S.S. (2001b) The sleeper *Bostrichthys sinensis* (Family Eleotridae) stores glutamine and reduces ammonia production during aerial exposure. ***J. Comp. Physiol.,* 171B:** 357-367.

Ip, Y.K., Lim, C.B., Chew, S.F., Wilson, J.M. and Randall, D.J. (2001c) Partial amino acid catabolism leading to the formation of alanine in *Periophthalmodon schlosseri* (mudskipper): A strategy that facilitates the use of amino acids as an energy source during locomotory activity on land. ***J. Exp. Biol.,* 204:** 1615-1624.

Ip, Y. K., Randall, D. J., Kok, T. K. T., Bazarghi, C., Wright, P. A., Ballantyne, J. S., Wilson, J. M. and Chew, S. F. (2003a) The mudskipper *Periophthalmodon schlosseri* facilitates active $NH_4^+$ excretion by increasing acid excretion and decreasing $NH_3$ permeability in the skin. ***J. Exp. Biol***., **207**: 787-801.

Ip, Y. K., Tam, W. L., Wong, W. P. Loong, A. I., Hiong, K. C., Ballantyne, J. S. and Chew, S. F. (2003b) A comparison of the effects of environmental ammonia exposure on the Asian freshwater stringray *Himantaru signifier* and the Amazonian freshwater stringray *Potamotrygon motoro*. ***J. Exp. Biol***., **206**: 3625-3633.

Ip, Y. K., Chew, S. F., Wilson, J. M., Randall, D. J. (2004a) Defences against ammonia toxicity in tropical air-breathing fishes exposed to high concentrations of environmental ammonia: a review. ***J. Comp. Physiol***., **174B**: 565-575.

Ip, Y.K., Zubaidah, R.M., Liew, P.C., Loong, A.M., Hiong, K.C., Wong, W.P. and Chew, S.F. (2004b) African sharptooth catfish *Clarias gariepinus* does not detoxify ammonia to urea or amino acids but actively excretes ammonia during exposure to environmental ammonia. ***Physiol. Biochem. Zool.,* 77:** 242-254.

Ip, Y. K. Tay, A. S. L., Lee, K. H., Chew, S. F. ( 2004c) Strategies for surviving high concentrations of environmental ammonia in the swamp eel *Monopterus albus*. ***Physiol. Biochem. Zool***., **77**: 390-405.

Ip, Y.K., Chew, S.F. and Randall. D. J. (2004d) Five tropical air-breathing fishes, six different strategies to defend against ammonia toxicity on land. ***Physiol. Biochem. Zool***. **77(5**): 768-782.

Ip, Y.K., Law, I.Y., Wong, W.P., Lee, S.L.M. and Chew, S.F. (2005a) The African sharptooth catfish *Clarias gariepinus* can tolerate high levels of ammonia in its tissue and organ during four days of aerial exposure. ***Physiol. Biochem. Zool.,*** **78:** 630-640.

Ip, Y.K., Peh, B.K., Tam, W.L., Wong, W.P. and Chew, S.F. (2005b) Effects of intra-peritoneal injection with $NH_4Cl$, urea, or $NH_4Cl$+urea on nitrogen excretion and metabolism in the African lungfish *Protopterus dolloi*. ***J. Exp. Zool.,*** **303:** 272-282.

Ip, Y.K., Yeo, P.J., Loong, A.M., Hiong, K.C., Wong, W.P. and Chew, S.F. (2005c) The interplay of increased urea synthesis and reduced ammonia production in the African lungfish *Protopterus aethiopicus* during 46 days of aestivation in a mucus. ***J. Exp. Zool.,*** **303:** 1054-1065.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B. and Ackerman, P. A. (1999) Heat shock proteins and physiological stress in fish. ***Am. Zool***. **39**: 901-909.

Iwata, K., Kakuta, I., Ikeda, M., Kimoto, S. and Wada, N. (1981) Nitrogen metabolism in mud skipper, *Periophthalmus cantonenesis*: A role of free amino acids in detoxification of ammonia produced during its terrestrial life. ***Comp. Biochem. Physiol***., **68A**: 589-596.

Iwata, K. (1988) Nitrogen metabolism in the mudskipper *Periophthalmus cantonensis*: Changes in free amino acids and related compounds in various tissues under conditions of ammonia loading, with special reference to its high ammonia tolerance. ***Comp. Biochem. Physiol.,*** **91A:** 499-508.

Iwata, K. and Deguchi, M. (1995) Metabolic fateand distribution of $^{15}$N-ammonia in an ammoniotelic amphibious fish, *Periophthalmus modestus*, following immersion in $^{15}$N-ammonium sulphate: a long term experiment. ***Zool. Sci***., **12**: 175-184.

Iwata, K., Kajimura, M. and Sakamoto, T. (2000a) Functional ureogenesis in the gobiid fish, *Mugilogobius abei*. ***J. Exp. Biol.*, 203:** 3703-3715.

Iwata, K., Kajimura, M., Sakamoto, T. (2000c) Functional ureogenesis in the gobiid fish, *Mugilogobius abei*. ***J. Exp. Biol***., **203**: 3703–3715.

Janicki, R. and Lingis, J. (1970) Mechanism of ammonia production from aspartate in teleost liver. ***Comp. Biochem. Physiol.*, 37A:** 101-105.

Janssens, P.A. (1964) The metabolism of aestivating African lungfish. ***Comp. Biochem. Physiol.*, 11:** 105-117.

Jenssens, P. A. and Cohen, P. P. (1968) Biosynthesis of urea in the aestivating African lungfish and in *Xenopus laevis* under conditions of water shortage. ***Comp. Biochem. Physiol***., **24**: 887-898.

Jow, L.Y., Chew, S.F., Lim, C.B., Anderson, P.M. and Ip, Y.K. (1999) The marble goby *Oxyeleotris marmoratus* activates hepatic glutamine synthetase and detoxifies ammonia to glutamine during air exposure. ***J. Exp. Biol.*, 202:** 237-245.

Jow, L.Y., Chew, S.F., Lim, C.B., Anderson, P.M., Ip, Y.K. (1999) The marble goby *Oxyeleotris marmoratus* activates hepatic glutamine synthetase and detoxifies ammonia to glutamine during air-exposure. ***J. Exp. Biol***., **202**: 237–245.

Julsrud, E.A., Walsh, P.J. and Anderson, P.M. (1998) N-Acetyl-L-glutamate and the urea cycle in the gulf toadfish (*Opsanus beta*) and other fish. ***Arch. Biochem. Biophys.*, 250:** 55-60.

Jürss, K., Bastrop, R., 1995. Amino acid metabolism in fish. In: *Biochemistry and Molecular Biology of Fishes*, Vol. 4. Elsevier, (Edited by Hochachka, P.W., Mommsen, T. P.), Amsterdam, pp. 159–189.

Kharbuli, Z. Y., Datta, S., Biswas, K., Sarma, D. and Saha, N. (2006) Expression of ornithine-urea cycle enzymes in early life stages of air-breathing walking catfish *Clarias batrachus* and induction of ureogenesis under hyper –ammonia stress. ***Comp. Biochem. Physiol***. **143B**:44-53.

Kloppick, E., Jacobasch, G. and Rapoport, S. (1967) Steigerung der glykolyse durch den einfluss von ammoniumionen auf die phosphofrukto kinaseaktivitat. (Enhancement of the glycolytic rate by action of ammonium ion on phosphofructokinase activity). ***Acta. Biol. Med. Ger***., **18**: 37-42.

Kok, T. W. K., Lim, C. B., Lam, T. J and Ip, Y. K. (1998) The mud skipper, *Periophthalmodon schlosseri* respires more efficiently on land than in water and vice versa for *Boleophthalmus boddaerti*. ***J. Exp. Zool***., **280**: 86-90.

Kong, H.K., Kahatapitiya, N., Kingsley, K., Salo, W.L., Anderson, P.M., Wang, Y.S. and Walsh, P.J. (2000) Induction of carbamoyl phosphate synthetase III and glutamine synthetase mRNA during confinement stress in gulf toadfish (*Opsanus beta*). ***J. Exp. Biol***., **203**: 311-320.

Korsgaard, B., Mommsen, T.P. and Wright, P.A. (1995) Nitrogen excretion in teleostean fish: adaptive relationships to environment, ontogenesis and viviparity. In: *Nitrogen Metabolism and Excretion* (Edited by Walsh, P.J. and Wright, P.A.), Boca Raton, CRC Press, pp. 259-287.

Kosenko, E., Kaminsky, Y. Kaminsky, A., Valencia, M., Lee, L., Hermenegildo, C. and Felipo, V. (1997) Superoxide production and antioxidant enzymes in ammonia intoxication in rats. ***Free Radic. Res***., **27**: 637-644.

Kosenko, E., Kaminsky, Y., Felipo, V., Minana, M. D. and Grisolia, S. (1993) Chronic hyperammonia prevents changes in brain energy and ammonia metabolites induced by acute ammonium intoxication. ***Biochem. Biophys. Acta***., **1180**: 321-326.

Kosenko, E., Kaminsky, Y., Grau, E., Minana, M. D., Grisolia, S. and Felipo, V. (1994) Brain ATP depletion induced by acute ammonia intoxication in rats is mediated by activation of the NMDA receptor and $Na^+$, $K^+$-ATPase. ***J. Nerochem***., **63**:2172-2178.

Kosenko, E., Kaminsky, Y., Grau, E., Minana, M. D., Grisolia, S. and Felipo, V. (1995) Nitroarginine, an inhibitor of nitric oxide synthetase, attenuates ammonia toxicity and ammonia-iduced alterations in brain metabolism. ***Neurochem. Res***., **20**: 451-456.

Kosenko, E., Kaminsky, Y., Lopata, O., Muravyov, N. and Felipo, V. (1999) Blocking NMDA receptors prevents the oxidative stress induced by acute ammonia intoxication. ***Free Radic. Biol. Med***., **26**: 1369-1374.

Kosenko, E., Kaminsky, Y., Stravroskaya, I. G. and Felipo, V. (2000) Alteration of mitochondrial calcium homeostasis by ammonia-induced activation of NMDA receptors in rat brain in vivo. ***Brain Res***., **880**: 139-146.

Krebs, H.A. and Henseleit, K. (1932) Untersuchungen über die Harnstoffbildung im Tierkörper. ***Hoppe Seylers Z. Physiol. Chem***., **210**: 33-66.

Lande, M.B., Priver, N.A. and Zeidel, M.L. (1994) Determination of apical membrane permeabilities of barrier epithelia. ***Am. J. Physiol***., **267C**: 367-374.

Lang, T., Peters, G., Hoffmann, R. and Meyer, E. (1987) Experimental investigations on the toxicity of ammonia: Effects on ventilation frequency, growth, epidermal mucous cells and gill structure of rainbow trout *Salmo gairdneri*. ***Dis. Aquat. Org***., **3:** 159-165.

Leach, G.J. and Taylor, M.H. (1982) The effects of cortisol treatment on carbohydrate and protein metabolism in *Fundulus heteroclitus*. ***Gen. Comp. Endocrinol***., **48**: 76-83.

Levi, G., Morisi, G., Coletti, A. and Catanzaro, R. (1974) Free amino acids in fish brain: normal levels and changes upon exposure to high ammonia concentrations in vivo, and upon incubation of brain slices. ***Comp. Biochem. Physio***l., **49A**: 623-636.

Liem, K.F. (1987) Functional design of the air ventilation apparatus and overland escursions by teleosts. ***Fieldiana. Zool***., **37**: 1-29.

Lim, C.B., Chew, S.F., Anderson, P.M., Ip, Y.K. (2001) Reduction in the rates of protein and amino acid catabolism to slow down the accumulation of endogenous ammonia: a strategy potentially adopted by mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*) during aerial exposure in constant darkness. ***J. Exp. Biol***., 204: 1605–1614.

Lindquist, S. and Craig, E. A. (1988) The heat-shock proteins. ***Annu. Rev. Genet***., **22**: 631-677.

Long, J. A. (1995) *The Rise of Fishes: 500 Million Years of Evolution*. John Hopkins University Press, Baltimore, pp. 223.

Loong, A.M., Hiong, K.C., Lee, S.M.L., Wong, W.P., Chew, S.F. and Ip, Y.K. (2005) Ornithine-urea cycle and urea synthesis in African lungfishes, *Protopterus aethiopicus* and *Protopterus annectens,* exposed to terrestrial conditions for six days. ***J. Exp. Zool***., **303**: 354-365.

Maetz, J. (1973) $Na^+/NH_4^+$, $Na^+/H^+$ exchanges and $NH_3$ movements across the gill of *Carassius auratus*. ***J. Exp. Biol***., **58**: 255-275.

Makarewicz, W. (1963) AMP-aminohydrolase and glutaminase activities in the kidneys and gills of some freshwater vertebrates. ***Acta. Biochem. Polon***., **10**: 363-369.

Makarewicz, W. and Zydowo, M. (1962) Comparative studies on some ammonia producing enzymes in the excretory organs of vertebrates. ***Comp. Biochem. Physiol***., **6**: 269-275.

Mans, A.M., Biebuyck, J.F. and Hawkins, R.A. (1983) Ammonia selectivity stimulates neutral amino acid transport across blood-brain barrier. ***Am. J. Physiol***., **245C**: 74-77.

Marcaggi, P. and Coles, J. A. (2001) Ammonium in nervous tissues: transport across cell membranes, fluxes from neurons to glial cells, and role in signaling. ***Prog. Neurobiol***., **64**: 157-183.

McBean, R. L. and Goldstein, L. (1967) Ornithine-urea cycle activity in *Xenopus laevis*: adaptation in saline. ***Science***, **157**: 931-932.

McDonald, M.D. and Wood, C.M. (2004) The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*). ***J. Comp. Physiol***., **174B**: 71-81.

McKenzie, D.J., Randall, D.J., Lin, H. and Aota, S. (1993) Effects of changes in plasma pH, $CO_2$ and ammonia on ventilation in trout. ***Fish Physiol. Biochem***., **10**: 507-515.

Meijer, A.J., Lamers, W.H. and Chamuleau, R.A.F.M. (1990) Nitrogen metabolism and ornithine cycle function. ***Physiol. Rev***., **70**: 701-748.

Michalak, A., Rose, C., Butterworth, J. and Butterworth, R. F. (1996) Neuroactive amino acids and glutamate (NMDA) receptors in frontalcortex of rats with experimental acute liver failure. ***Hepatology***, **24**: 908-914.

Milligan, C.L. (1997) The role of cortisol in amino acid mobilization and metabolism following exhaustive exercise in rainbow trout (*Oncorhynchus mykiss* Walbaum). ***Fish Physiol. Biochem***., **16**: 119-128.

Mommsen, T. P. and Walsh, P. J. (1989) Evolution of urea cycle synthesis in vertebrates: the piscine connection. ***Science***, **243**: 72-75.

Mommsen, T. P., Danulat, E. and Walsh, P. J., (1992) Metabolic actions of glucagons and dexamethasone in liver of the ureogenic teleost *Opsanus beta*. ***Gen. Comp. Endocrinol***., **85**: 316-326.

Mommsen, T. P., Vijayan, M. M. and Moon, T. W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. ***Rev. Fish Biol. Fish***., **9**: 211-268.

Mommsen, T.P., Busby, E.R., von Schalburg, K.R., Evans, J.C., Osachoff, H.L. and Elliott, M.E. (2003a) Glutamine synthetase in tilapia gastrointestinal tract: zonation, cDNA and induction by cortisol. ***J. Comp. Physiol***., **173B**: 419-427.

Mommsen, T.P., Danulat, E. and Walsh, P.J. (1992) Metabolic actions of glucagon and dexamethasone in liver of the ureogenic teleost *Opsanus beta*. ***Gen. Comp. Endocrinol***., **85**: 316-326.

Mommsen, T.P., Osachoff, H.L. and Elliott, M.E. (2003b) Metabolic zonation in teleost gastrointestinal tract: Effects of fasting and cortisol in tilapia. ***J. Comp. Physiol***., **173B**: 409-418.

Mommsen, T.P., Vijayan, M.M. and Moon, T.W. (1999) Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. ***Rev. Fish Biol. Fish***., **9**: 211-268.

Mommsen, T.P., Walsh, P.J. (1991) Urea synthesis in fishes: evolutionary and biochemical perspectives. In: *Biochemistry and Molecular Biology of Fishes.* Elsevier, (Edited by Hochachka, P.W., Mommsen, T.P.), Amsterdam, pp. 137–163.

Moon, T. W. and Johnston, I. A. (1981) Amino acid transport and interconversion in tissues of freshly caught and food-deprived plaice, *Pleuronectes platessa* L. ***J. Exp. Biol***., **19**: 653-663.

Moore, R. B. and Kauffman, N.G. (1970) Simultaneous determination of citrruline and urea using diacetyl monoxime. ***Anal. Biochem***., **33**: 263-272.

Morales,A. E., Garcia-Rejon, L. and de la Higuera, M. (1990) Influence of handling and/or anaethesia on stress response in rainbow trout: Effects of liver primary metabolism. ***Comp. Biochem. Physiol***., **95A**: 87-93.

Morii, H., Nishikata, K. and Tamura, O. (1978) Nitrogen excretion of mudskipper fish *Periophthalmus cantonensis* and *Boleophthalmus pectinirostris* in water and on land. ***Comp. Biochem. Physiol***., **60A**: 189-193.

Munshi, J. S. D. and Hughes, G.M. (1992) Air-breathing fishes: their structure and life history. Oxford Publication and IBH, New Delhi.

Murray, B. W., Busby, E. R., Mommsen, T. P., and Wright, P. A. ( 2003) Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expression in rainbow trout (*Oncorhynchus mykiss*). ***J. Exp. Biol***., **206**: 1511-1521.

Nener, J.C. (1988) Variable and constrained features of the ornithine-urea cycle. ***Can. J. Zool***., **66**: 1069-1077.

Oppong, K.N.W., Bartlett, K., Record, C. O. and Al Mardini, H. (1995) Synaptosomal glutamate transport in thioacetamide-induced hepatic encephalopathy. ***Hepatology,*** **22**: 553-558.

Paley, R.K., Twitchen, I.D. and Eddy, F.B. (1993) Ammonia, $Na^+$, $K^+$ and $Cl^-$ levels in rainbow trout yolk-sac fry in response to external ammonia. ***J. Exp. Biol***., **180**: 273-284.

Pasti, L., Zonta, M., Pozzan, T., Vicini, S. and Carmignoto, G. (2001) Cytolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. ***J. Neurosci***., **21**: 477-484.

Paulus, H. (1983) The evolutionary history of the ornithine cycle as a determinant of its structure and regulation***. Curr. Top Cell Regul***., **22**:177-200.

Peng, K.W., Chew, S.F., Lim, C.B., Kuah, S.S.L., Kok, W.K., Ip, Y.K., (1998) The mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* can tolerate environmental $NH_3$ concentrations of 446 and 36 µM, respectively. ***Fish Physiol. Biochem***., **19**: 59–69.

Person-LeRuyet, J., Boeuf, G., Zambonino-Infant, J., Helgason, S. and Le Roux, A. (1988) Short-term physiological changes in turbot and seabream juveniles exposed to exogenous ammonia. ***Comp. Biochem. Physiol***., **119A**: 511-518.

Pottinger, T. G. (1990) The effect of stress and exogenous cortisol on receptor-like binding of cortisol in the liver of rainbow trout, *Onchorynchus mykiss*. ***Gen. Comp. Endocrinol***., **78**: 194-203.

Powers-Lee, S.G. and Meister, A. (1988) Urea synthesis and ammonia metabolism. In: *The Liver Biology and Pathology*, 2nd edn. (Edited by Arias, I.M., Jakoby, W.B., Popper, H., Schachter, D. and Shafritz, D.A.), Raven Press, New York, pp. 317-329.

Rainboth, W. J. (1996) Fishes of the Cambodian Mekong, FAO species. In: *Identification Field Guide for Fishery Purposes. Rome: FAO*. pp. 265.

Randall, D. J. and Wright, P. A. (1989) The interaction between carbon dioxide and ammonia excretion and water pH in fish. ***Can. J. Zool***., **67**: 2936-2942.

Randall, D. J. and Wicks, B.J. (2000) Fish: ammonia production, excretion and toxicity. *Proceedings of the Fifth International Symposium on Fish Physiology, Toxicology and Water Quality*, 10-13 November, 1998, City University of Hong Kong, pp. 41-50.

Randall, D.J. and Wright, P.A. (1987) Ammonia distribution and excretion in fish. ***Fish Physiol. Biochem***., **3**: 107-120.

Randall, D. J. and Perry, S. F. (1992) Catecholamines. In: *Fish Biology, Vol. 12B*, (Edited by Hoar, W. S. and Randall, D. J.), Academic Press, New York, pp.255-300.

Randall, D.J., Wilson, J.M., Peng, K.W., Kok, T.W.K., Kuah, S.S.L., Chew, S.F., Lam, T.J. and Ip, Y.K. (1999) The mudskipper, *Periophthalmodon schlosseri* activively transports $NH_4^+$ against a concentration gradient. ***Am. J. Physiol***., **46**: R1562-R1567.

Randall, D.J., Wood, C.M., Perry, S.F., Bergman, H., Maloiy, G.M.O., Mommsen, T.P. and Wright, P.A. (1989) Urea excretion as a strategy for survival in a fish living in a very alkaline environment. ***Nature***, **337**: 165-166

Ratner, S. (1955) Enzymic synthesis of arginine (condensing and spitting enzymes). In: *Methods of Enzymology*, Vol. II (Edited by Colowick, S. P. and Kaplan, N. O.), Academic Press, New York, pp. 356-367.

Reichenbach-Klinke, H.H. (1967) Untersuchungen uber die Einwirkung des Fischorganismus auf den Fischorganismus. ***Archiv. Fischereiwissenschaft***, **17**: 122-132.

Reid, S. G., Bernier, N. J. And Perry, S. F. (1998) The adrenergic stress response in fish: control of catecholamine storage and release. ***Comp. Biochem. Physiol***. **120C**: 1-27.

Roa, V. L.R., Audet, R. M. and Butterworth, R. F. (1997) Portacaval shunting and hyperammonemia stimulate the uptake of L-[$^3$H] arginine but not of L -[$^3$H] nitroarginine into rat brain synoptosomes. ***J. Neurochem***., **68**: 337-343.

Rose, C.,Michalak, A., Ramarao, K. V., Quack, G., Kircheis, G. and Butterwoth, R.F. (1999) L-Ornithine-L-aspartate lowers plasma and cerebrospinal fluid ammonia and prevents brain edema in rat with acute liver failure. ***Hepatology***, **30**: 636-640.

Rose, C. (2002) Increased extracellular brain glutamate in acute liver failure: decreased uptake or increased release? ***Meta. Brain Disease***, **17**:251-261

Rozemeijer, M J. C. and Plaut, I. (1993) Regulation of nitrogen excretion of the amphibious blenniidae *Alticus kirki* (Guenther, 1868) during emersion and immersion. ***Comp. Biochem. Physiol***., **104A**: 57-62.

Schmalhausen, I. I. (1968) *The Origin of Terrestrial Vertebrates*. Academic Press, New York, pp. 314.

Saha, N. and Ratha, B.K. (1989) Comparative studies of ureogenesis in freshwater, air-breathing teleosts. ***J. Exp. Zool***., **252**: 1-8.

Saha, N. and Ratha, B.K. (1990) Alterations in excretion pattern of ammonia and urea in a freshwater air-breathing teleost, *Heteropneustes fossilis* (Bloch) during hyper-ammonia stress. ***Indian J. Exp. Biol***., **28**: 597-599.

Saha, N. and Ratha, B.K. (1994) Induction of ornithine-urea cycle in a freshwater teleost, *Heteropneustes fossilis*, exposed to high concentrations of ammonium chloride. ***Comp. Biochem. Physiol***., **108B**: 315-325.

Saha, N., Dkhar, J. and Ratha, B. K. (1995) Induction of ureogenesis in perfused liver of a freshwater teleost, *Heteropneustes fossilis*. ***Comp. Biochem. Physiol***., **116B**: 57-63.

Saha, N. and Ratha, B.K., (1998) Ureogenesis in Indian air-breathing teleosts: adaptation to environmental constraints. ***Comp. Biochem. Physiol***., **120A**, 195-208.

Saha, N., Das, L. and Dutta, S. (1999) Types of carbamyl phosphate synthetase and subcellular localization of urea cycle and related enzymes in air-breathing walking catfish, *Clarias batrachus*. ***J. Exp. Zool***., **283**: 121-130.

Saha, N., Das, L., Dutta, S. and Goswami, U.C. (2001) Role of ureogenesis in the mud-dwelled singhi catfish (*Heteropneustes fossilis*) under condition of water shortage. ***Comp. Biochem. Physiol***., **128A**: 137-146.

Saha, N., Kharbuli, Z.Y., Bhattacharjee, A., Goswami, C. and Häussinger, D. (2002a) Effect of alkalinity (pH 10) on ureogenesis in the air-breathing walking catfish, *Clarias batrachus*. ***Comp. Biochem. Physiol***., **132A**: 353-364.

Saha, S., Dutta, S. and Bhattacharjee, A. (2002b) Role of amino acid metabolism in an air-breathing catfish, *Clarias batrachus* in response to exposure to a high concentration of exogenous ammonia. ***Comp. Biochem. Physiol.*** **133 B**: 235–250.

Saha, N., Datta, S., Biswas, K. and Kharbuli, Z.Y. (2003) Role of ureogenesis in tackling problems of ammonia toxicity during exposure to higher ambient ammonia in the air-breathing walking catfish *Clarias batrachus*. ***J. Biosci***., **28**: 733-742.

Saha, N and Ratha, B. K. (2007a) Functional ureogenesis and adaptation to ammonia metabolism in Indian freshwater air-breathing catfishes. ***Fish Physiol. Biochem***., **33**: 283-295.

Saha, N., Datta, S., Kharbuli, Z. Y., Biswas, K. and Bhattacharjee, A. (2007b) Air-breathing catfish, *Clarias batrachus* upregulates glutamine synthetase and carbamyl phosphate synthetase III during exposure to high external ammonia. ***Comp. Biochem. Physiol***., **147B**: 520-530.

Salvatore, F., Zappia, V. and Costa, C. (1965) Comparative biochemistry of deamination of L-amino acids in elasmobranch and teleost fish. ***Comp. Biochem. Physiol***., **16**: 303-309.

Sayer, M.D.J. and Davenport, J. (1991) Amphibious fish: why do they leave water? ***Rev. Fish Biol. Fish***., **1**: 159-181.

Sayer, M. J. D. (2005) Adaptations of amphibious fish for surviving life out of water. ***Fish and Fisheries***, **6**: 186-211.

Sears, E.S., McCandless, D.W. and Chandler, M.D. (1985) Disruption of the blood-brain barrier in hyperammonemic coma and the pharmacologic effects of dexamethasone and difluoromethyl ornithine. ***J. Neurosci. Res***., **14**: 255-261.

Shankar, R. A. and Anderson, P. M. (1985) Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. ***Arch. Biochem. Biophys***., **239**: 248-259.

Shih, J. P. (1940) On the foods of *Monopterus*: ***Sinensia*** **11**: 573-576.

Smart, G.R. (1976) The effect of ammonia exposure on gill structure of the rainbow trout. ***J. Fish Biol***., **8**: 471-479.

Smart, G.R. (1978) Investigations of the toxic mechanisms of ammonia to fish-gas exchange in rainbow trout (*Salmo gairdneri*) exposed to acutely lethal concentration. ***J. Fish Biol***., **12**: 93-104.

Sousa, R.J. and Meade, T.L. (1977) The influence of ammonia on the oxygen delivery system of Coho salmon haemoglobin. ***Comp. Biochem. Physiol***., **58A**: 23-58.

Storer, G. H. (1967) Starvation and effects of cortisol in the gold fish (*Carassius auratus*). ***Comp. Biochem. Physiol***., **20**: 939-948.

Sugden, P. H. and Newsholme, E.A. (1975) The effect of ammonium, inorganic phosphate and potassium ions on the activity of phosphofructokinases from muscle and nervous tissues of vertebrates and invertebrates. ***Biochem. J***., **150**: 113-122.

Sukumaran, N. and Kutty, M. N. (1977) Oxygen consumption and ammonia excretion in the catfish *Mystus armatus*, with special reference to swimming speed and ambient oxygen. ***Proc. Indian Acad. Sci***., **86B**: 195-206.

Szatkowski, M., Barour, B. and Attwell, D. (1990) Nonvesicular release of glutamate from glial cells by reverved electrogenic glutamate uptake. ***Nature***, **384**: 443-446.

Tay, A.S.L., Chew, S.F. and Ip, Y.K. (2003) The swamp eel *Monopterus albus* reduces endogenous ammonia production and detoxifies ammonia to glutamine during 144h of aerial exposure. ***J. Exp. Biol***., **206**: 2473-2486.

Taylor, E. (2000) Effects of exposure to sublethal levels of copper on brown trout: Mechanisms of ammonia toxicity. *Proceedings of the Fifth International Symposium on Fish Physiology, Toxicology and Water Quality,* 10-13 Nov, 1998, City University of Hong Kong, pp. 51-68.

Thurston, R.V., Russo, R.C. and Smith, C.E. (1978) Acute toxicity of ammonia and nitrite to cutthroat trout fry. ***Trans. Am. Fish. Soc***., **107**: 361-368.

Thurston, R.V., Russo, R.C. and Vinogradov, G.A. (1981) Acute toxicity to fishes: The effect of pH on the toxicity of the un-ionized ammonia species. ***Environ. Sci. Tech***., **15**: 837-840.

Tsui, T.K.N., Randall, D.J., Chew, S.F., Jin, Y., Wilson, J.M. and Ip, Y.K. (2002) Accumulation of ammonia in the body and $NH_3$ volatilization from alkaline regions of the body surface during ammonia loading and exposure to air in the weather loach *Misgurnus anguillicaudatus* . ***J. Exp. Biol***., **205**: 651-659

van Waarde, A (1983) Aerobic and anaerobic ammonia production by fish. ***Comp. Biochem. Physiol***., **74B**: 675-684.

Vesce, S., Bezzi, P. and Volterra, A. (2001) Synaptic transmission with glia. ***News Physiol. Sci***., **16**: 178-184.

Vijayan, M. M. and Moon, T.W. (1994) The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. ***Gen. Comp. Encr***., **96**: 75-84.

Vijayan, M. M., Foster, G. D. and Moon, T. W. (1993) Cortisol implantation alters hepatic metabolism and hormonal responsiveness in the sea raven. ***Fish Physiol. Biochem***., **12**: 327-335.

Vijayan, M. M., Mommsen, T. P., Glemet, H. C. and Moon, T. W. (1996) Metabolic effects of cortisol treatment in a marine teleost, the sea raven. ***J. Exp. Biol***., **199**: 1509-1514.

Vijayan, M. M., Pereira, C., Grau, E. G. and Iwama, G. K. (1997) Metabolic responses associated with confinement stress in tilapia: the role of cortisol. ***Comp. Biochem. Physiol***., **116C**: 89-95.

Vijayan, M. M., Ballantyne, J. S. and Leatherland, J. F. (1991) Cortisol-induced changes in some aspects of the intermediary metabolism of *Salvelinus fontinalis*. ***Gen. Comp. Endocr***., **82**: 476-486.

Vijayan, M. M., Mommsen, T.P., Glemet, H. C. and Moon, T. W. (1996) Metabolic effects of cortisol treatment in a marine teleost, the sea raven. ***J. Exp. Biol***., **199**: 1509-1514.

Vijayan, M. M., Pereira, C., Grau, E.G. and Iwama, G. K. (1997) Metabolic responses associated with confinement stress in tilapia: the role of cortisol. ***Comp. Biochem. Physiol***., **116C**: 89-95.

Waarde, A. and Henegouwen, M. D. W. (1982) Nitrogen metabolism in goldfish, *Carassius auratus* (L.) pathway of aerobic and anaerobic glutamate oxidation in goldfish liver and muscle mitochondria. ***Comp. Biochem. Physiol***. **72B**: 133-136.

Waarde, A.V., Kesbeke, F. (1982) Nitrogen metabolism in goldfish, *Carassius auratus* (L): activities of amidase and amide synthetase in goldfish tissues. ***Comp. Biochem. Physiol***., **71B**: 599–603.

Walsh, P. J. and Milligan, C. J., (1995) Effects of feeding on nitrogen metabolism and excretion in the gulf toadfish (*Opsanus beta*). ***J. Exp. Biol***. **198**:1559-1566.

Walsh, P. J., Mayer, G.D., Medina, M., Bernstein, M. L., Barimo, J.F., and Mommsen, T. P. (2003) A second glutamine synthetase gene with expression in the gills of the Gulf toadfish (*Opsanus beta*). ***J. Exp. Biol***., **206**: 1523-1533.

Walsh, P. J., Tucker, B.C., Hopkins, T. E. (1994) Effects of confinement and crowding on ureogenesis in the gulf toadfish *Opsanus beta*. ***J. Exp. Biol***., **191**: 195–206.

Walsh, P.J. (1997) Evolution and regulation of urea synthesis and ureotely in (Batrachoidid) fishes***. Ann. Rev. Physiol***., **59**: 299-323.

Walsh, P.J. (1998) Nitrogen excretion and metabolism. In: *The Physiology of Fishes*, 2nd edn. (Edited by Evans, D.H.), CRC Press, Boca Raton, pp. 199-214.

Walsh, P.J., Tucker, B.C. and Hopkins, T.E. (1994) Effects of confinement/crowding on ureogenesis in the gulf toadfish *Opsanus beta*. ***J. Exp. Biol***., **191**: 195-206.

Walton, M.J. and Cowey, C.B. (1977) Aspects of ammoniogenesis in rainbow trout, *Salmo gairdneri*. ***Comp. Biochem. Physiol***., **57B**: 143-149.

Walton, M.J. and Cowey, C.B. (1982) Aspects of intermediary metabolism in salmonid fish. ***Comp. Biochem. Physiol***., **73B**: 59-79.

Wang, Y. and Walsh, P.J. (2000) High ammonia tolerance in fishes of the family Batrachoididae (toadfish and midshipmen). ***Aquat. Toxicol***., **50**: 205–219.

Watts, R.L. and Watts, D.C. (1974) Nitrogen metabolism in fishes. In: *Chemical Zoology*, Vol. 8 (Edited by Florkin M. and Scheer B.T.), Academic Press, New York, pp. 369-446.

Welch, W. J. (1993) How cells response to stress. ***Sci. Am***. **268**: 56-64.

Wendelaar Bonga, S. E., 1997. The stress response in fish. ***Physiol. Rev***. **77**: 591-625.

Whiting, S.J. and Wiggs, A.J. (1977) Effect of nutritional factors and cortisol on tyrosine aminotransferase activity in liver of brook trout, *Salvelinus fontinalis* Mitchill. ***Comp. Biochem. Physiol***., **58B**: 189-193.

Wilkie, M. P. and Wood, C. M. (1991) Nitrogeneous waste excretion, acid-base regulation and ion-regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. ***Physiol. Zool***., **64**: 1069-1086.

Wilkie, M. P. and Wood, C. M. (1994) The effect of extremely alkaline water (pH 9. 5) on rainbow trout gill function and morphology. ***J. Fish Biol***., **45**: 87-98.

Wilkie, M. P. and Wood, C. M. (1996) Recovery from high pH exposure in rainbow trout: white muscle ammonia storage, ammonia washout and the restoration of blood chemistry. ***Physiol. Zool***. **68**: 379-401.

Wilkie, M.P. (1997) Mechanisms of ammonia excretion across fish gills. ***Comp. Biochem. Physiol***., **118A**: 39-50.

Wilson, J. M., Iwata, K., Iwama, G. K., Randall, D. J. (1998) Inhibition of ammonia excretion and production in rainbow trout during severe alkaline exposure. ***Comp. Biochem. Physiol***., **121B**:99-100.

Wilson, J. M., Randall, D. J., Donowitz, M., Vogl, W. A. and Ip, Y. K. (2000) Immunolocalisation of ion-transport proteins to branchial epithelium mitochondrial-rich cells in the mudskipper (*Periophthalmodon schlosseri*). ***J. Exp. Biol***., **203**: 2297-2310.

Wilson, R.P. (1973) Nitrogen metabolism in channel catfish, *Ictalurus punctatus* I: tissue distribution of aspartate and alanine aminotransferases and glutamate dehydrogenase. ***Comp. Biochem. Physiol***., **46B**: 617–624.

Wilson, R.W., Wright, P.A., Munger, S. and Wood, C.M. (1994) Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for $Na^+$- $NH_4^+$ exchange. ***J. exp Biol***., **191**:37- 58.

Wood, C.M. (1993) Ammonia and urea metabolism and excretion. In: *The Physiology of Fishes* (Edited by Evans, D.H.), CRC Press, Boca Raton, pp. 379-425.

Wright, P. and Wood, C. M. (1985) An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockage. ***J. Exp. Bio***., **114**: 329-353.

Wright, P.A. (1995) Nitrogen excretion: Three end products, many physiological roles. ***J. Exp. Biol***., **198**: 273-281.

Wright, P.M., Wright, P.A. (1996) Nitrogen metabolism and excretion in bullfrog (*Rana catesbeiana*) tadpoles and adults exposed elevated environmental ammonia levels. ***Physiol. Zool***., **69**: 1057–1078.

Yesaki, T. Y. and Iwama, G. K. (1992) Some effects of water hardness on survival, acid-base regulation, ionregulation and ammonia excretion in rainbow trout in highly alkaline water. ***Physiol. Zool***., **65**: 763-787.

Yoshitaka, H., Yoshiki, S., Naota, F., Hiroe, N., and Hikiru, M. (2006) Preoparative glutamine administration induces heat-shock protein 70 expression and attenuates cardiopulmonary bypass-induced inflammatory response by regulating nitric oxide synthetase activity. ***Circulation***, **106**: 2601-2607.

Zhang, H. and Yooung, A. P. (1991) A single upstream glucocorticoid response element juxtaposed to an AP1/ATF/CRE-like site renders the chicken glutamine synthetase gene hormonally inducible in transfected retina. ***J. Biol. Chem***., **266**: 24332-24338.