ROLE OF UREOGENESIS IN A FRESHWATER AIR-BREATHING CATFISH, *Clarias batrachus* UNDER DIFFERENT ENVIRONMENTAL CONSTRAINTS

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Declaration

I, Ms. Lipika Das hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of my 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 degree in any other University /Institute.

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2. Saha, N. and Das, L. (1999) Stimulation of ureogenesis in the perfused liver of an Indian air-breathing catfish, *Clarias batrachus*, infused with different concentrations of ammonium chloride. ***Fish Physiology and Biochemistry***, **21**: 303-311.

INTRODUCTION

Development of diverse metabolic strategies has been the key to the evolution of living organisms under various environmental conditions. The success of a group under a particular niche, has been due to its having appropriate adaptational flexibility in the metabolic strategies. Nitrogen metabolism is considered to be one of the most sensitive physiological systems showing adaptive responses to environmental variations. Accordingly, the nature of major nitrogen excretory products in animals have altered with the evolution of vertebrates from water to the land habitat (Cohen, 1976, Campbell, 1991). Consistent with the aquatic habitat, teleosts excrete ammonia as the major nitrogen excretory product resulting from the catabolism of dietary or structural proteins, and amino acids for the purpose of energy production (Delaunay, 1931; Forster and Goldstein, 1969; Baldwin, 1970; Walton and Cowey, 1977, 1982; Randall and Wright, 1987; Saha *et al.*, 1988; Campbell, 1991; Wood, 1993). Dietary intake of proteins by animals provides amino acids in excess of the amount required for the synthesis of new proteins to sustain protein turnover. Therefore, excess of amino acids which cannot be stored as proteins, as can be carbohydrates as glycogen and lipids as fat, are metabolized. Excess amino acids are deaminated and the carbon residues are either oxidized via the TCA cycle for energy production or used in glycogenesis or lipogenesis. Ammonia, which is produced from deamination of amino acids, is highly toxic and due to its toxicity even at a low concentration *in vivo* it cannot be retained inside the body for longer time, so it is either to be excreted directly or converted to some less toxic compounds such as urea, uric acid or amino acids (Copper and Plum, 1987; Campbell, 1991; Wood, 1993).

Ammonia is a common pollutant in inland waters, and its toxicity to fishes has been a subject of extensive laboratory studies and also reviews (Alabaster and Lloyd, 1980; Haywood, 1983; Randall and Wright, 1987; Wood, 1993; Saha and Ratha, 1998).

Therefore, ammonia needs to be excreted out in a very diluted form due to its high toxicity. In general, however, aquatic animals can tolerate more elevated levels of blood ammonia than terrestrial animals. Plasma total ammonia (NH_3 +NH_4^+) normally remains between 0.05 and 2 mM in most teleosts fishes (Campbell and Anderson , 1991; Wood 1993; Saha and Ratha, 1998), with the exception of singhi catfish, where it has been reported at levels of up to 4 mM in higher ambient ammonia concentration (Saha and Ratha, 1990,1994,1998). In contrast, blood ammonia levels greater than 0.05 mM can be toxic to central nervous system of most mammals (Copper and Plum, 1987; Mommsen *et al.*, 1991).

In teleosts, ammonia is usually excreted out to ambient water medium by diffusion through the gills immediately after its formation (Smith, 1929; Forster and Goldstein, 1969; Watts and Watts, 1974; Kormanik and Cameron, 1981; Evans and Cameron, 1986; Campbell, 1991; Wood, 1993; Wilkie, 1997). Most of the endogenous ammonia are formed in liver and some in kidney of fish mostly by transdeamination process (Pequin and Serfaty, 1963; Vellas and Serfaty, 1974; Walton and Cowey, 1982). In terrestrial animals, where ammonia excretion become difficult, due to non-availability of sufficient water, ammonia is either converted to urea or some other compounds for temporary storage and are excreted out mainly through urine using lesser amount of water (Cohen, 1976; Hoar 1983; Campbell, 1991; Wood, 1993; Anderson, 1995a). Insoluble uric acid is found to be the excretory products of those animals where conservation of metabolic water is highly essential due to their arid environment (Hoar, 1983; Nener, 1988; Powers and Meister, 1988; Campbell, 1991; Wood, 1993).

Based on the type of primary nitrogenous excretory products, animals have been classified into three different groups:

(i) **<u>Ammoniotelic</u>** : Animals which excrete ammonia as the major excretory product as in most aquatic animals.

(ii) **Ureotelic** : Animals which excrete urea as the major excretory products as in mammals and amphibians.

(iii) **Uricotelic** : Animals which excrete uric acid as the major excretory products as in insects, birds and reptiles.

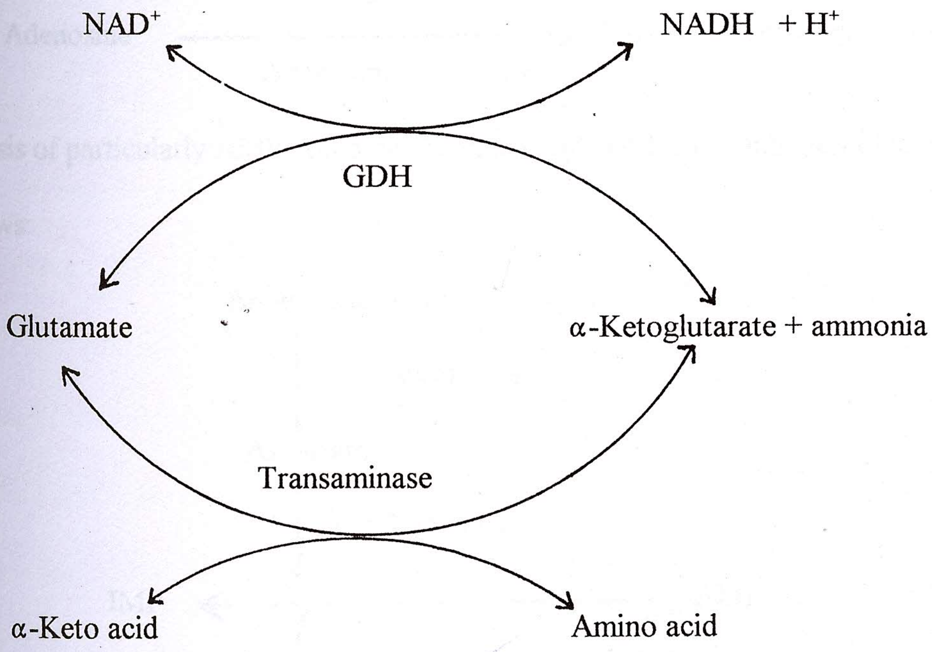
It is not necessary that all animals will fall neatly into one of these categories or another. They may have mixed patterns of nitrogen excretion, depending upon their physiological and environmental conditions. For example, amphibians, which can live both in land as well as in water, excrete both ammonia and urea. They are ammoniotelic in water and ureotelic on land. The tadpoles living in water are ammoniotelic, and when they metamorphose into adult, they become ureotelic especially during their stay in amphibious or land habitat.

Ammonia as a nitrogen excretory product has many advantages. There is no expenditure of energy for the conversion of protein nitrogen to ammonia. Instead, some of the reactions involved in the formation of ammonia such as the deamination of glutamate through glutamate dehydrogenase (GDH) ultimately produces energy (Bessman and Pal, 1976). Due to its small size, high solubility in water and higher partition coefficient, ammonia is easily eliminated by diffusion (Forster and Goldstein, 1969). Evans and Cameron (1986) have demonstrated the ability of NH_4^+ to exchange with Na^+ absorption by the gills of freshwater fishes. In freshwater fishes the exchange of NH_4^+ for Na^+ serves dual purpose of elimination of nitrogenous waste products as NH_4^+ and absorption of Na^+ from the external medium.

Formation of ammonia:

Ammonia can be formed by several pathways namely via deamination of amino acids, amides, purines, pyriminidines and hexosamines, and through transdeamination of amino acids (Cohen and Brown, 1960; Walton and Cowey, 1977, 1982; Randall and Wright, 1987).

Transdeamination: The amino group of most of the amino acids, with the exception of histidine, serine, cysteine, is transferred to another keto acid forming a new amino acids. The dissociated amino group tends to be channelized directly or indirectly through the formation of glutamate. Glutamate undergoes oxidative deamination catalyzed by GDH to form ammonia and α-ketoglutarate (Krebs *et al.*, 1978). The overall reaction in the liberation of ammonia from amino acids via glutamate formation is known as transdeamination (Braunstein, 1939), which may be summarized in the following reaction:



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

Deamination: Van slyke *et al.* (1943) showed that glutamine, an amide, helps for temporary storage and transport of ammonia in animals. Glutamine is deaminated through hydrolytic removal of secondary amino group by the enzyme glutaminase which is found either as phosphate dependent (PDG) or phosphate independent (PIG) forms.

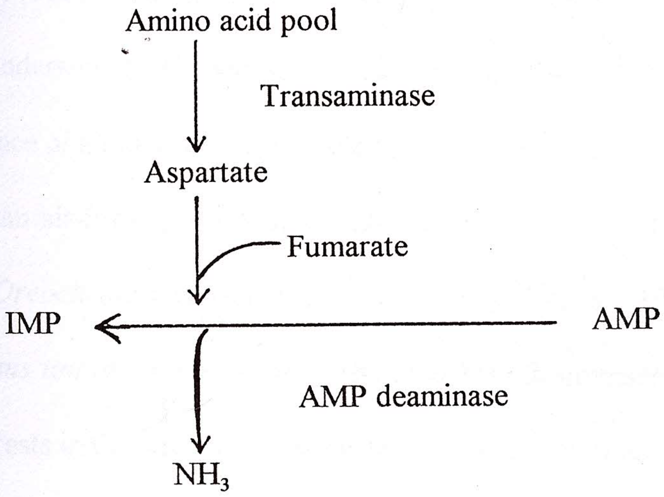
$$\text{Glutamine} + H_2O \xrightarrow[\text{PDG/PIG}]{\text{Glutaminase}} \text{Glutamate} + \text{ammonia}$$

Nucleodeamination: Nucleodeaminases catalyse the deamination of nucleosides and nucleotides to liberate ammonia (Cohen and Brown, 1960).

$$\text{Guanylic acid} \xrightarrow[\text{Guanylic acid deaminase}]{} \text{Xanthylic acid} + \text{ammonia}$$

$$\text{Adenosine} \xrightarrow[\text{Adenosine deaminase}]{} \text{Inosine} + \text{ammonia}$$

Hydrolysis of particularly AMP could be ultimately utilized for deamination of amino acid as follows:



The role of AMP deaminase has been shown to be more important in ammonia production in some fishes (Makarewicz and Zydowo, 1962; Makarewicz, 1963) and glutaminase in some others (Walton and Cowey, 1977).

***Urea synthesis in fish*:**

Although teleosts are primarily ammoniotelic, some amount of urea have been reported to excrete as nitrogenous excretory product (Holmes and Donaldson, 1969; Saha *et al.*, 1988; Saha and Ratha, 1989, 1998) and also in tissues of several fish species (Smith, 1929; Burrows, 1964; Alexander *et al.*, 1968; Goldstein and Forster, 1971; Brett and Groves, 1979; Vellas, 1981; Hoar, 1983; Ramaswamy and Reddy, 1983; Saha and Ratha, 1989, Campbell, 1991; Anderson, 1995a) besides marine fishes (where urea production and retention serves the purpose of osmoregulation). The formation of urea in fish has been suggested to be through either one or more of these pathways such as (i) the ornithine-urea cycle (here after referred to as urea cycle), (ii) the uricolytic pathways, and (iii) the catabolism of dietary arginine (Fig. 1).

The sources of urea in teleosts and the involvement of urea cycle is still under useful debate. Until recently, the presence of a functional urea cycle, which appeared to be the major source of urea formation in higher vertebrates was not known to exist in teleosts (Campbell and Anderson, 1991; Mommsen and Walsh, 1991; Wood, 1993; Anderson, 1995a). The presence of a functional urea cycle has been reported in various teleosts, such as, in some Indian air-breathing teleosts (Saha and Ratha, 1987, 1989), alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami* (Randall *et al.*, 1989), and in marine toadfishes, *Opsanus tau and Opsanus beta* (Read, 1971; Mommsen and Walsh, 1989). Accordingly, interests in the study of urea cycle, expression of urea cycle during early

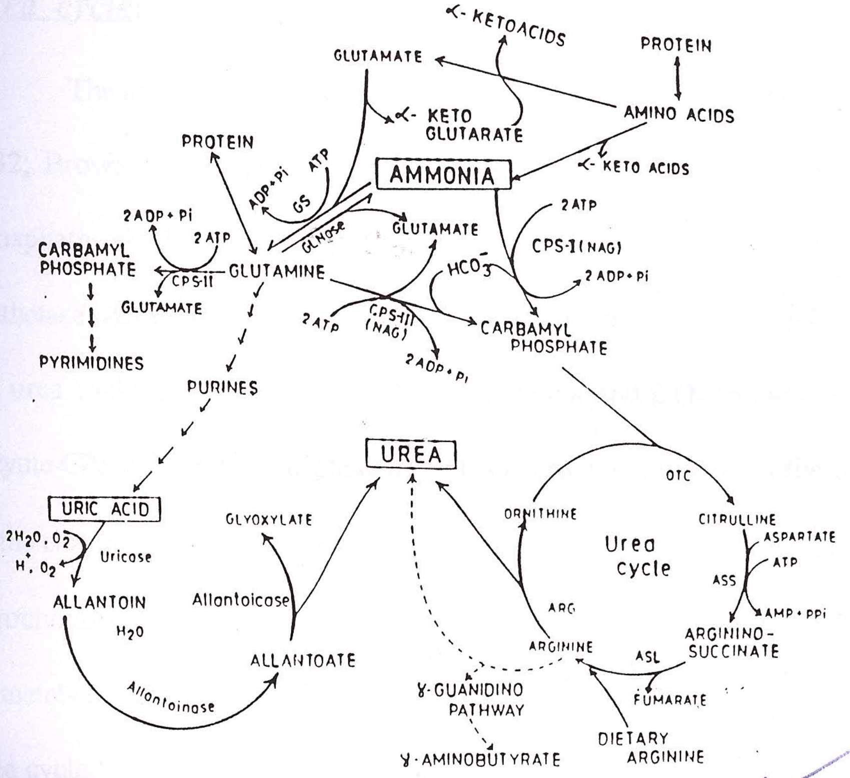


Fig. 1. Outlines of different pathways of urea synthesis in fish.

CPS - carbamyl phosphate synthetase; OTC - ornithine trans-carbamylase; ASS - argininosuccinate synthetase; ASL - arginino succinate lyase; ARG - arginase; GDH - glutamate dehydrogenase; GS - glutamine synthetase; GLNase - glutaminase; NAG - N-acetyl-L-glutamate embryonic developmental stages, regulation of expression of urea cycle enzymes, and nitrogen excretion patterns under different environmental constraints in different teleosts have recently been increased.

Urea cycle:

The urea cycle involves a series of five enzymatic reactions (Krebs and Henseleit, 1932; Brown and Cohen, 1959) (Fig. 1). The five enzymes of the urea cycle are carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG). The first reaction of urea cycle involves the fixation of ammonia and CO_2 to carbamyl phosphate by the enzyme CPS. Carbamyl phosphate is then converted to citrulline in the presence of ornithine by the enzyme OTC. Both these reactions in the ureotelic vertebrates take place inside the mitochondria, and the citrulline produced is transported to the cytosol. It is converted ultimately to urea and ornithine by other three cytosolic enzymes (ASS, ASL and ARG) of urea cycle.

The presence of a functional urea cycle in elasmobranchs and lungfishes (Brown and Cohen, 1959; Forster and Goldstein, 1966; Huggins *et al.*, 1969; Schooler *et al.*, 1966; Janssens and Cohen, 1966), and in marine teleosts (Huggins *et al.*, 1969; Read, 1971; Mommsen and Walsh, 1989) have been reported. Brown and Cohen (1960) could not detect the CPS and OTC activity in several species of freshwater teleosts studied. Huggins *et al.* (1969) could detect all the enzymes of the urea cycle enzymes in some freshwater teleosts, but their activities were so low that no physiological significance could be attributed to them. They divided the urea producing animals into three categories depending on the role of urea synthesis. These three groups are:

Ureogenic : Species which have the potential for urea synthesis via urea cycle due to the presence of all the urea cycle enzymes activity, although for various reasons, its synthesis may be repressed in freshwater teleosts.

Ureotelic : These animals are ureogenic and synthesize sufficient urea by urea cycle to account for the bulk of nitrogen excretion.

Ureosmotic : These animals produce urea via urea cycle for maintaining the osmotic equilibrium with environment.

Uricolytic pathway :

Another source of urea in teleosts could be purine degradation or uricolytic pathways which was first reported by Brunel (1937) in fish species (Fig. 1) . Adenine and guanine produce uric acid as a catabolic product, which further breaks down in a three step uricolytic pathway involving three enzymes such as uricase, allantoinase and allantoicase to produce urea in most of the teleosts (Forster and Goldstein, 1969; Watts and Watts, 1974). Cvancara (1969a) could find relatively high levels of activity of uricase in nineteen species of freshwater teleosts and suggested that degradation of purines and nucleic acids might account for urea production at the levels of which it is found in the blood and excreted in teleosts. Saha and Ratha (1987) reported the presence of all the three uricolytic enzymes at least in liver of a freshwater air-breathing teleost, *Heteropneustes fossilis* and suggested that uricolysis could be one of the pathways for the formation of urea in this fish in addition to urea cycle.

Dietary arginine :

Arginase (ARG), the last enzyme of the urea cycle, which converts arginine to urea and ornithine, has been reported to be present in various tissues of freshwater teleosts such as in liver (Hunter, 1929; Brown and Cohen, 19 0; Huggins *et al.*, 1969; Cvancara, 1969b, 1971; Wilson, 1973), kidney and heart (Hunter, 1929; Cvancara, 1969b), and to a lesser extend in spleen, gills, ovaries, testes and muscle of some teleosts (Cvancara, 1969b). Therefore, it was suggested by various workers that the dietary arginine could be one of the major sources of urea in freshwater teleosts.

Active ureogenesis through urea cycle has been confirmed in amphibians and terrestrial animals (Krebs and Henseleit, 1932; Cohen, 1976), and in marine fishes (Read, 1971; Cohen, 1976; Pang *et al.*, 1977; Read, 1971; Mommsen and Walsh, 1989; Campbell, 1991; Wood, 1993; Anderson, 1995a). However, in freshwater teleosts the presence of a functional urea cycle enzymes could not be detected in many of the teleosts studied (Manderscheid, 1933; Brown and Cohen, 1960; Huggins *et al.*, 1969; Wilson, 1973). Brown and Cohen (1960) could not detect CPS and OTC activity in several species of freshwater teleosts studied by them and therefore, suggested that the genes responsible for synthesizing some of these urea cycle enzymes, whose activities could not be detected got deleted and proposed the "gene deletion" hypothesis. Huggins *et al.* (1969) reported a full complement of urea cycle in a variety of freshwater teleosts but with very low activities, and suggested that the expression of genes responsible for the synthesis of enzymes of urea cycle might have been altered as a result of an adaptational change in freshwater teleosts when the excretion of ammonia was facilitated by diffusion. The presence of a regulatory physiological system for converting ammonia to urea via urea cycle has been well documented in lungfishes (Janssens, 1964; Goldstein *et al.*, 1967), mudskippers (Gordon *et al.*, 1969, 1978), and aquatic amphibians (Janssens and Cohen, 1968; Baldwin, 1970; Balinsky, 1970; Janssens, 1972) during their terrestrial life when the excretion of ammonia is not possible. Goldstein *et al.* (1973) could also detect the activities of all the urea cycle enzymes in a well preserved sample of coelacanth liver which were comparable to those in elasmobranchs.

The lake Magadi (Kenya) tilapia, *O. a. grahami*, which lives in alkaline 'Soda' lake having the water pH of 10 and osmolarity of 525 mOsm/kg, is reported to excrete large amounts of urea rather than ammonia due to having a functional urea cycle (Randall *et al.*, 1989; Wood *et al.*, 1989). This is the only known instance of complete ureotelism in a complete aquatic teleost fish. However, in Lahontan cutthroat, *Oncorhynchus clarki henshawi,* which also live in alkaline water of pH 9.4, the activities of urea cycle enzymes in liver were found too low (Wilkie *et al.*, 1993). High activities of all the urea cycle enzymes in liver of at least four species of freshwater Indian air-breathing teleosts such as *H. fossilis, Clarias batrachus*, *Anabas testudineus* and *Amphipnous cuchia,* and in kidney of three species (except *A. testudineus*) have been reported from our laboratory (Saha and Ratha, 1987, 1989). These fishes are primarily aquatic but breathe predominantly air by frequent surfacing. They usually inhabit stagnant and slow flowing shallow water bodies of ponds and lakes, and live inside the mud during drought conditions and also frequently are being exposed to the air (Beavan, 1982; Jhingran, 1983; Saha and Ratha, 1989). When they get exposed to outside water, an accumulation of toxic ammonia takes place *in vivo,* since ammonia excretion into the surrounding environment is very difficult due to lack of water (for review, see Saha and Ratha, 1998). At least one of the above mentioned species (*H. fossilis*) was found to tolerate very high ambient ammonia (up to 75 mM NH_4Cl) which is unuasual among freshwater teleosts and even for many amphibians (Saha and Ratha, 1990, 1991, 1994, 1998). This singhi catfish appears to be the champion among teleosts to tolerate such a high ambient ammonia. In addition to the presence of a functional and regulatory urea cycle, various other adaptations related to nitrogen metabolism mainly to avoid the ammonia toxicity have also been reported in singhi catfish (details mentioned below).

Subcellular localization of the urea cycle enzymes and the types of CPSs:

Urea is synthesized in different groups of animals via the urea cycle, but for different purposes. In ureotelic species such as in mammals and amphibians, urea is synthesized from ammonia, a toxic metabolite, which is formed by the catabolism of amino acids and proteins, as a readily excretable form (Campbell, 1991; Wood, 1993). In ureo-osmotic marine elasmobranchs (sharks, skates and rays) urea is synthesized via the urea cycle and retained inside the body for osmoregulatory purposes (Perlman and Goldstein, 1988; Goldstein and Perlman, 1995). Nener (1988) has postulated that the urea cycle is highly constrained in terms of enzyme composition and tissue localization among organisms that produce urea for different purposes.

The synthesis of urea via the urea cycle needs both the mitochondrial and cytosolic enzymes. Some differences in the isoenzymic forms and the subcellular localization of some of the urea cycle enzymes in vertebrates have been reported by various workers, and have been correlated with their physiological functions in different groups of animals. There are three different types of carbamyl phosphate synthetase (CPSs) which have been identified till date (for review, see Anderson, 1995a,b) (Fig. 1). The carbamyl phosphate formed by CPS is the precursor for two major metabolic pathways, the urea cycle (and/or arginine biosynthesis) and pyrimidine nucleotide biosynthesis. The first step of the urea cycle (ammonia fixation) in mammalian and amphibian ureotelic species is catalyzed by CPS I by the following reaction:

$$\mathrm{NH_3} + 2\mathrm{ATP} + \mathrm{HCO_3^-} \xrightarrow[\mathrm{NAG,\ Mg_2^+}]{\mathrm{CPS\ I}} \text{Carbamyl phosphate} + 2\mathrm{ADP} + \mathrm{Pi}$$

CPS I is localized in the mitochondrial matrix and utilizes only ammonia as the nitrogen donating substrate for carbamyl phosphate synthesis and requires the presence of N-acetyl-L-glutamate (NAG) as an allosteric activator for activity. CPS II, which is responsible for pyramidine nucleotide biosynthesis, utilizes glutamine as the physiologically significant nitrogen-donating substrate, does not require NAG for activity (and activity is not affected by the presence of NAG), is subjected to allosteric inhibition by UTP, and is localized in the cytosol of many tissues. The reaction takes place as follows:

$$\text{Glutamine} + \mathrm{H_2O} + 2\mathrm{ATP} + \mathrm{HCO_3^-} \xrightarrow[\mathrm{Mg_2^+}]{\mathrm{CPS\ II}} \text{Carbamyl phosphate} + 2\mathrm{ADP} + \mathrm{Pi} + \text{Glutamate}$$

Another type of CPS, the CPS III was first reported by Trammel and Campbell (1970,1971) in several species of invertebrates. Like CPS I, CPS III is a mitochondrial enzyme, requires NAG for activity, and is not affected by allosteric effectors common to CPS II. However, like CPS II, CPS III utilizes glutamine as the nitrogen-donating substrate (Campbell and Anderson, 1991; Anderson, 1994, 1995a,b). The reaction takes place as follows:

$$\text{Glutamine} + \mathrm{H_2O} + \mathrm{HCO_2^-} + 2\mathrm{ATP} \xrightarrow[\mathrm{NAG,\ Mg_2^+}]{\mathrm{CPS\ III}} \text{Carbamyl phosphate} + 2\mathrm{ADP} + \mathrm{Pi} + \text{Glutamate}$$

Earlier studies aimed at identifying CPS activity in fish reasonably assumed that activity related to urea cycle would be a CPS I. However, Anderson (1976) reported the presence of CPS III activity in liver of *Micropterus salmoides* (largemouth bass), a freshwater fish and, at much higher levels, in liver of ureosmotic marine elasmobranchs and a holocephalan (Anderson, 1980). The latter studies also established the presence of CPS III activity in freshwater elasmobranch, *Potamotrygon circularis* (freshwater stingray) and the marine teleost, *Porichthys notatus* (plainfin midsipman).

CPS III from the spiny dogfish (*Squalus acanthias*) and largemouth bass (*M. salmoides*), representatives of marine ureosmotic elasmobranchs and freshwater ammoniotelic teleosts, respectively, have been isolated and characterized (Anderson, 1980; Casey and Anderson, 1983). The properties of both are very similar to CPS I, except that glutamine serves as the nitrogen-donating substrate. Ammonia can replace glutamine as the nitrogen-donating substrate, but the K_m is quite high; unlike CPS II and most other amidotransferases, however, the V_{max} with ammonia as substrate is less than one-fourth that attained with glutamine.

CPS III activity has been reported in several other teleost species and in coelacanth (Mommsen and Walsh, 1989; Randall *et al.*, 1989), and also in the Indian air-breathing singhi catfish (*H. fossilis*) from our laboratory (Saha *et al.*, 1997). In ureosmotic elasmobranchs the function of CPS III is clearly reasonable to assume from this and from its structural similarity to CPS I that its function in other fish species (where it is present) is also related to urea cycle. Thus, establishing the presence of CPS III activity is of considerable significance for understanding the nature and function of urea cycle in fish. The limited data available in the literature suggest that the levels of CPS activity in most fish are very low or undetectable. It is possible that in some circumstances the gene may be expressed only during certain portions of a life cycle or only during certain stressful environmental situations, and there may also be considerable individual variation within a given species.

A question related to the foregoing observations is whether CPS III (as opposed to CPS I) is a functional evolutionary trait of all fish. Mommsen and Walsh (1989) reported the presence of CPS III activity in all fish species they examined, which represented a broad range of fish systematics. The levels of activity were not reported, however, except for two toadfish species (*O. beta* and *O. tau*); identification of CPS activity as CPS III was based on the observation of higher activity with glutamine than with ammonia as nitrogen-donating substrate. This definitive study, and the demonstration of CPS III activity in a tilapia fish (*O. a. grahami*) adapted to an alkaline environment (Randall *et al.*, 1989), has led to the current assumption in the literature that CPS III (as opposed to CPS I) activity, is, in fact, an evolutionary trait of all fish (Mommsen and Walsh, 1989, 1991, 1992; Wood, 1993). Current speculation is that CPS I evolved from CPS III (Mommsen and Walsh, 1989; Campbell and Anderson, 1991; Hong *et al.*, 1994; Anderson, 1995a,b)

The second enzyme of the urea cycle, the ornithine transcarbamylase (OTC) has always been localized within the mitochondrial matrix in all ureotelic, ureosmotic, uricotelic and ammoniotelic vertebrates including certain fish species (Ratner, 1973; Gamble and Lehninger, 1973; Vorhaben and Campbell, 1977; Casey and Anderson, 1985; Campbell and Anderson 1991; Dkhar *et al.*, 1991). The third, fourth and fifth enzymes of the urea cycle, argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG), respectively, have been reported to be cytosolic in several ureotelic species (Ratner, 1973; Skrzypek-Osiecka *et al.*, 1980; Jackson *et al.*, 1986). In contrast to ureotelic species, ARG in uricotelic and ammoniotelic species is reported to be mitochondrial (Tsuyama *et al.*, 1980; Taylor and Stewart, 1981; Carvajal *et al.*, 1987; Dkhar *et al.*, 1991). Casey and Anderson (1985) have reported the mitochondrial localization of ARG in ureosmotic elasmobranchs, *S. acanthias*. Mitochondrial localization of ARG has also been reported in the liver of ureogenic gulf toadfish, *O. beta* (Mommsen and Walsh, 1989; Anderson and Walsh, 1994).

Mommsen and Walsh (1989), after studying the urea cycle enzymes in different groups of fish species, suggested that the urea cycle, which is a monophyletic trait in vertebrates, underwent two key changes during the course of vertebrate evolution - i) a switch over from CPS III to CPS I and, ii) replacement of mitochondrial ARG by a cytosolic equivalent.

***Hyper-ammonia stress*:**

As mentioned above, ammonia is highly toxic to most of the living organisms. Therefore, ammonia has to be either eliminated or converted to less toxic compounds to prevent any build-up to harmful concentrations within the body. In teleosts, ammonia is being continuously excreted out as a major nitrogenous waste into the surrounding water mostly through the gills. Therefore, they have been classified as ammoniotelic. In terrestrial animals since excretion of ammonia as such is difficult, ammonia formed from different metabolic processes gets converted to some other less toxic compounds such as urea, uric acid or amino acids for detoxification.

Ammonia toxicity to fish has been primarily attributed to the un-ionized form (NH_3) and the ionized form (NH_4^+) being relatively less toxic (EIFAC, 1970; Alabaster and Lloyd, 1982; Erickson, 1985; WHO, 1986; Hickey and Vickers, 1994). The proportion of un-ionized ammonia increases with increase in pH and temperature (Emerson *et al.*, 1975). Acute ammonia toxicity includes 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), disturbances of ionic balance and acid-base balance (Maetz, 1973; Cameron and Hiesler, 1983; Cameron, 1986; Paley *et al.*, 1993) in fish. Acute toxicity of un-ionized ammonia to mysids and larval inland silversides was influenced by pH and salinity in a specific manner (Miller *et al.*, 1990). Sousa and Meade (1977) proposed that the mechanism of ammonia toxicity involved stimulation of glycolysis by the ammonium ion (NH_4^+) and the simultaneous suppression of krebs cycle due to depletion of α-ketoglutarate, which removes ammonia by amination to form first glutamate, and then glutamine. These two concurrent actions would result in an increase of acidic metabolites from glycolysis and krebs cycle, and would lower blood pH due to accumulation of pyruvate and lactate (Campbell, 1991). The resulting acidemia would shift the oxygen saturation of haemoglobin and cause death by suffocation. The toxic action of ammonia might also involve an osmoregulatory disturbance in channel catfish (Tomasso *et al.*, 1980), as it has been reported to increase the permeability of tissue to water (Dennis, 1966; Lloyd and Orr, 1969). The uncoupling of oxidative phosphorylation by NH_4^+ ion as suggested by Smart (1978) could be another adverse effect of ammonia to inhibit ATP production. Ammonia also affects the membrane potential and excitability of neurons (Cooper and Plum, 1987). Due to this wide ranging toxic effects, ammonia is either immediately excreted out or converted to some less toxic substances such as urea, uric acid or amino acids for temporary storage *in vivo*.

Ammonia toxicity in various ammoniotelic teleosts has been studied extensively and the 96 hr LC_{50} value for unionized ammonia was formed to be well below 0.1 mmol/litre (Haywood, 1983; Thurston *et al.*, 1983 a,b; Dabrowska and Wlasow, 1986; Campbell, 1991).The 48 hr LC_{50} value of total ammonia (TA) for *Cyprinus carpio* was 0.28 mmol/l (Dabrowska and Wlasow, 1986), and the 24 hr LC_{50} value for TA was 0.15 mmol/l for the trout, *Salmo gairdneri* (Olson and Fromm, 1971), whereas for the ureotelic alkaline lake Magadi tilapia, *O. a. grahami* the 24 hr LC_{50} value for TA was 0.75 mmol/ litre (Walsh *et al.*, 1993). The mudskipper, *Periophthalmus cantonensis* is reported to tolerate 15 mmol/litre NH_4Cl (Iwata, 1988).

Reports on the effect of higher ambient ammonia on nitrogen excretion pattern in teleosts are very much limited. Fromm and Gillette (1968) demonstrated that an increase in ambient ammonia from 0 to 8 µg/ml caused an increase in blood ammonia of trout from 40 to 70 µg/ml. At some critical level of blood ammonia, if the fish has to survive, it must either decrease its sensitivity to ammonia or convert the ammonia to a less toxic nitrogenous compound which can either be excreted immediately or stored temporarily until conditions are favourable for excretion. Olson and Fromm (1971) found that goldfish, *Carassius auratus* subjected to increased ambient ammonia level showed increase in urea excretion rate. When the purely ammoniotelic largemouth bass (*M. salmoides*), where the levels of activity of various urea cycle enzymes are very low, was exposed to 0.25 and 1 mmol/litre NH_4Cl, no change in urea-N excretion was observed (Kong *et al.*, 1998). However, in the ammoniotelic, but potentially ureogenic singhi catfish (*H. fossilis*), the ambient TA tolerance limit was found to be many fold higher than that any of these ureogenic and non-ureogenic teleosts (Saha and Ratha, 1990, 1994). To date, this catfish appears to have the maximum capacity of tolerating the external TA (up to 75 mM NH_4Cl) for weeks without mortality (Saha and Ratha, 1990, 1994). One of the major reasons for tolerating such a high ambient ammonia by singhi catfish was suggested to be due to the presence of a functional urea cycle both in hepatic and in some extra-hepatic tissues (Saha and Ratha, 1987) together with the capacity to stimulate ureogenesis under hyper- ammonia stress (Saha and Ratha , 1986, 1990, 1994; Saha *et al.*, 1995). This facultative ureogenic air-breathing singhi catfish also shows a rapid transition from ammoniotelism to ureotelism when exposed to high ambient ammonia (Saha and Ratha, 1986, 1990, 1994). This was accompanied with the higher accumulation of ammonia and urea in different tissues and also the stimulation of some of the urea cycle enzymes both in liver and kidney.

In addition to conversion of toxic ammonia to urea, there could be other means of detoxification of ammonia such as the conversion of ammonia to various non-essential free amino acids (FAAs). Enhanced synthesis and accumulation of non-essential FAAs have recently been shown in the perfused liver of another ureogenic air-breathing catfish, *C. batrachus* under higher ammonia load (Saha *et al.*, 2000). In other fish species, where the ureogenesis is reported to play any significant role for detoxification of ammonia, such as the mudskipper, *P. cantonensis* during exposure to air (Iwata *et al.*,1981) and in higher ambient ammonia (Iwata, 1988), in the carp, *C. carpio* during exposure to higher ambient ammonia (Dabrowska and Wlasow, 1986), and more recently in the marble goby, *Oxyeleotris marmoratus* during exposure to air for a shorter period (Jow *et al.*, 1999), the synthesis of various non-essential FAAs from the accumulated ammonia are reported to play significant role to avoid the accumulation of ammonia to a toxic level *in vivo.*

Adaptation to dehydration stress:

Since excretion of ammonia *per se* is extremely difficult for most animals living outside water, the changes in the pattern of end product of nitrogen metabolism or excretion is absolutely necessary for any aquatic animal to migrate from water to land or for living temporarily outside water for various periods (Gordon, 1970). However, as an exception, some terrestrial snails (Speeg and Campbell, 1968), crabs (DeVries and Wolcott, 1993; Greenaway and Nakamura, 1991) and isopods (Wright and O'Donnel, 1993) excrete significant portions of their nitrogenous wastes by ammonia volatilization.

Gordon (1970) suggested that "nitrogen metabolism is one of the most sensitive physiological systems in its responses to environmental changes". One of the important enzymatic pathways involved in nitrogen metabolism is the urea cycle. The highly toxic nitrogenous metabolic end product ammonia is converted to urea through the urea cycle. Environmental factors such as temperature, water availability and diet have been shown to alter the activity of the urea cycle enzymes (Millman, 1951; Mandelstam and Yudkin,1952; Tillinghast *et al.*, 1969; Nuzum and Snodgrass, 1971).

The effect of water shortage on the urea cycle enzymes and nitrogen excretion patterns have been studied by various workers in amphibians. Purely aquatic frog, *Xenopus laevis* excretes predominately ammonia as the major nitrogenous excretory product while remaining in water. It accumulates large amount of urea when kept out of water or in dilute saline solution (Balinsky, 1981). Janssens and Cohen (1968) reported increase synthesis of urea in *X. leavis.* under conditions of water shortage. Both the rate of urea production and the levels of CPS activity were increased when *X. laevis* was desiccated by exposure to slightly hyper-osmotic saline solutions (McBean and Goldstein, 1970). Balinsky (1970) also reported the increased activity of the urea cycle enzymes in aestivating *X. laevis.* McClanahan (1972) reported the elevation of plasma concentration in *Scaphiopus cauchi* due to urea accumulation while the soil dried up. Urea accumulation in plasma and other tissues was observed during their burrowing period inside the soil in other terrestrial amphibians such as *Bufo viridis* (Katz, 1973; Rick *et al.*, 1980; Degani *et al.*, 1981), *Ambystoma tigrinum* (Delson and Whitford, 1973), *Salamandra salamandra* (Degani, 1981a) and in *Pelobates syriacus* (Degani, 1982)

The African lungfish (*Protopterus aethiopicus*) is entirely dependent on aerial respiration. During drought periods the fish can survive for long periods of time by aestivation in the mud surrounded by a hardened mucous cocoon, which is connected by a tube to the surface for breathing (Smith, 1930). While in water the fish excrete approximately equal amounts of ammonia and urea as end products of nitrogen metabolism. However, during aestivation, to conserve water and preclude ammonia accumulation, ammonia formation ceases and only urea is formed, which is stored in body tissues (accumulating to levels of as high as 3% of the body weight during long periods of aestivation) and releases when the fish returns to an aqueous environment (Smith, 1930). Although all enzymes of both the uricolytic and urea cycle pathways are present, virtually all urea is formed by the urea cycle, the rate of urea formation does not change significantly during the switch from an aqueous environment to aestivation (Janssens, 1964; Brown *et al.*, 1966; Forster and Goldstein, 1966; Janssens and Cohen, 1966, 1968). Mommsen and Walsh (1989) reported that the CPS activity is a CPS I, not a CPS III, in this species of lungfish and that the glutamine synthetase and arginase activities are localized in the cytosol. Janssens and Cohen (1968) were not able to detect glutamine synthetase activity in liver. These observations seem to clearly indicate that the ammonia-dependent urea cycle characteristic of higher vertebrates operates in lungfish.

The Australian lungfish *(Neoceratodus forsteri)* uses its lung only as an accessory breathing organ and cannot survive deprivation of water by aestivation. Accordingly, the level of the urea cycle enzymes and the rate of urea synthesis are dramatically lower than in the African lungfish (Goldstein *et al.*, 1967). Results similar to those described for the African lungfish have been reported for the South American lungfish (*Lepidosiren paradoxa*), which aestivates in a moist cocoon, except that the rates of urea synthesis and levels of urea cycle enzymes were lower, which is consistent with the intermediate environmental position between the African lungfish (aestivates in a dry cocoon) and the Australian lungfish (does not aestivate) (Carlisky and Barrio, 1972; Funkhouser *et al.*, 1972). The latter authors suggested that accumulation of urea during aestivation may serve a second useful function of elevating the vapour pressure and thereby minimizing water loss by evaporation.

Gordon and coworkers (Gordon, 1970; Gordon *et al.*, 1969, 1970, 1978) investigated the changes in nitrogen excretion in an East African mudskipper (*P. cantonensis*) and the Chilean clingfish (*Sicyases sanguineus*). Their findings indicated a shift towards ureotelism while out of water. Subsequent studies by Morii and coworkers (Morii, 1979; Morii *et al.*, 1978, 1979) with two mudskipper species *(P. cantonensis and Boleophthalmus pectinirostris)* concluded that a shift from ammoniotelism to ureotelism during the period out of water does not occur. The explanation for these differences has not been resolved. In both series of studies, however, urea was formed and excreted, but the source of urea was not investigated. Chew and Ip (1987) reported that glutamine synthetase activity could not be detected in two mudskipper species (*P. schlosseri and B. boddaerti*), which would seem to rule out uricolysis or glutamine-dependent CPS III and the urea cycle as sources of urea. Gregory (1977) found that two members of the mudskipper family (*Periophthalmus expeditonium and Periophthalmus gracilis*) and one member of the amphibious *Scartelaos* family (*Scartelaos histophorus*) excreted both urea (up to 33% of total nitrogen excreted) and ammonia, and that liver extracts of the two mudskippers had sufficient uricolytic enzymes activity to account for the urea formed, but that of the five required urea cycle enzymes only arginase and ornithine transcarbamylase could be detected . However, CPS is not active under the assay conditions described (10 mM ATP, 6 mM

Mg^{2+}) (Anderson, 1981; Casey and Anderson, 1983). Another amphibious marine teleost, *Blennius pholis* (L.) (blenny), was found to be predominately ammoniotelic in seawater and during periods of aerial exposure (Davenport and Sayer, 1986). The major route of nitrogen elimination during aerial exposure was via ammonia in mucous secretions. Also, in contrast to reports from studies of other amphibious fish, the blenny (1) apparently does not store nitrogen during aerial exposure and release it as a burst of ammonia and urea after re-immersion, and (2) continues to excrete nitrogen waste when exposed to air.

The Indian air-breathing teleost species are also amphibious according to the definition of Gordon *et al.* (1969): "Amphibious fishes are those which spend periods of time out of water, on or above the ground surface as normal parts of their life histories". Many species of the Indian air-breathing teleosts are known to live for months in a semidry condition inside mud in response to habitat drying (Sayer and Davenport, 1991) and are also able to survive totally outside water for hours ranging from 10 to 100 hr (Saha and Ratha, 1989). Therefore, various adaptations related to nitrogen metabolism are also anticipated in this group of Indian air-breathing fish especially during their amphibious life. Ramaswamy and Reddy (1983) demonstrated a marked shift towards ureotelism from ammoniotelism in two Indian obligatory air-breathing teleosts (*Anabas scandens and Channa gachua*) but not in the facultative air-breathing teleost, *Mystus vittatus* when exposed to air for 5 or 10 hr. A similar transition to ureotelism from ammoniotelism has been reported in singhi catfish (*H. fossilis)*, when exposed to air for 24 hr (Ratha *et al.*, 1995).

Adaptation to alkaline water:

An alkaline environment (pH 8.5-10) causes severe physiological disturbances for most fish, initially inhibiting diffusion of ammonia across the gills, resulting in an increase in plasma ammonia concentration, among other effects (Wright *et al.*, 1990; Wood, 1990; Wilkie and Wood, 1996). In natural waters, exceptionally high pH (above pH 9) are measured in many of the saline lakes world-wide, among them the so called "soda lakes". As a function not only of decreased proton concentration, but also characteristically high alkalinity and rather unusual ionic compositions, biological diversity in soda lakes is generally very poor. Survival of a particular species will depend on a whole set of factors, i.e., the specific physical, chemical and biological characteristics of the habitat must be perfectly matched by numerous adaptations at the biochemical, physiological, anatomical and behavioural levels.

pH values above 9 may also be encountered in marine environments such as intertidal rockpools (Truchot and Duhamel-Jouve, 1980) as well as in freshwater lakes and streams (Jordon and Lloyd , 1964; Murray and Ziebell, 1984; Falter and Cech, 1991). In addition, fish in aquaculture may be accidentally exposed to high pH for instance when calcium carbonate is added to pond water for improved fish production (Bandt, 1935) or unusual phytoplankton blooms occur due to nutrient input in fish ponds (Schreckenbach *et al.*,1975).

Fishes living in highly alkaline waters have had to evolve major modifications with respect to nitrogenous waste excretion. Decreased ambient proton concentration impedes ammonia excretion via the allegedly main route (i.e, the gills) and mechanisms found normally in teleosts. At circumneutral pH (pH 6-8), about 90% of the total nitrogenous waste produced by fish is excreted across the gills, and ammonia excretion usually accounts for about 85% of this total (for review, see Wood, 1993). Urea excretion generally makes up the remaining 10-20% of total nitrogenous waste. Although many researchers have argued that ammonia excretion in freshwater takes place via branchial Na^+/NH_4^+exchange (Maetz and Garcia, 1964; Krogh, 1965; Payan, 1978; Wright and Wood, 1985), recent evidence suggests that ammonia is primarily excreted in the unionized, NH_3 form (Cameron and Heisler, 1983; Avella and Bornancin, 1989; Wood, 1993) . At present, it appears that ammonia excretion is dependant upon the presence of suitable NH_3 partial pressure gradients (ΔP_{NH3}) between the blood and the unstirred boundary layers of the gill (also referred to as the gill water; Randall and Wright, 1987). A model has been proposed by Wilkie and Wood (1996) to explain the mechanism of ammonia excretion by the fish at circumneutral pH water across the gill (Fig. 2A).

In their model, ammonia excretion is facilitated by the hydration of CO_2 in the gills unstirred boundary layers, which results in the production of protons that trap NH_3 as NH_4^+, as it possibly diffuses across the branchial epithelium (Wright *et al.*, 1989). This effectively creates a "sink" that continuously favour NH_3 diffusion under circumneutral pH (about pH 6-8 in the bulk water) conditions.

When freshwater teleosts are exposed to alkaline water there is an immediate reduction in ammonia excretion rate and a corresponding increase in plasma ammonia concentration (Brett and Zala, 1975; Cameron and Heisler, 1983; Wright and Wood, 1985; Wilkie and Wood, 1991., Yesaki and Iwama, 1992; Wright, 1993; Wilkie and Wood, 1995). To understand how this occurs one only has to look at the $NH_3 \rightleftharpoons NH_4^+$ equilibrium, which is described by the following equation:

$$NH_3 + H^+ \rightleftharpoons NH_4^+$$

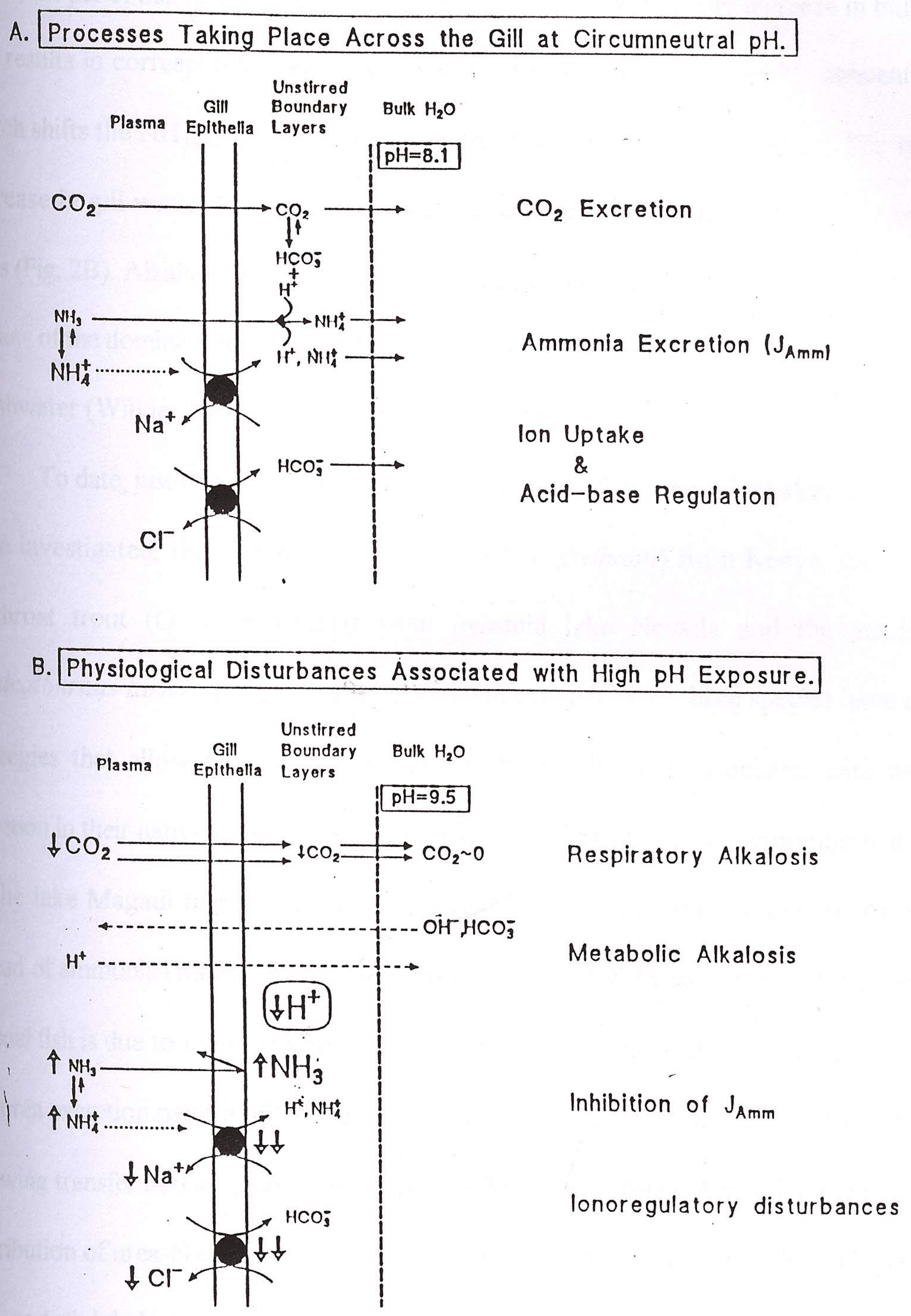


Fig. 2. **A model to describe (A) physiological processes which take place across the gills of freshwater fish and (B) to describe physiological disturbances which occur across the branchial epithelium at high pH (pH 9.5) as proposed by Wilkie and Wood (1996).**

Since, the pK of this relationship is approximately 9.5 (at 15 ^{0}C), any increase in bulk water pH results in corresponding elevation of gill water pH (or decreased H^+ concentration), which shifts the $NH_3 \rightleftharpoons NH_4^+$ equilibrium towards NH_3 formation. The resultant increase in gill water P_{NH} reduces ΔP_{NH} and is reflected by lower ammonia excretion rates (Fig. 2B). Alkaline water may act directly upon branchial Na^+/NH_4^+ transporters, but in view of the dominant role that NH_3 diffusion likely plays in facilitating NH_3 excretion in freshwater (Wilkie, 1994; Wilkie and Wood, 1994).

To date, just three teleostean species thriving in their natural alkaline habitats have been investigated, the lake Magadi Tilapia, (*O. a. grahami*) from Kenya, the Lahontan cutthroat trout (*O. c. henshawi)* from pyramid lake Nevada and the anadromous *Chalcalburnus tarichi* endemic to lake Van Turkey. All these three species have evolved strategies that allow them to circumvent problems that are associated with ammonia excretion in their native alkaline waters. Perhaps, the most dramatic adaptation is exhibited by the lake Magadi tilapia, which excretes virtually all of its nitrogenous waste as urea, instead of ammonia (Randall *et al*., 1989; Wood *et al*.,1989). In fact, urea production in this unusual fish is due to the presence of a fully active complement of the urea cycle enzymes. The urea excretion pattern of the Lahontan cutthroat trout (*O. c. henshawi*) are not altered following transfer into alkaline pyramid lake (Wilkie *et al*., 1994). Although, the percentage contribution of urea-N excretion to total nitrogenous waste is higher in this fish, about 25-30%, and slightly higher than percentages reported for "typical" freshwater teleosts (Wright, 1993; McGeer *et al*., 1994), activities of key urea cycle enzymes, such as CPS III, are too low to suggest the presence of a functional urea cycle (Wilkie and Wright, 1993; Wilkie *et al*., 1994). It was, however, suggeted that the urea production occurs via the typical teleost pathway of uricolysis due to the presence of significant levels of uricolytic enzyme activities

(Wilkie and Wright, 1993; Wilkie *et al.*, 1994). In *C. tarichi*, on the other hand, excrete about 37% of its nitrogenous waste as urea, but it too has no functional urea cycle (Danulat and Kempe, 1992). Thus, urea production in this fish is probably via uricolysis (Wilkie and Wood, 1996).

Interestingly, transiently elevated rates of urea-N excretion appear to be a common ammonia detoxification response of salmonids to acute elevation in environmental pH (Wilkie and Wood, 1991; Wilkie and Wright, 1993). Wilkie *et al.* (1993) demonstrated that Lahontan cutthroat trout, acclimated to pH 9.4, but challenged at pH 10, increased their reliance on urea excretion presumably through enhanced rates of uricolysis. Similarly rainbow trout (*Oncorhynchus mykiss*), which also lack a functional urea cycle, doubled their urea excretion rates at pH 9.5 (Wilkie and Wood, 1991). It should be emphasized that these elevation in urea and excretion were temporary and did not persist beyond 2 or 3 days of high pH exposure at which time ammonia excretion had been fully re-established (Wilkie and Wood, 1991). Recently, Wright *et al.* (1995) reported that embryonic rainbow trout larvae increased urea excretion by six times, following acute (4 hr) exposure to pH 9.5.

Clearly, another key adaptation that would benefit fish living at high pH would be unusual high tolerance to ammonia. It has been reported that the LC_{50} value for ammonia in the Lahontan cutthroat trout is six-fold higher than values reported for most teleost fish (Walsh *et al.*, 1993). *C. tarichi* is also reported to have the capacity for high tolerance to ammonia (Wood, 1993).

Several species of freshwater air-breathing teleosts exist on the Indian subcontinent. All of them have accessory air-breathing structures (for reviews, see Dutta and Munshi, 1985; Munshi and Ghosh, 1994; Munshi and Hughes, 1992), which are thought to have evolved as an adaptation to hypoxic water conditions during severe periodic droughts

(Johansen, 1970; Randall *et al.*,1981). Obligatory air breathers include climbing perch (*A. testudineus*), cuchia eel (*A. cuchia*), two snakeheads (*Channa striata* and *Channa marulis*), and the facultative air-breathers include singhi catfish (*H. fossilis*), walking catfish (C. *batrachus*), and the two snakeheads (*Channa punctatus* and *C. gachua*) (Munshi and Ghosh, 1994). Both obligatory and facultative air-breathers usually inhabit stagnant, slow flowing swampy water bodies or wet lands, which are usually unhabitable to purely aquatic fishes such as carp. These swamps, which are often covered with macrovegetation like water hyacinth, are characterized by a low dissolved oxygen (P_{O_2} = 2.5-30 torr), a water pH range of 6.5-10, more free carbon dioxide gas (0.24-1.7 mmol/1), bicarbonate concentrations of 0.6-2.5 mmol 1^{-1}, high ammonia level (evolved mostly as a degradable product of micro and macrovegetation), and a water temperature of 20-30 °C (Chatrath, 1992). During summer, when the swamps dry up, fishes face more adverse ecological conditions, and most of the air-breathing fishes burrow inside mud to avoid total dehydration. Some of them, such as cuchia eel, live inside mud almost throughout the year. Among other things, an exceptional tolerance to high ambient ammonia and low ambient oxygen has made some of these species successful candidates for aquaculture. Under laboratory conditions, these fishes have been shown to tolerate periods of total dehydration; cuchia eel (*A. cuchia*) survives for 90-100 hr, singhi (*H. fossilis*) and walking catfish (*C. batrachus*) for 60-70 hr, and climbing perch (*A. testudineus*) and one snake head (*C. punctatus*) for 8-12 hr (Saha and Ratha, 1989). Therefore, it would also be interesting to study the role of ureogenesis as a physiological adaptational strategy in other freshwater Indian air-breathing teleosts while living in amphibious or aerial habitat mainly to avoid the ammonia toxicity.