INDUCTION OF UREA CYCLE ENZYMES AND CHARACTERIZATION OF ARGINASE IN A FRESHWATER AIR-BREATHING TELEOST, *Heteropneustes fossilis* (Bloch)

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CERTIFICATE

This is to certify that the thesis entitled
"**Induction of urea cycle enzymes and characterization of arginase in a freshwater air-breathing teleost,** *Heteropneustes fossilis* (Bloch)"
submitted by
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for the degree of
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embodies the record of original investigations
carried out by her under our supervision.

The thesis presented is worthy of being considered for the award of the Ph. D. degree.
This work has not been submittted for any degree of any other university.

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INTRODUCTION

Nitrogen metabolism is one of the major pathways of energy supply in all animals. Dietary intake of proteins by animals provides amino acids in excess of the amount required for protein synthesis. Therefore, excess of amino acids which cannot be stored as proteins, as can be carbohydrates as glycogen and lipids as fat, are metabolized. They are deaminated releasing ammonia and the carbon residues which are either oxidized via the TCA cycle for energy production or used in gluconeogenesis or lipogenesis. Ammonia is highly toxic and cannot be stored in the body even in low concentrations (Smith, 1929; Jackson et al, 1986; Cooper and Plum, 1987; Campbell, 1991).

Ammonia toxicity to fish has been primarily attributed to the un-ionized form (NH_3) with the ionized form (NH_4^+) being relatively less toxic (EIFAC, 1970; Alabaster and Lloyd, 1982; Erickson, 1985; WHO, 1986; Hickey and Vikers, 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 Heisler, 1983; Cameron, 1986; Paley et al, 1993; Wajsbrot 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 species 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 the Krebs cycle due to the 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 early Krebs cycle and would lower blood pH due to accumulation of pyruvate and lactate (Campbell, 1991). The resulting acidemia would shift the oxygen dissociation curve (Bohr effect) to reduce maximal 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 these wide ranging toxic effects , ammonia is either immediately excreted out or converted to some less toxic substances such as urea or uric acid for temporary storage in vivo.

In teleosts, ammonia usually is excreted out to the ambient water medium by diffusion through the gills (Smith, 1929; Forster and Goldstein, 1969; Watts and Watts, 1974; Kormanik and Cameron, 1981; Evans and Cameron, 1986;

Campbell, 1991; Wood,1993). In terrestrial animals, ammonia *in vivo* is converted either to urea or to some other compounds, which are excreted out mainly through urine utilizing lesser amount of water (Cohen, 1976; Hoar,1983; Campbell, 1991; Wood, 1993; Anderson, 1994a). Insoluble uric acid is found to be the excretory product of those animals where conservation of metabolic water is highly essential due to their arid environment (Hoar, 1983; Nener, 1988; Powers-Lee 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) *Ammoniotelic* : Animals which excrete ammonia as the major excretory product as in most aquatic animals.

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

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

However, not all animals fall neatly into one category or another because many exhibit mixed patterns of nitrogen excretion, depending upon their physiological and environmental conditions. Amphibians, which can live in land as well as in water, excrete both ammonia and urea. They are ammoniotelic in water and ureotelic on land.

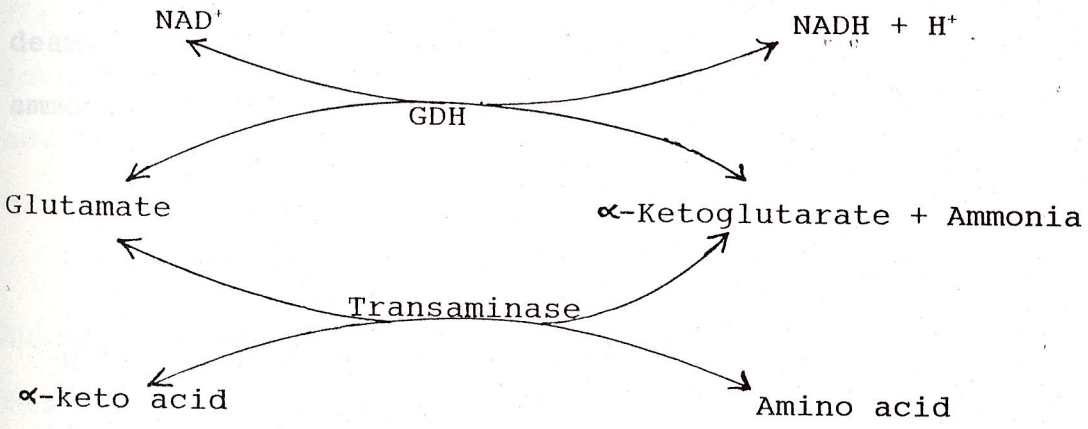
Tadpoles change from being ammoniotelic during early stages of development to ureotelic during later stages of development.

Ammonia has many advantages as a nitrogen excretory product. 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 deamination of glutamate through glutamate dehydrogenase ultimately produce 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 fish. In freshwater fishes the exchange of NH_4^+ for Na^+ serves the dual purpose of elimination of nitrogenous waste product NH_4^+ and absorption of Na^+ from the external water medium.

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

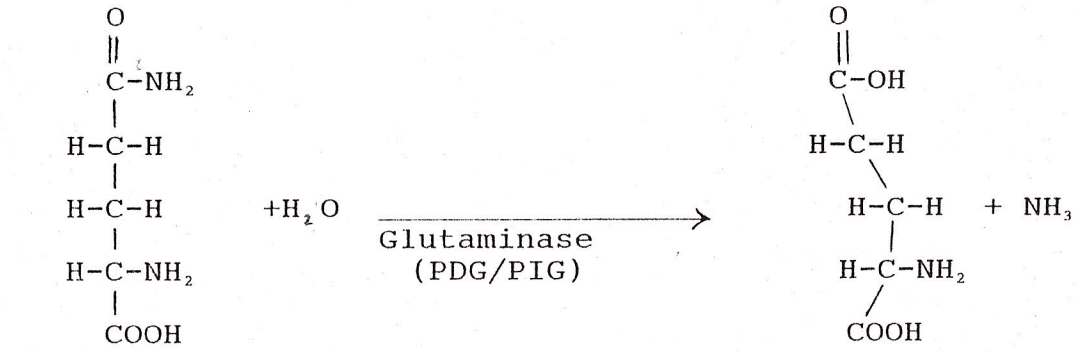
Transdeamination : The amino group from most of the amino acids with the exception of histidine, serine, cysteine, is transferred to another keto acid forming a new amino acid. The dissociated amino group tends to be channelized

directly or indirectly through the formation of glutamate. Glutamate undergoes oxidative deamination catalyzed by glutamate dehydrogenase (GDH) to form ammonia and ∝-keto-glutarate (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



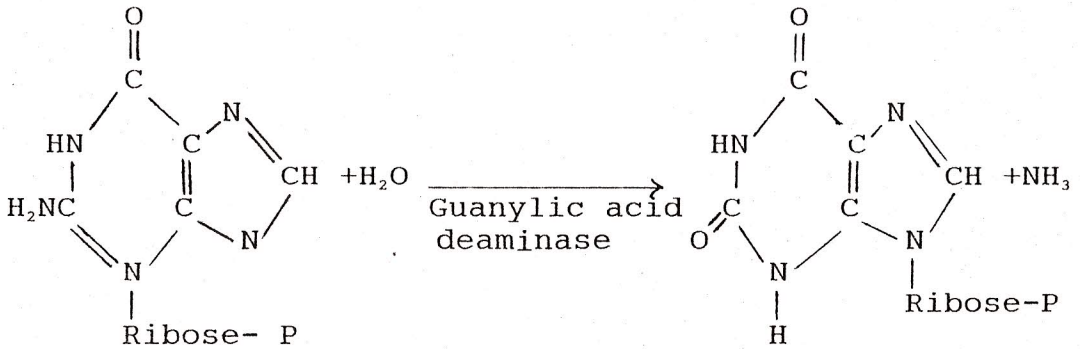
Transdeamination has been reported as the major pathway for ammoniogenesis in the liver of freshwater teleosts (Janssens, 1964;Campbell et al 1983; Campbell, 1991), and in the mudskippers, Boleopthalmus boddaerti and Periopthalmodon schlosseri (Chew and Ip, 1987).

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.

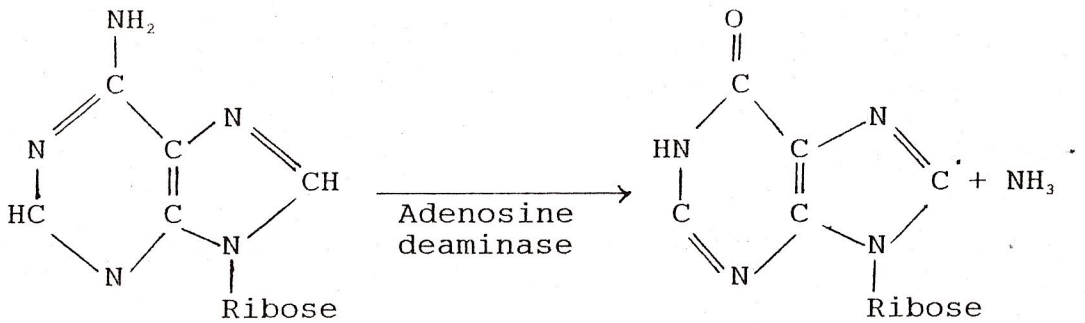


Glutamine Glutamate

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

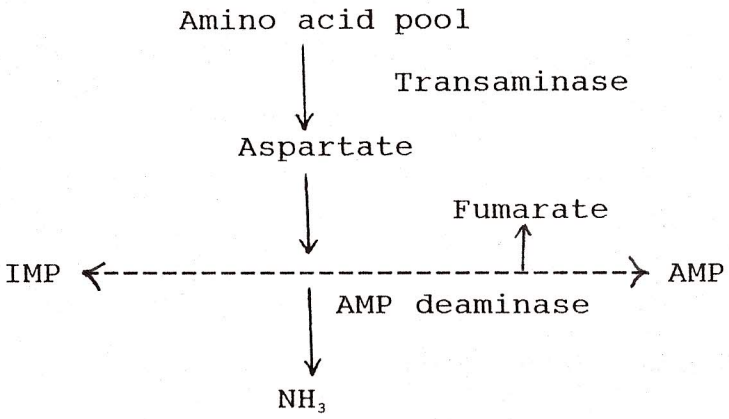


Guanylic acid Xanthylic acid



Adenosine Inosine

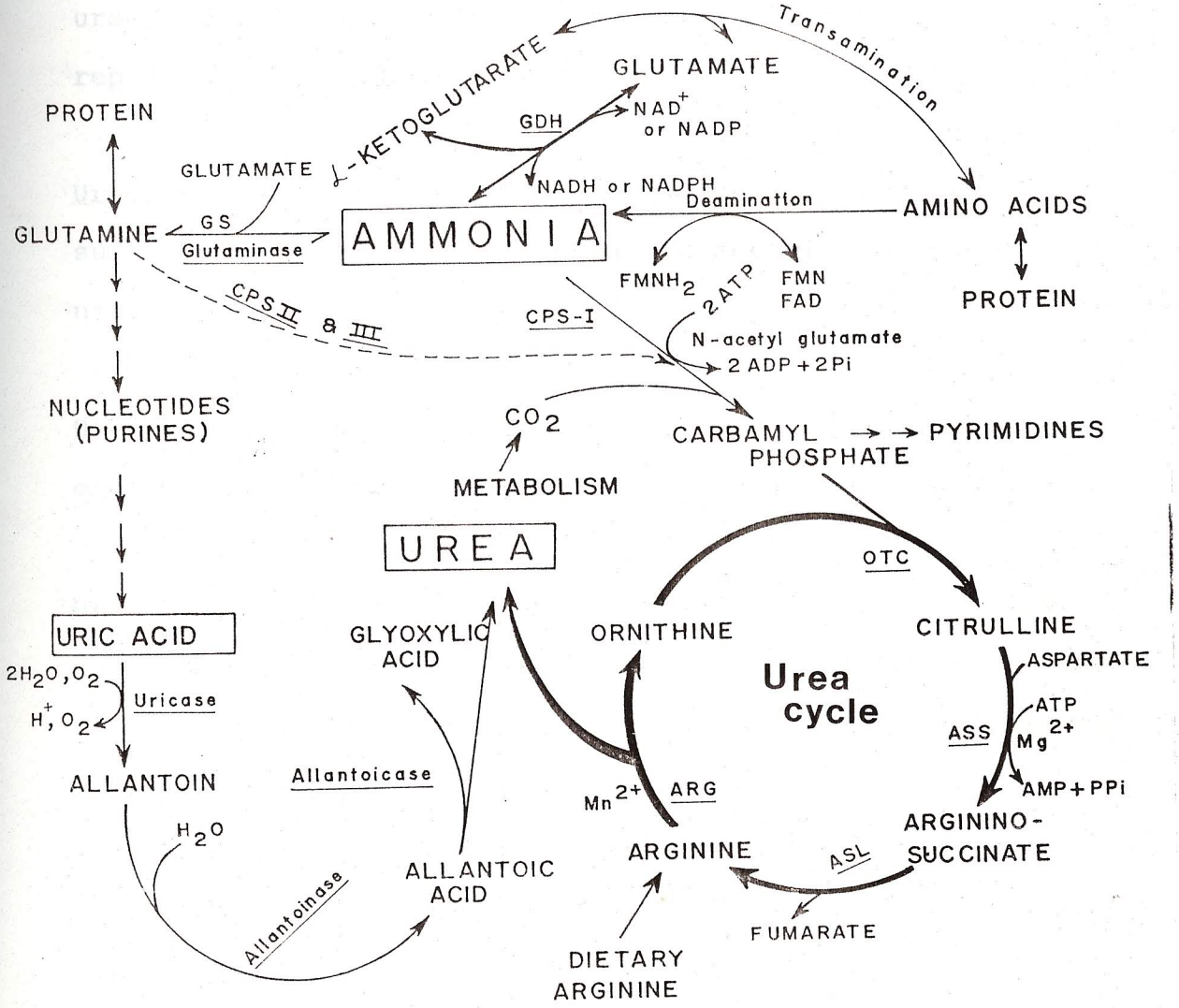
Hydrolysis of particularly AMP could be ultimately utilized for deamination of amino acids as follows :



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).

Formation of urea : Although teleosts are primarily ammoniotelic, some amount of urea has been reported both in the excretory products (Holmes and Donaldson, 1969; Saha *et al*, 1988; Saha and Ratha, 1989) as well as in the tissues of several fishes (Smith, 1929; Burrows, 1964; Brett and Groves, 1979; Vellas, 1981; Ramaswamy and Reddy, 1983; Saha and Ratha, 1989) besides marine fishes (where urea production and retention serves the purpose of osmoregulation) (Alexander *et al*, 1968; Goldstein and Forster, 1971; Hoar, 1983; Campbell, 1991; Anderson,1994a). The formation of urea in fish has been suggested to be through either one or more of these pathways such as (i) Ornithine-urea (o-u) cycle (ii) uricolytic pathway and (iii) catabolism of dietary arginine.

O-u cycle : The o-u cycle involves a series of 5 enzymatic reactions (Krebs and Henseleit, 1932; Brown and Cohen, 1959). The five enzymes of the urea cycle are carbamyl phosphate synthetase (CPS), Ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG)(Fig. 1). The first reaction of the o-u cycle involves the fixation of ammonia and CO_2 to carbamyl phosphate by the enzyme CPS. Carbamyl phosphate is then converted to citrulline in presence of ornithine by the enzyme OTC. Both the reactions in ureotelic vertebrates take place inside the mitochondria and citrulline produced is transported to the cytosol. It is converted ultimately to urea and ornithine by the other three cytosolic enzymes (ASS, ASL and ARG) of o-u 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 CPS and OTC in several species of freshwater teleosts studied. Huggins et al (1969) could detect all the enzymes of the o-u cycle enzymes in some freshwater teleosts but their activites were so low that no physiological significance could be attributed to them. They divided the urea producing animals into 3 categories on the basis of the role of urea synthesis. These are ureogenic, ureotelic and ureosmotic.



[CPS - Carbamylphosphate synthetase-I (ammonia and N-acetylglutamate dependent); - II (glutamine dependent and N-acetyl glutamate independent); - III (glutamine and N-acetyl glutamate dependent); OTC - Ornithine transcarbamylase; ASS - Argininosuccinate synthetase; ASL - Argininosuccinate lyase; ARG - Arginase; GS - Glutamine synthetase]

Fig.1. A brief diagramatic representation of nitrogen metabolic pathways in animals with special reference to ammonia and urea.

Ureogenic : The species having full complements of the o-u cycle enzymes indicating the potential for synthesizing urea, although for various reasons, its synthesis may be repressed in freshwater fishes.

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

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

Uricolytic pathway : Another source of urea in teleosts could be purine degradation or uricolytic pathway which was first reported by Brunel (1937). Adenine and guanine produce uric acid as a catabolic product which further breakdown in a 3 step uricolytic pathway involving three enzymes - uricase, allantoinase and allantoicase to produce urea in most of the teleosts (Forster and Goldstein, 1969; Watts and Watts, 1974)(Fig.1). Cvancara (1969a) could find relatively high 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 the liver tissue of a freshwater air-breathing teleost, Heteropneustes fossilis and suggested that uricolysis could

be one of the pathway for the formation of urea in this fish in addition to the o-u cycle.

Dietary arginine : Arginase the last enzyme of the o-u cycle which converts arginine to urea and ornithine (Fig.1) has been reported to be present in various tissues of freshwater teleosts such as in liver (Hunter, 1929; Brown and Cohen, 1960; Huggins et al, 1969; Cvancara, 1969b; 1971; Wilson, 1973), kidney and heart (Hunter, 1929; Cvancara, 1969b), and to a lesser extent in spleen, gills, ovaries, testes and muscle of some teleosts (Cvancara, 1969b). Cvancara (1969b), therefore, has suggested that dietary arginine could be one of the major sources of urea in freshwater teleosts.

Active ureogenesis through o-u cycle has been confirmed in amphibians and terrestrial animals (Krebs and Henseleit, 1932; Cohen, 1976) and in marine fishes (Cohen, 1976; Pang et al, 1977; Hoar, 1983; Read, 1971; Mommsen and Walsh, 1989; Campbell, 1991; Wood,1993; Anderson,1994a). However, in freshwater teleosts the presence of a functional o-u cycle was not confirmed since some of the o-u cycle enzymes could not be detected in many of the teleosts studied (Manderscheid, 1933; Brown and Cohen, 1960; Wilson, 1973). Brown and Cohen (1960) could not detect CPS and OTC activity in several freshwater teleosts studied by them and therefore, suggested that the genes responsible for synthesizing some of these enzymes of o-u cycle, whose activities could not be detected, got deleted

and proposed the 'deletion' hypothesis. Huggins et al (1969) reported a full complement of o-u cycle enzymes in a variety of freshwater teleosts but with very low activity and suggested that the expression of the genes responsible for the synthesis of enzymes of the o-u cycle might have been altered as a result of an adaptational change in the freshwater teleosts when the excretion of ammonia was facilitated by diffusion. The presence of a regulatory physiological system for converting ammonia to urea via the o-u cycle has been well-documented in lungfishes (Janssens, 1964; Goldstein et al, 1967), mudskippers (Gregory, 1977; Gordon et al, 1969, 1978), and aquatic amphibians (Janssens and Cohen, 1968; Baldwin, 1970; Janssens, 1972; Balinsky, 1970) during their terrestrial life when the excretion of ammonia is not possible. Goldstein et al (1973) could also detect the activities of all o-u cycle enzymes in a well preserved sample of coelacanth liver which were comparable to those in elasmobranchs.

The lake Magadi (Kenya) tilapia (freshwater) Oreochromis alcalicus 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 o-u cycle (Randall et al, 1989; Wood et al, 1989). This is the only known instance of complete ureotelism in a completely aquatic teleost fish. However, in Lahontan cutthroat trout, Oncorhynchus clarki henshawi, which also live in alkaline water of pH 9.4, the activities of o-u enzymes in

the liver were found to be low (Wilkie *et al*, 1993). High activities of all the o-u cycle enzymes in the liver of at least four species of freshwater air-breathing teleosts such as *Heteropneustes fossilis*, *Clarias batrachus*, *Anabas testudineus* and *Amphipnous cuchia* and in the 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 in the mud during drought conditions and also frequently are being exposed to the air (Jhingran, 1983; Beavan, 1982). They are capable of tolerating temporary dehydration when kept outside water (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 (Saha, 1986). At least in one of the above mentioned species, *H. fossilis* has been shown to tolerate a very high ambient ammonia (upto 75 mM NH_4Cl) which is unusual among freshwater teleosts and even for many amphibians (Saha, 1986; Saha and Ratha, 1990, 1991, 1994). The induction of o-u cycle enzymes in liver and kidney of *H. fossilis* and stimulation of urea production from accumulated ammonia, when these fishes were exposed to higher ambient ammonia (Saha and Ratha, 1986, 1991, 1994) and also when kept outside water (Saha, 1986, unpublished data), have been reported. In addition to the presence of

functional and regulatory o-u cycle, various other adaptations to nitrogen metabolism mainly to avoid ammonia toxicity have been reported in H. fossilis. The presence of higher physiological activity of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) (reductive amination) in various tissues, and the induction of activities of these two enzymes under hyper-ammonia stress have been reported (Chakravorty, 1990; Chakravorty et al, 1989; Das, 1991; Das et al, 1991).

Adaptations to nitrogen metabolism, mainly a shift from ammoniotelism towards ureotelism, have been reported in various vertebrates such as amphibians during water shortage (Janssens and Cohen, 1968; McBean and Goldstein, 1970; Balinsky, 1970, 1981; Golstein, 1972), African lungfish during aestivation (Janssens, 1964; Goldstein et al, 1967), and mudskippers when exposed to air (Gordon et al, 1969, 1978) to avoid ammonia toxicity. Induction of o-u cycle and a shift towards ureotelism has been reported in a purely aquatic frog, Xenopus laevis when exposed to 10 mM NH_4Cl (Janssens, 1972), which is primarily ammoniotelic while living in water. Olson and Fromm (1971) found that goldfish, Carassius auratus when subjected to increased ambient ammonia level there was an increase in urea excretion rate. However, there is no report on the presence of a functional o-u cycle in goldfish. Three fold increase in urea production rate has been observed in ureotelic alkaline lake tilapia O. a. grahami when exposed to 0.5 mM ammonia at pH 10 (Wood et al, 1989) where the presence of

functional o-u cycle has been reported (Randall et al, 1989).

Induction of o-u cycle enzymes both in the liver and kidney tissues of H. fossilis was studied only when the fish was exposed to 50 mM NH_4Cl and also when exposed to the air in our laboratory. (Saha, 1986; Saha and Ratha, 1986, 1991, 1994).In both the cases the tissue ammonia concentration was significantly enhanced followed by the induction of the activity of the enzymes of o-u cycle (ureogenesis), suggesting that enhanced ammonia level in vivo was one of the factors to induce the activity of o-u cycle enzymes. However, the threshold concentration and maximum concentration of ammonia in vivo which was needed to cause initiation and maximum induction of all the o-u cycle enzymes could not be determined. This could be done by infusing different concentrations of NH_4Cl in perfused liver of H. fossilis and monitoring the level of activity of the o-u cycle enzymes.

Sub-cellular localization of o-u cycle enzymes and different isoenzymic forms of CPS :

Urea is synthesized in different groups of animals via o-u 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). In ureosmotic marine elasmobranchs (sharks, skates and rays) urea is

synthesized via o-u cycle not for excretion but for retention for osmoregulation (Perlman and Goldstein, 1988). Nener (1988) have postulated that the o-u cycle is highly constrained in terms of enzyme composition and tissue localization among organism that produce urea for different purposes.

The synthesis of urea via the o-u cycle needs both mitochondrial and cytosolic enzymes. Some differences in the isoenzymic forms and the sub-cellular localization of some of the enzymes of o-u cycle have been reported, and correlated with their physiological functions in different groups of animals. In ureotelic species such as in mammals and amphibians, the first enzyme of the o-u cycle, carbamyl phosphate synthetase called CPS I is located in the mitochondrial matrix and utilizes ammonia as a nitrogen donating substrate for carbamyl phosphate synthesis, and requires the presence of N-acetyl glutamate (NAG) for catalytic activity (Ratner, 1973; Jackson et al, 1986). Another isozymic form of CPS (CPS II) found in ureotelic species is located in the cytosol and utilizes glutamine instead of ammonia as a nitrogen donating substrate for carbamyl phosphate synthesis. CPS II does not require NAG for its catalytic activity. Carbamyl phosphate formed by the cytosolic CPS II is utilized for pyrimidine synthesis and by the mitochondrial CPS I for urea synthesis (Ratner, 1973; Hager and Jones, 1967; Jones, 1980; Jackson et al, 1986; Campbell and Anderson, 1991). A third type of CPS, called CPS III was reported for the first time by Tramell

and Campbell (1970, 1971) in several species of invertebrates. CPS III requires NAG for catalytic activity like CPS I, but utilizes glutamine as the nitrogen donating substrate like CPS II and is located in the mitochondrial matrix (Campbell and Anderson, 1991; Anderson, 1994b). The role of CPS III has been reported to be in urea biosynthesis. The presence of CPS III has been reported in the liver of large mouth bass, Micropterus salmoides, a freshwater teleost (Anderson, 1976), alkaline lake Magadi tilapia, O. a. grahami (Randall et al, 1989), toad fish, O. beta (Mommsen and Walsh, 1989) and at much higher level in the liver of marine elasmobranch, Squalus acanthias (Anderson, 1980; 1981; Casey and Anderson, 1983). The second enzyme of the o-u cycle, ornithine transcarbamylase (OTC) has always been localized within the mitochondrial matrix in all ureotelic and ureosmotic vertebrates (Ratner, 1973; Gamble and Lehninger, 1973; Vorhaben and Campbell, 1977; Casey and Anderson, 1985; Campbell and Anderson,1991). The third, fourth and fifth enzyme of o-u cycle , arginino-succinate 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 elasmobranch, S. acanthias. Mitochondrial localization of ARG has also been reported in the liver of gulf toadfish, O. beta (Mommsen and Walsh, 1989; Anderson and Walsh, 1994).

Mommsen and Walsh (1989) 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 arginase by a cytosolic equivalent. The presence of a functional o-u cycle with comparatively higher physiological level of all the enzymes and mitochondrial ARG in H. fossilis are unique features among freshwater teleosts (Saha and Ratha, 1987,1989; Dkhar et al, 1991). The mitochondrial localization of glutamine synthetase (GS) in both liver and kidney of H. fossilis (Chakravorty et al, 1989) resembles those of elasmobrachs (Webb and Brown, 1976, 1980; Anderson, 1982, Smith et al, 1987) and uricotelic species (Vorhaben and Campbell, 1977; Campbell et al, 1983, 1984) where glutamine dependent CPS III isoenzyme have been reported to help in urea synthesis. However, the isoenzymic patterns of CPS has not yet been known in H. fossilis.

Annual variation of o-u cycle enzymes:

Induction of o-u cycle enzymes by hormones such as thyroxine, glucagon, glucocorticosteroid, corticosterone have been reported (Schultheiss, 1977; Balinsky et al,

1972; Husson et al, 1987; Kumar and Kalyankar , 1984; Marti et al, 1988; Lamers and Mooren, 1981). The extent of induction is dependent on the concentration of the hormones, time of exposure, temperature and the age of the animal. The levels of steroid hormones in vivo alter during various stages of reproductive cycle in fish in a year. The levels of these hormones are high during the pre-spawning and spawning periods and low during post-spawning phase (Sundararaj, 1959; Liley, 1969; Sundararaj and Goswami, 1969; Lamba et al, 1983). Bryla et al (1977) has reported that in perfused rat liver, glucagon increased citrulline production by about 1.6 times over the period from September to October, whereas from October to February, the rate of citrulline synthesis were reported to be less. Cohen et al (1982) have reported that isolated rat liver mitochondria showed extremely low rate of citrulline synthesis during the month of February. The activity of ARG from the liver of Clarias batrachus was found to be maximum in the month of July whereas from the month of September to October the activity was relatively low and did not change much during this period (Singh and Singh, 1988). GDH, which is one of the important enzymes in nitrogen metabolic pathway , has been reported to show annual variation in its activity in different tissues of H. fossilis (Das, 1991). GDH activity in reductive amination direction were found to be maximum in summer (May to June) and minimum in winter. However, in oxidative deamination direction it was found maximum in winter

(November to March) and minimum in summer (June to July). However, there is no report on the annual variation of the activity of the o-u cycle enzymes in ureogenic tissues of H. fossilis which is a seasonal breeder.

Molecular and kinetic properties of arginase :

Arginase is a ubiquitous enzyme and has been studied in various groups of organisms. This enzyme catalyzes the hydrolysis of arginine into urea and ornithine, and plays an important role in nitrogen metabolism (Lund and Wiggins, 1986; Morris, 1992). Ornithine is the precursor of polyamine synthesis in animals. Arginase occurs in almost all organisms including plants and bacteria (Ratner, 1973; Jackson et al, 1986; Nener, 1988) besides all ureogenic and non-ureogenic species and tissues (Baby et al, 1976; Huggins et al, 1969; Singh and Singh, 1988; Cvancara,1969b; Blachier et al, 1991; Dhanakoti et al, 1992; Jenkinson and Grigor. 1994). The universal distribution of arginase suggests that it appeared very early in the process of biochemical evolution. In insects, arginase helps in the conversion of arginine to proline via the formation of ornithine (Reddy and Campbell, 1969). Proline serves as the substrate for energy production in insects (Bursell, 1981).

Significant differences in arginase from ureotelic, uricotelic and ammoniotelic animals have been reported with respect to their molecular weight, substrate specificity, stability etc. (Reddy and Campbell, 1970; Hirsch-Kolb et

al, 1970; Rossi and Grazi, 1969). The Km value for arginine reported for several mammalian arginases lie in the range of 6 to 20 mM (Hirsch-Kolb et al, 1970). The pH optima for mammalian arginases lie in the range of 9.3 to 10.5 (Hirsch-Kolb et al, 1970). The pH optima for hepatic arginase from the teleost fish, Genypterus maculatus (Carvajal et al, 1987), Merluccius gayi (Carvajal et al, 1989) and from the freshwater air-breathing teleost, Clarias batrachus (Singh and Singh, 1990) was reported to be 9.5

Arginase requires Mn^{2+} for its catalytic activity and stability, although its oligomeric composition remains obscure (Hirsch-Kolb et al, 1971, Maggini et al, 1992; Turkoglu and Ozer, 1992). It has been reported that deficiency of hepatic Mn^{2+} in rat is associated with a decrease in arginase activity (Visek et al, 1992; Brock et al, 1994). Mg^{2+}, Co^{2+} and Ni^{2+} ions also serve as activators for arginase in addition to Mn^{2+}, whereas Zn^{2+} and Cd^{2+} ions act as inhibitors of arginase. Most mammalian arginases with the exception of beef liver arginase were inhibited by Ni^{2+} and Co^{2+} (Hirsch-Kolb et al, 1970). Rat hepatocyte plasma membrane bound arginase was inhibited by Cu^{2+}, Zn^{2+} and to a lesser extent by Co^{2+} (Fuentes et al, 1991). In the teleost fish, M. gayi, the metal ion requirement of arginase was accomplished by Mn^{2+}, and to a much lesser extent by Cd^{2+} and Co^{2+} (Carvajal et al, 1989). Mg^{2+}, Ca^{2+} and Co^{2+} activated the enzyme activity purified from the liver of guinea pig (Farooqui et al, 1978). Hepatic arginase of

freshwater air-breathing teleost, C. batrachus was activated by Co^{2+} and Ni^{2+} and inhibited by Cd^{2+} (Singh and Singh, 1988).

Bovine liver arginase was inhibited by many amino acids (Hunter and Downs, 1945). Ornithine, lysine and proline were competitive inhibitors of sheep liver arginase. On the other hand, activation of arginase with divalent cations altered the kinetics of inhibition of the enzyme by amino acids (Rao et al, 1973). Ornithine showed a mixed type of inhibition for the enzyme from rat kidney (Gasiorowska et al, 1970) and human liver (Bascur et al, 1966). Partial inhibition by proline, leucine, valine and isoleucine observed in the liver as well as in kidney arginase, indicated the existence of allosteric sites on both hepatic and renal enzymes (Carvajal and Cederbaum, 1986). Branched chain amino acids leucine, isoleucine, valine, ornithine, lysine and proline inhibited the enzyme from the liver of M. gayi (Carvajal et al, 1989). Ornithine and leucine acted as competitive inhibitors, whereas valine and isoleucine acted as non-competitive inhibitors for arginase purified from the liver of C. batrachus (Singh and Singh, 1990).Besides amino acids, polyamines also influence the activity of purified arginase (Subramanyam and Reddy, 1986).

Mammalian liver arginase is composed of 4 sub-units each having the molecular weight of 30,800 giving the enzyme an overall molecular weight of 120,000 (Hirsch-Kolb and Greenberg, 1968; Hirsch-Kolb et al, 1970;1971). However,

some data suggested that arginase from rat liver, though a mammal, was reported to be a trimer (Kanyo et al, 1992; Penninckx et al, 1974). The molecular weight of arginase reported from the gut of earthworm was 27,000 (Reddy and Campbell, 1968), from the land planarian was 2,40,000, from gull liver was 1,20,000 (Reddy and Campbell, 1970), from human erythrocyte was 1,05,000 (Ikemoto et al, 1989), from the liver of Squalus acanthias was 1,05,000 (Casey and Anderson, 1982), from Xenopus laevis liver was 76,000 (Peiser and Balinsky, 1982) and from the liver of C. batrachus was 87,000 (Singh and Singh, 1990). The existence of heterogeneity in size and charge of arginase sub-units from mouse liver have been reported (Spolarics and Bond, 1988). Different isozymic forms of arginase has been reported from the liver, kidney, sub-maxillary gland, intestine and pancreas on the basis of their tissue distribution, chromatographic behaviour, electrophoretic mobility and intereaction with antiserum (Herzfeld and Raper, 1976; Kaysen and Strecker, 1973; Venkatakrishnan and Reddy, 1983; Gasiorowska et al, 1970; Reddi et al, 1975; Porembska et al, 1971; Glass and Knox , 1973; Singh and Singh, 1988, 1990; Turkoglu and Ozer, 1991; Jenkinson and Grigor, 1994). The physiological level of arginase enzyme activity in the liver of ureogenic fish, H. fossilis has been reported to be quite high (Saha and Ratha, 1987)and about 60% of this activity is localized in the mitochondria and 40% in the cytoplasm. However, nothing is known about the molecular properties and isoenzymic pattern

of arginase in H. fossilis.

Objective :

It can be seen from the foregoing reports that the freshwater air-breathing teleost, Heteropneustes fossilis, is unique having ureogenic potential and adaptibility to shift from ammoniotelism to ureotelism under hyper-ammonia stress. It has mitochondrial glutamine synthetase and arginase activity in its liver and kidney. All these characters are unusual for a freshwater teleosts and show affinity with marine elasmobranchs, aquatic amphibians and reptiles. This requires a lot of additional studies on the effect of ammonia as an inducer for o-u cycle enzymes, possibility of glutamine besides ammonia being used as a substrate for ureogenesis, and the molecular and kinetic properties of the enzymes. Ubiquitous arginase was selected for purification and study of its properties in detail. To clarify these points,the following plan of work was made.

Plan of Work:

The work was planned as follows with the above objective in mind :

1. The liver of H. fossilis was perfused with haemoglobin-free media and different concentrations of NH_4Cl (0.01 to 1 mM) was infused into the liver for 60 min and the following observations were made.
   a) The level of ammonia in the perfused liver after infusing different concentrations of NH_4Cl for 60

min.

b) The amount of ammonia coming out into the effluent out of the total infused into the liver at every 2 min interval.

c) The activity of all the five o-u cycle enzymes such as CPS (ammonia dependent), OTC, ASS, ASL and ARG) in perfused liver.

d) The amount of urea-N coming out into the effluent at every 2 min interval of infusing the NH_4Cl.

2) In another set of experiment L-glutamine (1 mM and 2mM) instead of ammonia was infused into the perfused liver of H. fossilis for 60 min and the following observations were made:

a) The amount of urea-N coming out into the effluent at every 2 min interval of infusing L-glutamine.

b) The activity of CPS III (glutamine dependent) activity in the perfused liver after infusing L-glutamine for 60 min.

3) The occurence of three different isoenzymes of carbamyl phosphate synthetase (CPS I, II and III) were confirmed, and the sub-cellular localization of all the three isoenzymes of CPS and other four enzymes of o-u cycle such as OTC, ASS, ASL and ARG were studied both in the liver and kidney of H. fossilis.

4) The activity of all the o-u cycle enzymes both in the liver and kidney of H. fossilis were studied every month for one year to find out annual variation, if

any.

5) The enzyme arginase was purified from the liver of H. fossilis, and the fold of purification and percentage recovery were determined.

6) The molecular weight and various physico-chemical properties of purified arginase were determined.

7) The kinetic and the effect of different regulators such as metal ions and amino acids on the activity of purified arginase were studied.