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.

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

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Place: Shillong 793022

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CONTENTS

APPENDICES

1. Saha, N., Das, L. and Dutta, S. (1999) Types of carbamyl phosphate synthetases and subcellular localization of urea cycle and related enzymes in air-breathing walking catfish, *Clarias batrachus*. ***Journal of Experimental Zoology***, **283**: 121-130.

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) **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 products as in mammals and amphibians.

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

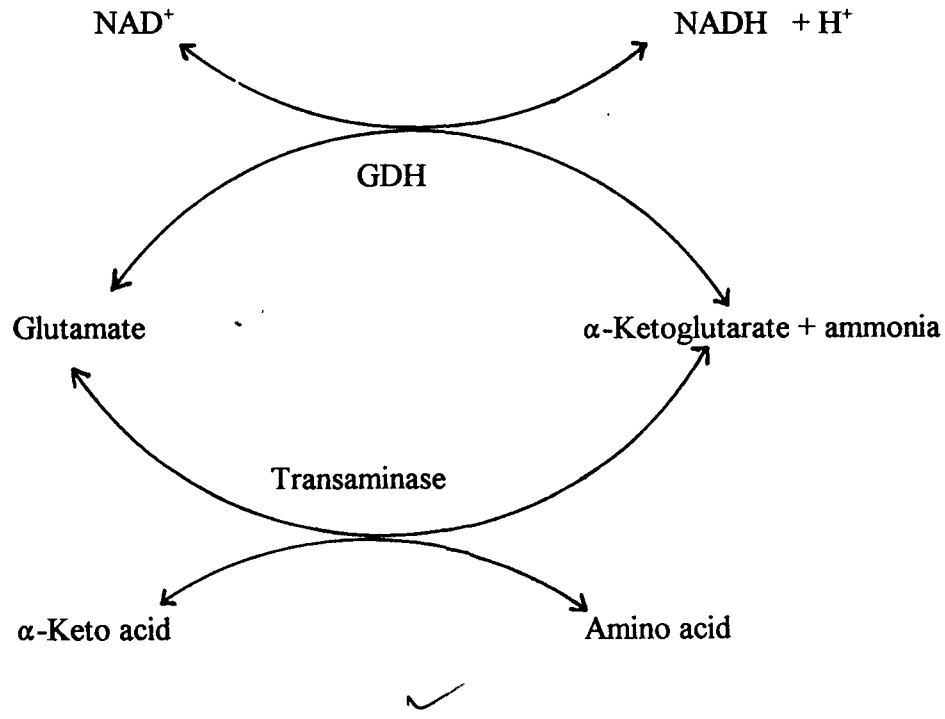
It is not necessary that all animals will fall neatly into one of these categories or another. They may have mixed patterns of nitrogen excretion, depending upon their physiological and environmental conditions. For example, amphibians, which can live both in land as well as in water, excrete both ammonia 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 *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.

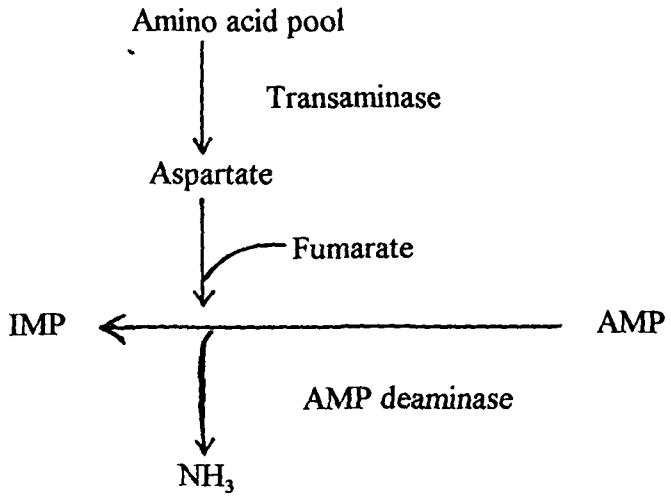
$$\text{Glutamine} + H_2O \xrightarrow[\text{PDG/PIG}]{\text{Glutaminase}} \text{Glutamate} + \text{ammonia}$$

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

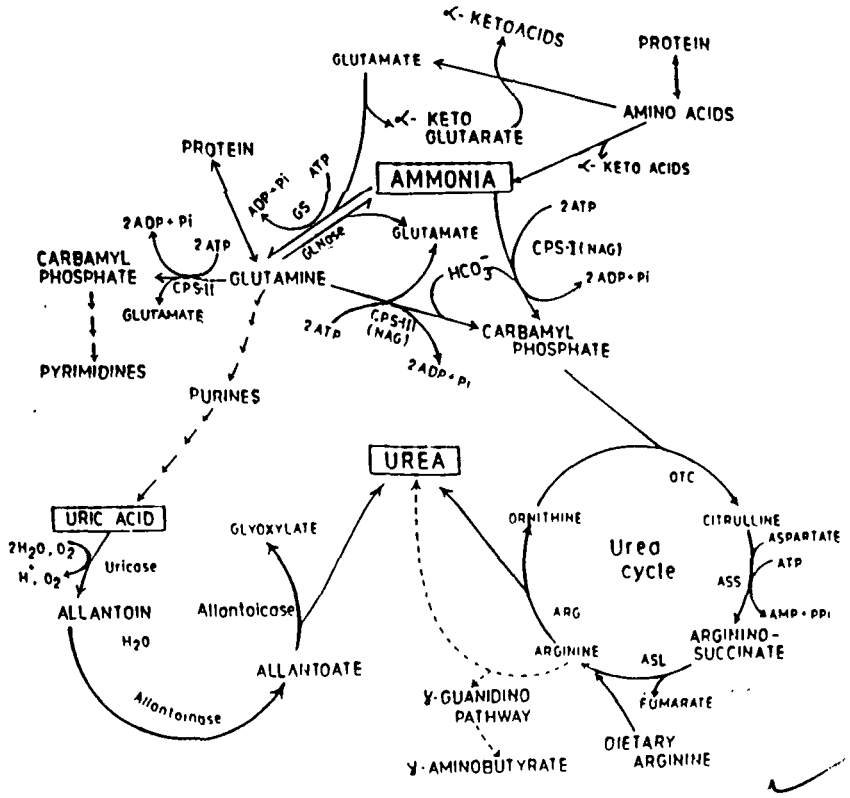


Fig. 1. Outlines of different pathways of urea synthesis in fish.

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:

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

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:

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

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:

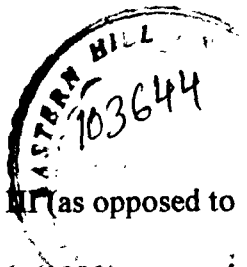
$$\text{Glutamine} + H_2O + HCO_2^- + 2ATP \xrightarrow[NAG,\ Mg_2^+]{CPS\ III} \text{Carbamyl phosphate} + 2ADP + Pi + \text{Glutamate}$$

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

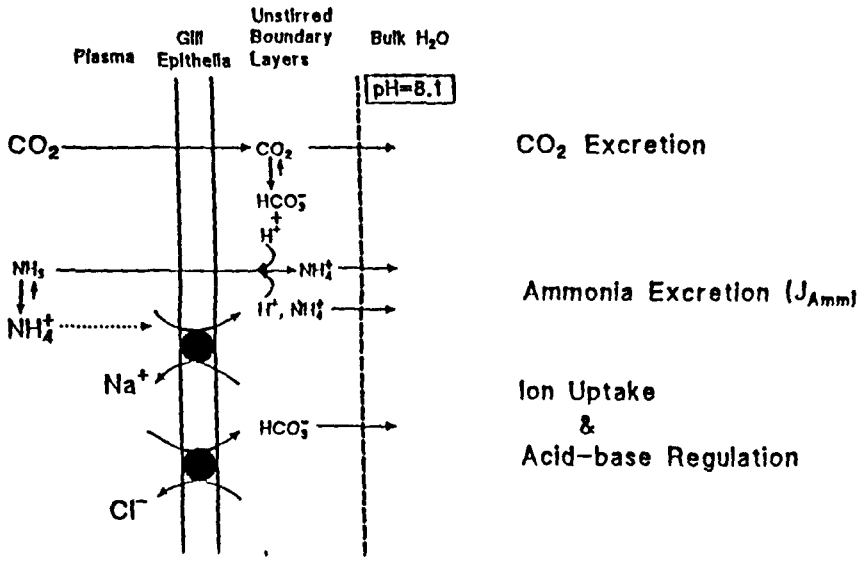
waste produced by fish is excreted across the gills, and ammonia excretion usually accounts for about 85% of this total (for review, see Wood, 1993). Urea excretion generally makes up the remaining 10-20% of total nitrogenous waste. Although many researchers have argued that ammonia excretion in freshwater 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:

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

A. Processes Taking Place Across the Gill at Circumneutral pH.



B. Physiological Disturbances Associated with High pH Exposure.

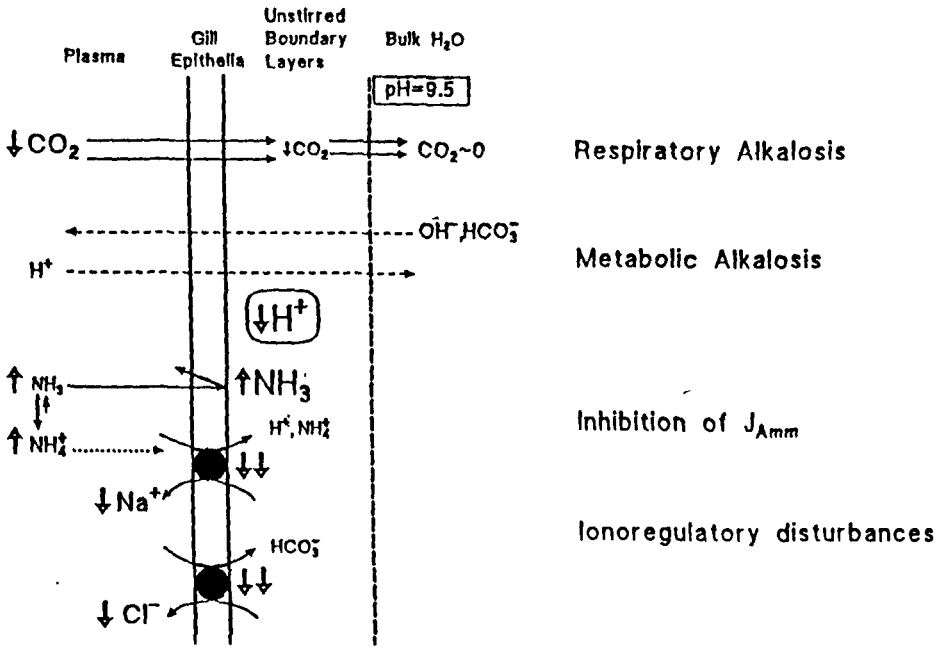


Fig. 2. A model to describe (A) physiological processes which take place across the gills of freshwater fish and (B) to describe physiological disturbances which occur across the branchial epithelium at high pH (pH 9.5) as proposed by Wilkie and Wood (1996).

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 l^{-1}, high ammonia level (evolved mostly as a degradable product of micro and macrovegetation), and a water temperature of 20-30 ^{0}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.

Objectives:

Thus, from the foregoing studies and reports about the extreme habitat of different species of Indian air-breathing freshwater teleosts, and also from reports mainly from our laboratory and some from other laboratories on various aspects of nitrogen metabolism, it would be interesting to study the role of ureogenesis in different groups of Indian air-breathing fishes to survive under various stressful conditions. Further, the studies on the pattern of subcellular localization of all the urea cycle enzymes along with the types of CPSs present in these air-breathing fishes may have some evolutionary significance with the relation to urea synthesis in lower vertebrates especially in pisces. Therefore, I proposed to study the subcellular localization of different urea cycle enzymes at least in two most ureogenic tissues (liver and kidney), the types of CPSs (CPS I, II and III) and also the abundance of different urea cycle enzymes in some other extra-hepatic tissues in one of these Indian air-breathing fishes, the walking catfish (*C. batrachus*). Further, I proposed to study the effect of hyper-ammonia stress, dehydration stress and the effect of high alkaline water on ureogenesis in *C. batrachus*, which they face regularly in some part of life cycle in their natural habitat.

PLAN OF WORK:

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

1. The possibility of occurrence of activity of different types of CPSs (CPS I, II, and III) were studied in the mitochondrial and cytosolic fractions of liver and kidney (most ureogenic tissues) of *C. batrachus*.
2. *C. batrachus* liver were perfused with haemoglobin-free isotonic medium containing

5 mM glucose and 2 mM ornithine, followed by the infusion of 2 mM ammonia, 5 mM glutamine, and in combination of 2 mM ammonia and 5 mM glutamine separately in the perfused liver mainly to find out the incorporation of ammonia and glutamine via the involvement of different types of urea cycle-related CPSs.

3. The subcellular localization of all the urea cycle enzymes (excluding CPS) such as OTC, ASS, ASL and ARG were studied in liver and kidney of *C. batrachus.*
4. The possibility of occurrence of activity of different urea cycle enzymes were also studied in some extra-hepatic tissues such as in muscle, intestine and brain of *C. batrachus.*
5. *C. batrachus* were initially exposed to different concentrations of NH_4Cl starting from 10 to 100 mM for several days to find out the maximum tolerance limit of ammonia and also to find out the percentage of survibility of this fish. Further experiments to study the effect of higher ambient ammonia concentration on ureogenesis in *C. batrachus,* 25 mM NH_4Cl concentration was chosen; fishes were exposed in this concentration of NH_4Cl for 7 days, and the following parameters were measured at different time intervals:
   a) The rate of excretion/absorption of ammonia and urea-N by the fish.
   b) The changes of ammonia and urea levels in plasma and in other tissues such as liver, kidney, muscle and brain.
   c) The changes of activity of all the urea cycle enzymes (both tissue and specific) such as CPS (urea cycle-related), OTC, ASS, ASL and ARG in liver and kidney.
6. *C. batrachus* liver was perfused with isotonic haemoglobin-free medium containing 5 mM glucose and 2 mM ornithine, followed by the infusion of different concentrations of NH_4Cl (0.1 to 5 mM) into the perfused liver for 60 min, and the following parameters

were measured at different time intervals:

a) The changes of ammonia level in the perfused liver after infusing different concentrations of NH_4Cl for 60 min in different sets of perfusion experiment.

b) The efflux of ammonia and urea-N in the effluent by the perfused liver at 2 min intervals.

c) The changes of activity of all the urea cycle enzymes (both tissue and specific) mentioned above in the perfused liver

7. *C. batrachus* were exposed to air for 12, 24 and 48 hr, and the following parameters were measured:

a) The rate of excretion of ammonia and urea-N both during emersion and during re-immersion in water at different time intervals

b) The changes in the levels of ammonia and urea in plasma and in different tissues such as liver, kidney, muscle and brain after different hours of emersion.

c) The changes of activity of all the urea cycle enzymes (both tissue and specific) as mentioned above both in liver and kidney after different hours of emersion.

8. *C. batrachus* were exposed to alkaline water (pH 10) for one week, and the following parameters were measured at different time intervals:

a) The rate of excretion of ammonia and urea-N.

b) The changes in the levels of ammonia and urea in plasma and in different tissues such as liver, kidney, muscle and brain.

c) The changes of activity of all the urea cycle enzymes (both tissue and specific) as mentioned above in liver and kidney.

MATERIALS AND METHODS

Animal:

Clarias batrachus weighing 50-100 g were purchased from commercial sources. They were maintained in the laboratory at about 30 ^{0}C in plastic aquaria containing natural stream water with 12 hr : 12 hr light and dark period. Minced pork liver and rice bran (5% of the body weight) was supplied as food on every alternate day and water was also changed on alternate days. Fishes were used after at least four weeks of acclimatization under the laboratory conditions when the mortality rate became zero and the food consumption was normal. They were always used for experiments after 24 hr of their last feeding, and no food was given during the experimental periods. No sex differentiation of fish was done in any of the experimental set up.

Experimental Set Up:

All the exposure experiments were carried out under the same environmental conditions at which the fishes were acclimatized. Fishes of similar size were used a day after the last feeding and no food was provided to the fish during the experimental periods. No sex differentiation was done in all the exposure experiments.

Ammonium chloride exposure experiment:

A set of fish (preweighed) were exposed individually in plastic buckets containing 1 litre of 25 mM NH_4Cl in each bucket. NH_4Cl solution was prepared in bacteria free filtered stream water. Another set of fish were kept individually in plastic buckets containing 1 litre of bacteria free filtered stream water, which served as control. Both the NH_4Cl solution and the water from each bucket were replaced with a fresh medium on every 12 hr after collecting the sample for the measurement of the rate of ammonia and urea-N excreted by the fish. A decrease in ammonia level from the NH_4Cl solution was

taken as uptake of ammonia by the fish. On day 1, 3 and 7, three fishes each from the NH_4Cl solution and from the water were removed, killed by decapitation after collection of blood from the caudal vein, and tissues such as liver, kidney, muscle and brain were dissected out. Tissues were immediately plunged into liquid nitrogen and stored at -60 ^0C for the analysis of tissue ammonia and urea level, and also for assaying the activity of the urea cycle enzymes.

Aerial exposure experiment:

A set of fish (preweighed) were kept individually in plastic buckets containing no water. Each bucket was covered with bilayer of cheese cloth and the humidity around the fish was about 70 - 80% which was measured with a humidity meter. After 12, 24 and 48 hr, a set of three fishes were given a quick washing with 100 ml of distilled water along with the plastic buckets, where the fishes were kept, for the measurement of the rate of ammonia and urea-N excretion by the fish during aerial exposure. Immediately after washing, fishes were killed by decapitation after collection of blood from the caudal vein, and tissues such as liver, kidney, muscle and brain were dissected out, plunged into liquid nitrogen and stored at -60 ^0C for analysis of tissue ammonia and urea level, and also for assaying the activity of the urea cycle enzymes. A control was also run side by side, where the fishes were kept individually in different buckets containing 1 litre of bacteria free filtered stream water in each bucket. The rate of excretion of ammonia and urea-N were also measured by the control fish from the water at the same time intervals used for the experimental animals. A set of control fish was also killed by decapitation after collection of blood from the caudal vein, and tissues such as liver, kidney, muscle and brain were dissected out for the measurement of tissue ammonia and urea level, and also for assaying the activity of the urea cycle enzymes.

Another set of fish were kept individually in plastic buckets without water in the same way as mentioned above. After 12, 24 and 48 hr, a set of fish were given a quick wash in 100 ml of distilled water to remove the mucous secreted during aerial exposure and re-immersed individually in plastic buckets containing 1 litre of bacteria free filtered stream water in each bucket. On re-immersion, a small amount of water sample was collected from each bucket after 0.5, 1, 2, 6 and 12 hr for the measurement of the rate of ammonia and urea-N excreted by the fish.

Alkaline water (pH 10) exposure experiment:

A set of fish (preweighed) were exposed individually in plastic buckets containing 1 litre of bacteria free filtered stream water in each bucket having the pH of 10 (adjusted with NaOH). Another set of fish were kept individually in plastic buckets containing 1 litre of bacteria free filtered natural stream water (pH 7), which served as control. Both the alkaline water of having pH 10 and the normal water having pH 7 from each bucket were replaced with fresh water at every 12 hr interval after collecting the sample for measurement of the rate of ammonia and urea-N excreted by the fish. On day 1, 3 and 7, three fishes from each water medium were removed, killed by decapitation after collection of blood from the caudal vein, and tissues such as liver, kidney, muscle and brain were dissected out. Tissues were immediately plunged into liquid nitrogen and stored at -60 ^{0}C for the analysis of tissue ammonia and urea level and also for assaying the activity of the urea cycle enzymes.

Liver Perfusion Technique:

C. batrachus (50-100 g body wt), which were acclimatised under the laboratory conditions mentioned above, were used for the perfusion of liver with different

concentrations of NH_4Cl and L-glutamine. Livers were perfused via the portal vein in a non-circulating manner with the haemoglobin free media as used by French *et al.* (1981) with certain modifications made by Saha *et al.* (1995). The medium contained 119 mM NaCl , 5 mM $NaHCO_3$, 5.4 mM KCl, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4$ and 1.25 mM $CaCl_2$ as a basic solution for perfusion. The osmolarity of the perfusing medium was 265mOsmol/ litre since the osmolarity of the blood of *C. batrachus* was also found to be 265 mOsmol/litre determined by the freezing point depression method (Camlab osmometer, Model 2000). The medium also contained 5 mM D-glucose and 2 mM L-ornithine. Different concentrations of NH_4Cl (0.1, 0.2, 0.5, 1, 2 and 5 mM) were infused along with the perfusion medium to study the effect of ammonia on the activity of urea cycle enzymes, its accumulation rate, and also its conversion to urea-N by the perfused liver. In another set of experiment, ammonia was replaced by L-glutamine (5 mM) to study its incorporation to urea-N via the urea cycle and also while studying the types of carbamyl phosphate synthetase enzymes present in this fish. The medium was gassed with O_2/CO_2 (99:1, v/v) before perfusion into the liver, pH adjusted to 7.5 and temperature of the medium was maintained at 30 ^{0}C. The medium was infused into the liver at a flow rate of 4 - 5 ml/g liver/min. The effluent, which was coming out from the perfused liver, was collected through a canula catheterised at the superior vena cava for analysis of ammonia and urea-N.

Livers were perfused for 20 min with the standard isotonic media containing 5 mM D-glucose and 2 mM L-ornithine prior to infusion of NH_4Cl or L-glutamine. Different concentrations of ammonium chloride and L-glutamine were infused separately in different sets of perfused liver for 60 min. Effluent of 2 ml each were collected between 16 to 20 min of perfusion with the standard medium (control), and between 55 to 60 of

infusion of NH_4Cl or L-glutamine for the measurement of ammonia and urea-N coming out into the effluent from the perfused liver. Immediately after 60 min of infusion of ammonium chloride the perfused liver was plunged into liquid nitrogen and stored at -60 ^0C until used for the assay of urea cycle enzymes activity and also the tissue ammonia level.

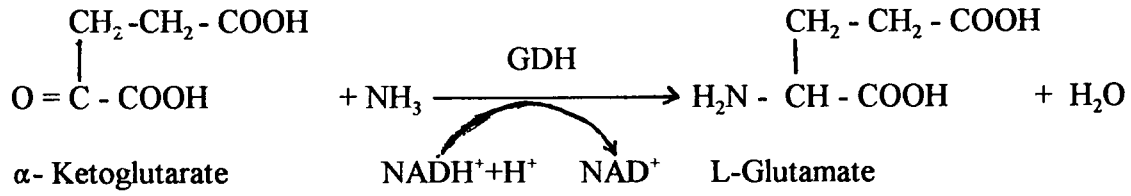
Estimations:

Estimation of ammonia and urea-N in the effluent:

Concentrations of ammonia and urea-N in the effluent were measured enzymatically based on the procedure of Kun and Kearney (1974). To each 2 ml sample of effluent, collected during the perfusion, 40 µl 2M PCA was added to precipitate out the protein present in the sample. The precipitate was separated out by centrifugation and the supernatant was neutralised by adding 40 µl 2M NaOH before the measurement of ammonia and urea-N.

Ammonia:

For measurement of ammonia in the effluent, all the ammonia was converted to glutamate by the enzyme glutamate dehydrogenase (GDH) in presence of α-ketoglutarate and NADH. The amount of NADH oxidized was equivalent to the amount of ammonia present in the effluent. The reaction takes place as follows:



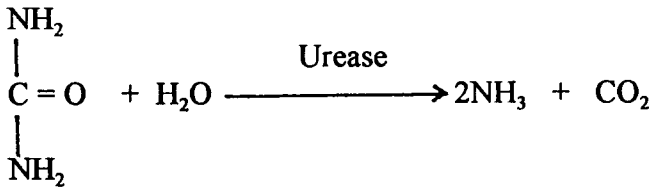
The reaction mixture of 1 ml contained the following:

Tris-HCl buffer (pH 8.0)	66 μmoles
α-Ketoglutarate	2.5 μmoles
EDTA	0.2 μmole
ADP	1.0 μmole
NADH	0.4 μmole
GDH	20 units
Effluent	0.2 ml

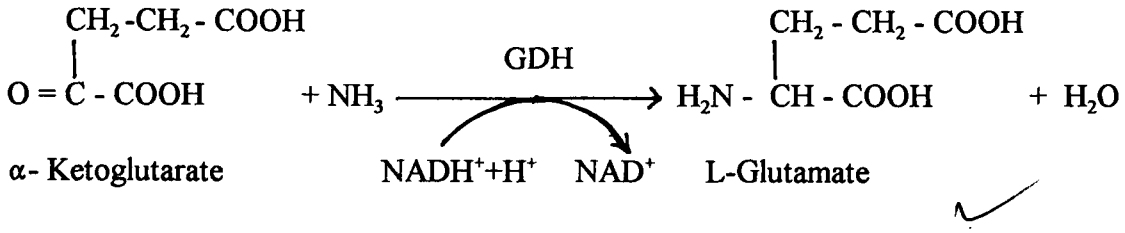
The reaction mixture was incubated at 37 ^{0}C for 30 min time sufficient to convert all the ammonia to L-glutamate. A control was also run simultaneously which contained everything in the reaction mixture as mentioned above except the effluent, which was replaced by 0.2 ml of distilled water. O.D was measured at 340 nm in a quartz 1 ml cuvette having 1 cm light path in a uv-visible spectrophotometer (Beckman, Model 640) both in the control and in the experimental sample. The differences in O.D values obtained between these two were used to calculate the concentration of ammonia present in the effluent taking 6.22×10^3 as molar extinction coefficient for NADH.

Urea-N:

For measurement of urea-N in the effluent, 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 urea-N present in the effluent. The enzymatic reaction takes place as follows:



Urea



The reaction mixture of 1 ml contained the following:

Tris-HCl buffer (pH 8.0)	66 μmoles
α- Ketoglutarate	2.5 μmoles
EDTA	0.2 μmole
NADH	0.4 μmole
GDH	20 units
Urease	20 units
Effluent	0.2 ml.

The reaction mixture was incubated at 37 ^{0}C for 30 min. A control was run simultaneously which contained everything as mentioned above except the effluent, which was replaced by 0.2 ml of distilled water. O.D. was measured at 340 nm in a quartz 1 ml cuvette having 1 cm light path in a uv-visible spectrophotometer (Beckman, Model 640) both in the control and in the experimental sample. The differences in O.D. value obtained between these two was used to calculate the concentration of urea-N present in the effluent taking 6.22×10^3 as molar extinction coefficient for NADH.

Blood Sampling:

Blood was collected from the caudal vein with a heparinized syringe, centrifuged immediately at 10,000 x g for 10 min at 0 $\pm$ 2 ^{0}C to settle the cells. To 0.5 ml of the supernatant (plasma) 0.5 ml of ice-cold 2M PCA was added to deproteinise the plasma and the protein was precipitated out by centrifugation. The supernatant was neutralised

with 2N NaOH and used for the estimation of ammonia and urea-N enzymatically following the same method as used for the measurement in the effluent (mentioned above).

Tissue Processing:

After collection of blood from the caudal vein, at least from those fishes used for different exposure experiments, fishes were killed by decapitation and tissues such as liver, kidney, brain and muscle were immediately removed, plunged into liquid nitrogen and deep frozen at -60 ^{0}C until used for all estimations and enzymatic assays. All estimations were completed within one week of preserving the tissue.

The frozen tissue was thawed on ice and a 10% homogenate of each was prepared in 0.1% cetyltrimethyl ammonium bromide (CTB) using a motor-driven Potter-Elvehjem type of glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 x g for 15 min and the supernatant was used for measurement of tissue levels of ammonia and urea and for assaying the activity of the urea cycle enzymes such as carbamyl phosphate synthetase (CPS, urea synthesis-related), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) and also for the measurement of protein. All the steps were carried out at 4 ^{0}C.

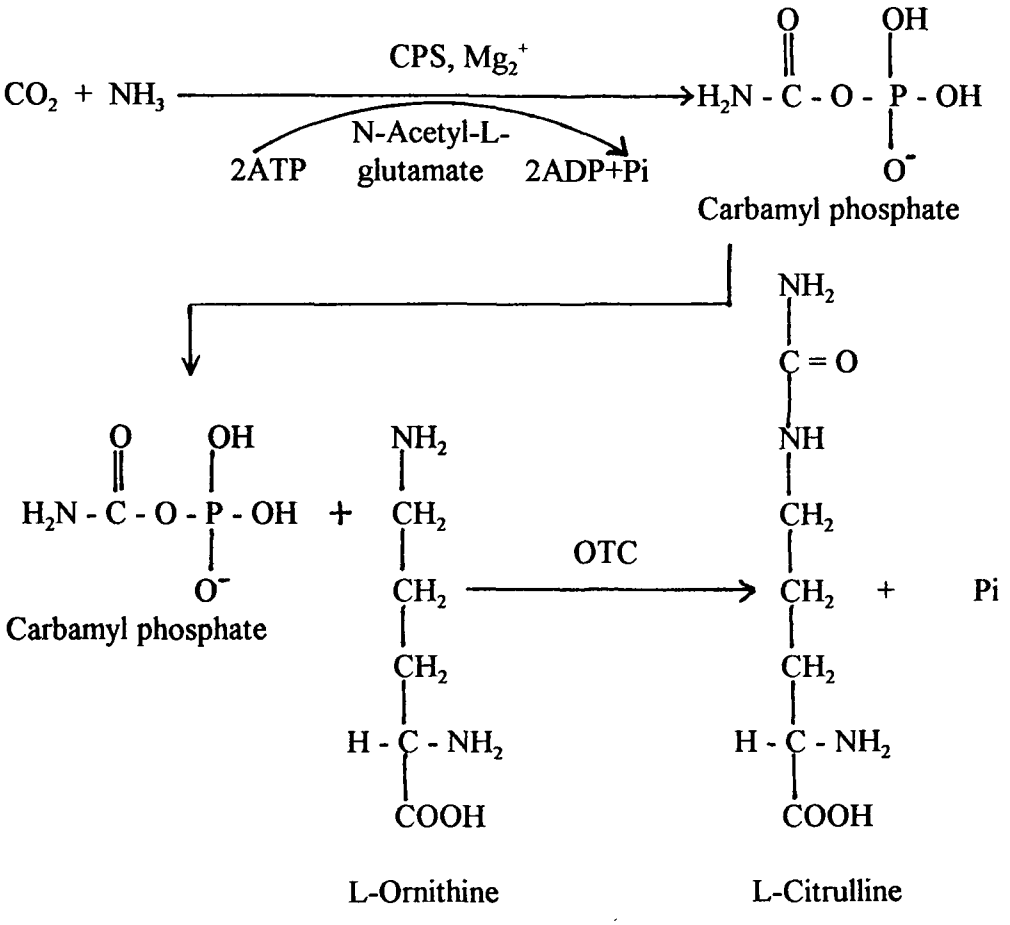
For estimation of ammonia and urea-N in various tissues, the same homogenate prepared in 0.1% CTB was used. The homogenate was treated with 2M PCA in 1:0.5 ratio and centrifuged at 10,000 x g for 10 min to precipitate out the protein. The supernatant was neutralised with 2N NaOH and the ammonia and urea-N levels were measured enzymatically following the same method as used for the effluent.

Subcellular Fractionation:

Subcellular fractionation for studying the subcellular localization of various urea cycle enzymes and also for studying the isoenzymic forms of CPSs present in this fish was carried out following the method of Dkhar *et al.* (1991) with minor modification. A 20% homogenate of freshly excised liver and kidney was prepared in a fractionating buffer containing 10 mM Tris-HCl buffer (pH 7.5), 300 mM mannitol, 1 mM EDTA, 1 mM DTT and 100 mM KCl with a motor-driven Potter-Elvehjem glass homogeniser with a loosely fitted Teflon pestle. The subcellular fractions such as nuclear, mitochondrial and cytosolic (soluble) were separated out by differential centrifugation of the homogenate. The homogenate was first centrifuged at 600 x g for 10 min to pellet out the cell debris and nuclei. The supernatant was decanted and saved. The loose pellet was again resuspended in equal volume of the fractionation buffer, homogenized for the second time as described above to facilitate breakage of unbroken cells, and centrifuged at 600 x g for 10 min. The supernatant was decanted, combined with the first supernatant and centrifuged at 20,000 x g for 20 min to give a well-defined and firm pellet (mitochondrial fraction). The supernatant obtained from this centrifuge step was the cytosolic fraction, which was decanted and poured through four layers of cheesecloth. The nuclear and mitochondrial fractions were resuspended in the same fractionation buffer as described above but without mannitol. All the fractions as well as a portion of the crude homogenate (kept separately before centrifugation) was treated with 0.5% Triton X-100 in 1:1 ratio. The crude homogenate and the mitochondrial fractions were also sonicated to facilitate proper breakage of mitochondria. All the steps were carried out at 4 ^{0}C.

Enzyme Assay:

Carbamyl phosphate synthetase (E.C.2.7.2.5) (CPS, urea synthesis-related): CPS was assayed following the method of Saha and Ratha (1987) with certain modifications. In the reaction mixture, 1 mM UTP was added so as to inhibit the pyrimidine synthesis-related CPS (CPS II) activity, which is known to be a potent inhibitor for this enzyme (Anderson, 1995a), while assaying the urea synthesis-related CPS activity. Carbamyl phosphate so formed by the CPS activity during the period of incubation was converted to citrulline in the presence of excess OTC and L-ornithine. The resultant citrulline formed was estimated for expressing the activity of CPS (urea synthesis-related). The enzymatic reaction takes place as follows:



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

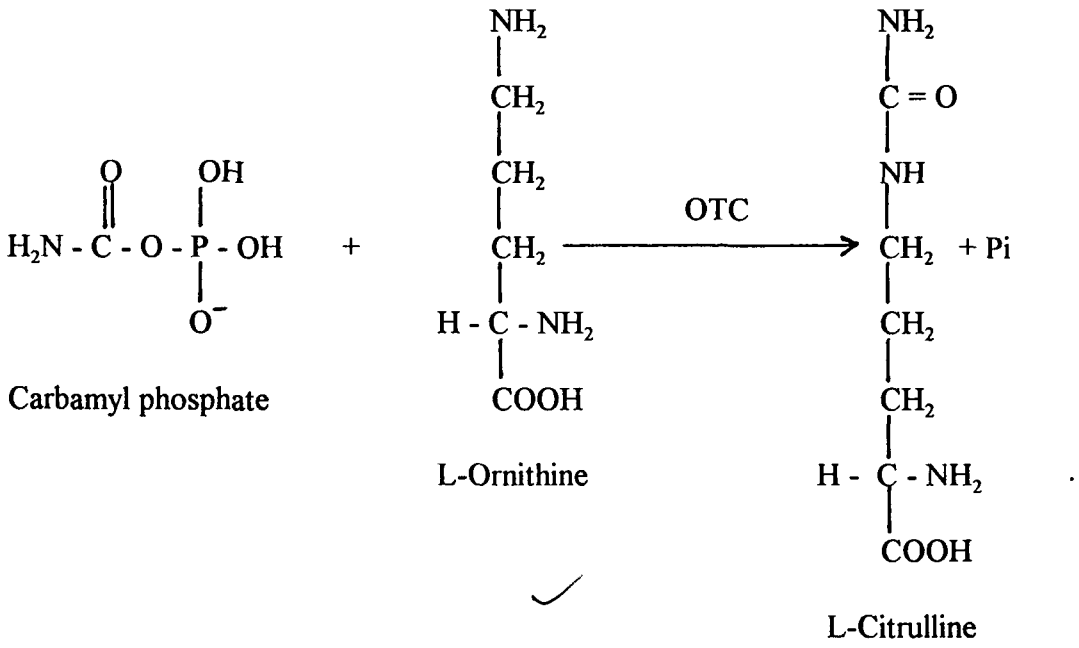
Potassium phosphate buffer (pH 7.5)	50 μmoles
Ammonium chloride	50 μ moles
Sodium bicarbonate	50 μmoles
ATP	20 μmoles
L-Ornithine	5 μmoles
N-Acetyl L- glutamate	5 μmoles
$MgSO_4$	10 μmoles
UTP	1 μmole
OTC	10 units
Tissue extract	0.3 ml

The assay mixture without the tissue extract and UTP, and the tissue extract with UTP were preincubated separately for 5 min at 30 ^{0}C. The reaction was initiated by mixing these two mixtures. After 30 min the reaction was stopped by the addition of 0.5 ml 10% PCA. A tissue extract was prepared simultaneously by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The supernatant was used for the estimation of citrulline following the method of Moore and Kauffman (1970). To 1.0 ml of suitably diluted supernatant 2.5 ml of acid mixture (prepared by mixing 300 ml conc. H_3PO_4, 100 ml H_2SO_4, 0.237 g $MnSO_4$ and 1.8 ml of 0.1 M $FeCl_3$ in 398 ml of distilled water) and 0.25 ml of 3% (w/v) diacetyl monoxime were added. The mixture was kept for boiling for 30 min, cooled and O.D was measured at 490 nm in a uv-visible spectrophotometer (Beckman, Model 640) 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 (0.04 - 0.1μmole) which was linear. One unit of CPS activity

was expressed as that amount of enzyme which catalyzed the formation of 1μmole of citrulline per hr at 30 ^{0}C.

Ornithine transcarbamylase (E.C.2.1.3.3) (OTC):

OTC was assayed spectrophotometrically following the method described by Brown and Cohen (1959) by estimating the product (citrulline) formed by the enzyme. The enzymatic reaction takes place as follows:



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

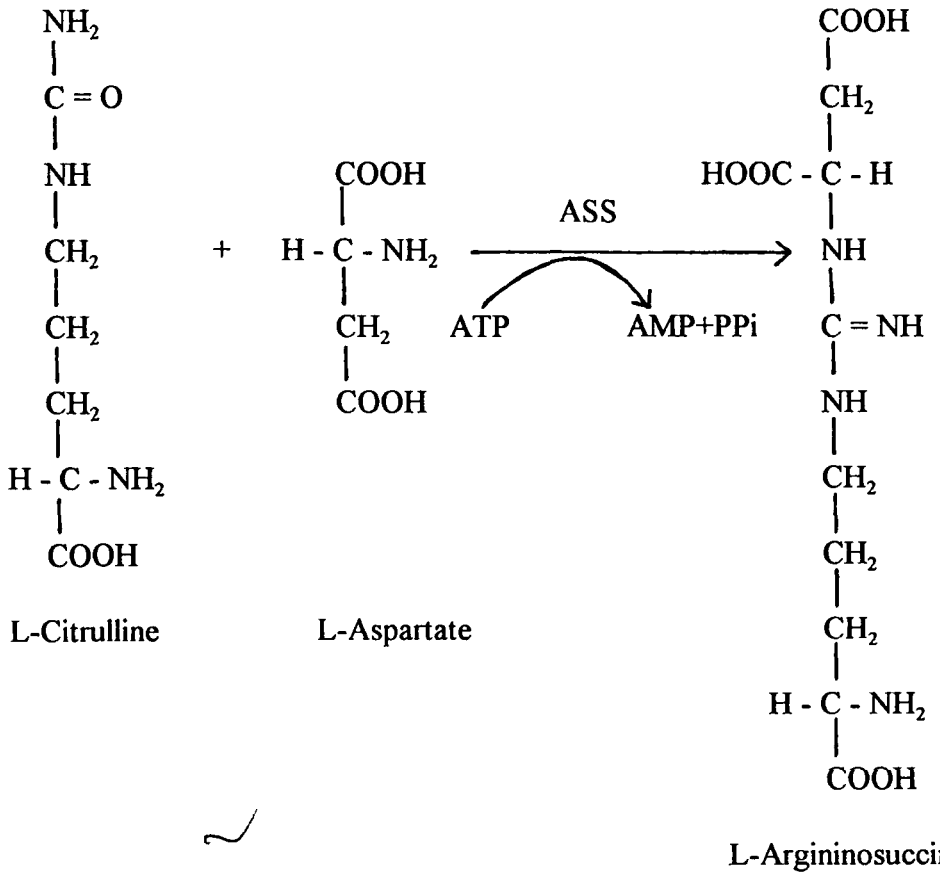
Glycylglycine buffer (pH 8.3)	90 μmoles
L-Ornithine	20 μmoles
Dilithium carbamyl phosphate	20 μmoles
Tissue extract (suitably diluted)	0.3 ml

The assay mixture without the tissue extract was preincubated for 5 min at 30 ^{0}C. The reaction was initiated by the addition of preincubated (separately) tissue extract to the assay mixture. After 20 min of incubation the reaction was stopped by the addition of 0.5 ml of 10% PCA to the reaction mixture. A tissue blank was also prepared by adding PCA to the reaction mixture prior to the addition of the tissue extract. The precipitated protein

was separated out by centrifugation. 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 1μmole of citrulline per hr at 30 ^{0}C.

Argininosuccinate synthetase (E.C.6.3.4.5) (ASS):

The ASS activity was assayed following the method of Ratner (1955) with certain modifications made by Saha and Ratha (1987). In the assay mixture, 20 units of urease was taken to convert all the urea present or formed to ammonia to avoid the interference while estimating citrulline estimation. This time, however, the amount of citrulline utilized by the enzyme per unit time was expressed ASS activity. The reaction takes place as follows:



The assay mixture (pH 7.0) in a final volume of 1.0 ml contained the following:

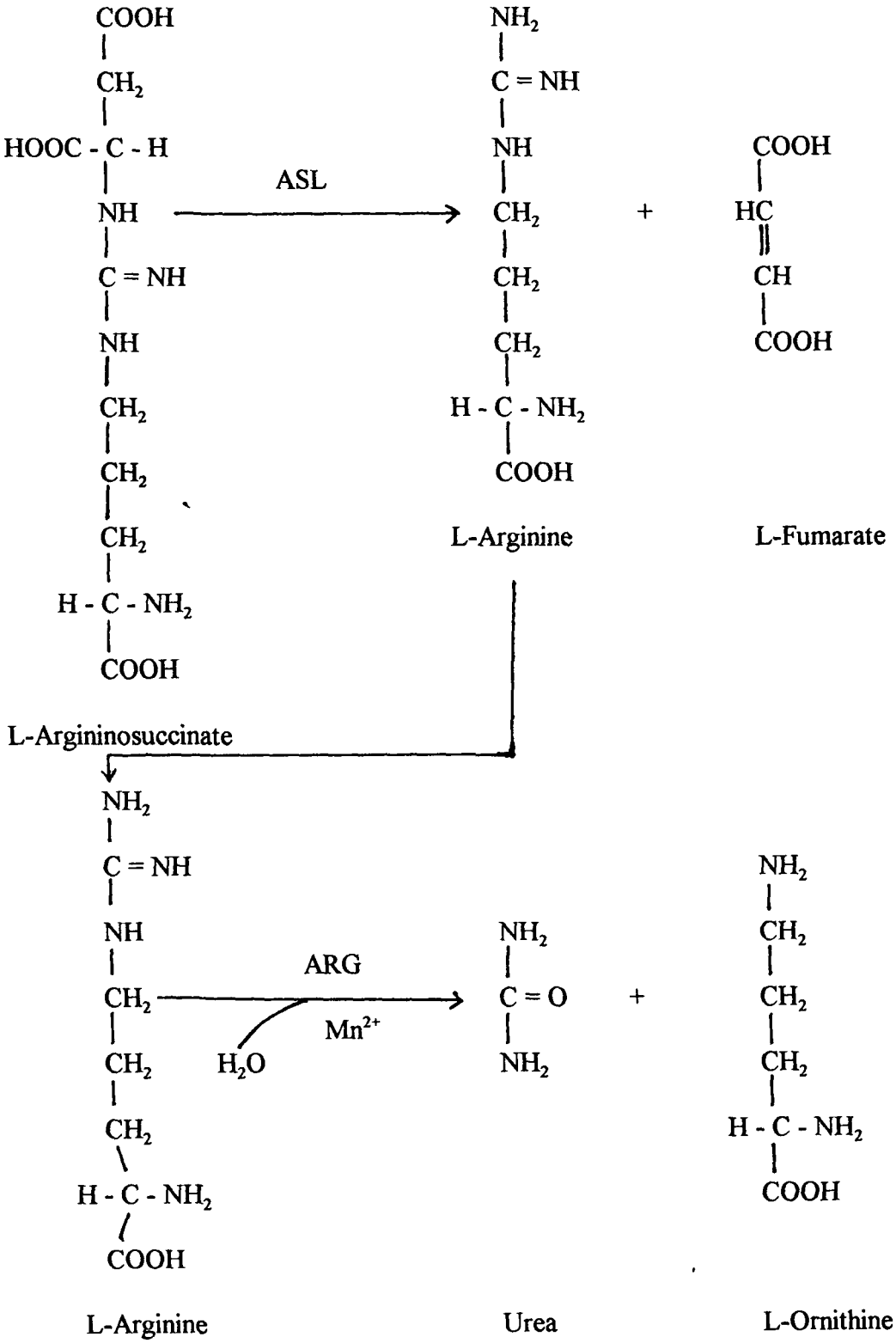
Potassium phosphate buffer (ph 7.0)	50 μmoles
L-Citrulline	3 μmoles
L-Aspartate	5 μmoles
$MgSO_4$	8.75 μmoles
ATP	5 μmoles
Urease	20 units
Tissue extract (suitably diluted)	0.2 ml

The reaction mixture without citrulline was preincubated for 5 min at 30 ^{0}C. The reaction was initiated with the addition of citrulline and incubated for 30 min at 30 ^{0}C. 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. 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 1μmole of citrulline per hr at 30 ^{0}C.

Argininosuccinate lyase (E.C.4.3.2.1) (ASL):

ASL activity was assayed following the method of Brown and Cohen (1959) with certain modifications made by Saha and Ratha (1987). The concentration of L-argininosuccinate was increased from 2 μmoles to 4 μmoles and 20 units of arginase (from Sigma) was added in each reaction mixture. Arginine formed by the ASL activity in the reaction mixture was converted further to urea in presence of excess arginase and the amount of urea formed was estimated to express the ASL activity. The reaction takes place as

follows:


COOH
CH2
HOOC - C - H
NH
C = NH
NH
CH2
CH2
CH2
H - C - NH2
COOH
L-Argininosuccinate
ASL
NH2
C = NH
NH2
CH2
CH2
CH2
H - C - NH2
COOH
L-Arginine
+
COOH
HC
CH
COOH
L-Fumarate
NH2
C = NH
NH
CH2
CH2
CH2
H - C - NH2
COOH
L-Arginine
ARG
Mn2+
H2O
NH2
C = O
NH2
Urea
+
NH2
CH2
CH2
CH2
H - C - NH2
COOH
L-Ornithine

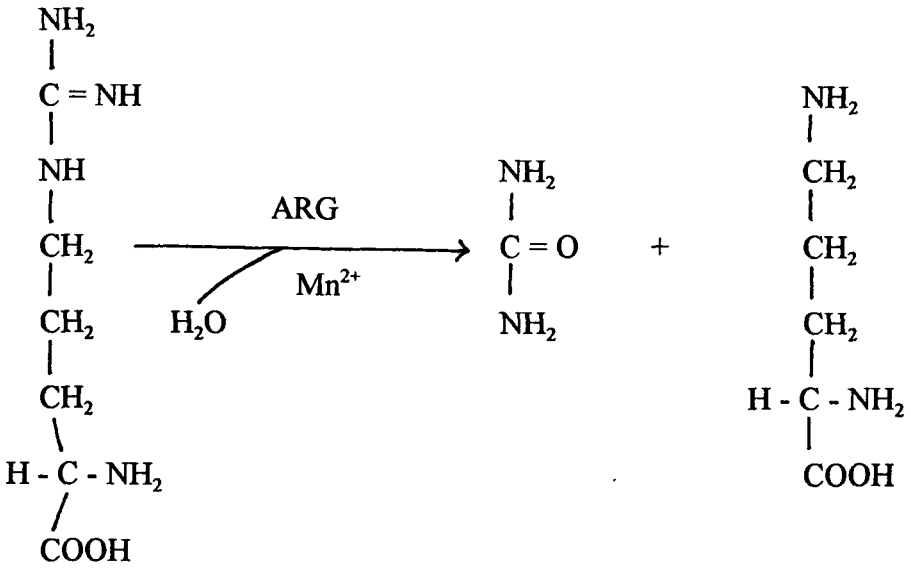
The assay mixture in a final volume of 1.0 ml contained the following:

Potassium phosphate buffer (pH 7.3)	50 μmoles
L-Argininosuccinate	4 μmoles
Arginase	20 units
Tissue extract (suitably diluted)	0.2 ml

The reaction mixture without the tissue extract was preincubated for 5 min at 30 ^{0}C. The reaction was initiated by the addition of tissue extract (preincubated separately) and the reaction was stopped after 30 min by the addition of 0.5 ml 10% PCA. A tissue blank was prepared for each assay by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. Urea formed by the enzymatic reaction was estimated following the method of Moore and Kauffman (1970). The method was same as described for citrulline estimation except that 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 0.05 μmoles) of urea. One unit of ASL activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of urea per hr at 30 ^{0}C.

Arginase (E.C.3.5.3.1) (ARG):

ARG activity was assayed following the method of Brown and Cohen (1959) with certain modifications made by Saha and Ratha (1987). The concentration of L-arginine was increased to 50 μmoles per assay. The urea formed during the incubation was estimated to express the ARG activity. The enzymatic reaction takes place as follows:



L-Arginine Urea L-Ornithine

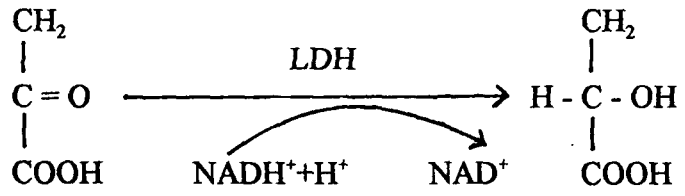
The assay mixture in a final volume of 2.0 ml contained the following:

Sodium glycinate buffer (pH 9.5)	50 μmoles
L- Arginine	50 μmoles
$MnCl_2$	0.5 μmole
Tissue extract (suitably diluted)	0.1 ml

The reaction mixture without L-arginine was preincubated for 5 min at 30 ^{0}C. The reaction was initiated by the addition of L-arginine and incubated for 15 min at 30 ^{0}C. The reaction was stopped by adding 1.0 ml 10 % PCA. A tissue blank was prepared for each assay by adding 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 in ASL assay. One unit of arginase activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of urea per hour at 30 ^{0}C.

Lactate dehydrogenase (E.C. 1.1.1.27) (LDH):

LDH activity was assayed following the method of Vorhaben and Campbell (1972). The enzymatic reaction takes place as follows:



L-Pyruvate L-Lactate

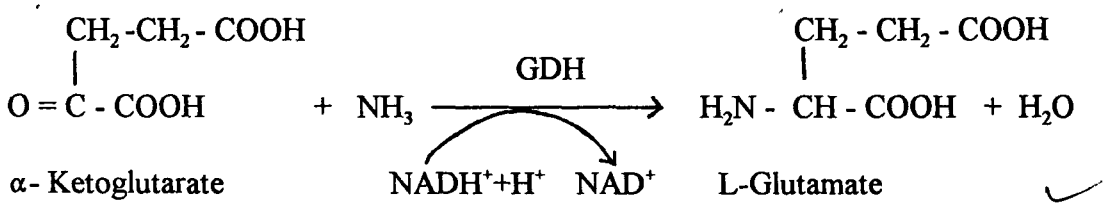
The reaction mixture in a total volume of 1 ml contained the following:

Potassium phosphate buffer (pH 7.0)	100 μmoles
Sodium pyruvate	10 μmoles
NADH	0.3 μmole
Tissue extract (suitably diluted)	0.05 ml

The assay mixture was incubated at 30 ^{0}C in a 1 ml quartz cuvette having 1 cm light path directly in a uv-visible spectrophotometer (Beckman, Model 640) having a Peltier temperature control system fitted to it The reaction mixture without Na-pyruvate was preincubated at 30 ^{0}C for 5 min. The reaction was started by the addition of Na-pyruvate 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 LDH activity. The amount of NADH utilized per hr was calculated taking 6.22 x 10^3 as molar extinction co-efficient value for NADH. One unit of LDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to NAD^+ per hr at 30 ^{0}C.

Glutamate dehydrogenase (E.C. 1.4.2.3) (GDH):

GDH was assayed following the method of Olson and Anfinsen (1952) with certain modifications made by Das *et al.* (1991). The enzymatic reaction takes place as follows:



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

Potassium phosphate buffer (pH 8.5)	100 μmoles
Ammonium chloride	50 μmoles
α- Ketoglutarate	25 μmoles
NADH	0.3 μmole
ADP	1.0 μmole
EDTA	0.2 μmole
Tissue extract (suitably diluted)	0.05 ml

The assay mixture was incubated at 30 ^{0}C in a 1 ml quartz cuvette having 1 cm light path directly in a uv-visible spectrophotometer (Beckman, Model 640) having a Peltier temperature control system fitted to it The reaction mixture without ammonium chloride was preincubated at 30 ^{0}C for 5 min. The reaction was started by the addition of ammonium chloride 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 GDH activity. The amount of NADH utilized per hr was calculated taking 6.22 x 10^3 as molar extinction co-efficient value for NADH. One unit of GDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to NAD^+ per hr at 30 ^{0}C.

Protein:

Protein was estimated following the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Estimation of Water Content in Muscle:

A piece of fresh muscle tissue was weighed and kept in oven at 60°C for drying. The weight of the dry muscle was taken at various time intervals till a constant weight was obtained. The difference in weight between the fresh muscle and dry muscle was considered as the water content and expressed as percentage of wet weight of muscle.

Chemicals:

All the enzymes, substrates, co-enzymes, uridine-5'-triphosphate (UTP), cetyl trimethyl ammonium bromide (CTB) and bovine serum albumin were either obtained from Sigma chemical Co., St. Louis, MO, USA or from Boeringher Mannheim, Mannheim, Germany. All other chemicals used were of analytical grades and obtained from local sources. 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 four observations for each point and presented as mean $\pm$ S.E.M.. The levels of significance between two sets of data were calculated by Students' '*t*' test (Croxton *et al.*, 1982) and *p* value above 0.05 was taken as non-significant (N.S.). Besides the presentation of data in a tabulated form, line graphs and histograms were also prepared to highlight the results.

RESULTS

Types of CPSs and the Sub-cellular Localization of Urea Cycle Enzymes in Different Tissues of C. batrachus:

CPS III activity in mitochondrial extracts of liver and kidney:

As noted by Anderson (1995a), both CPS III and CPS II can be identified by the fact that CPS III would be localized in the mitochondria (whereas CPS II would be localized in the cytosol), and although CPS III (like CPS II) utilizes glutamine as substrate, unlike CPS II, it would be activated by NAG and would not be inhibited by UTP. The same strategy was employed in the present study to identify these two enzymes both in liver and kidney. As shown in tables 1 and 2, a significant level of glutamine-dependent CPS activity was present in the cytosolic fraction of both liver and kidney. This activity was significantly inhibited by UTP and was not affected by the presence of NAG. In addition, little activity was observed when glutamine was replaced by 5 mM ammonia, whether or not NAG was present. These kinetic and specificity properties are characteristic of CPS II. It should be noted that CPS II is also active with ammonia as substrate, but at pH 7.5 the ammonia concentration must be much higher (e.g., 100 mM) than the 5 mM used here to attain a maximum velocity comparable to that attained with glutamine (Anderson, 1995a). Significant glutamine-dependent activity was also found in the mitochondrial fraction of both liver and kidney, but only when NAG was present; this glutamine- and NAG-dependent CPS activity was not significantly affected by the presence of UTP. These properties are characteristic of CPS III. Several observations made from the activity in liver mitochondrial extracts indicate that the glutamine-dependent activity is not an artifact in which activity is actually due to ammonia as a substrate, present as a contaminant of glutamine or arising from hydrolysis

of glutamine by a glutaminase present in the extract. Product formation with either ammonia or glutamine as substrate was linear with time up to 45 min (data not shown). Some ammonia was formed with time in reaction mixtures with glutamine (e.g. 0.15, 0.34 and 0.51 mM after 15, 30 and 45 min, respectively), but the levels were too low to account for the rate observed with glutamine as substrate (note the Km values of 2.2 and 0.46 mM for ammonia and glutamine, respectively, below). Thus, these results appear to confirm the expectation that these fish have a urea cycle-related mitochondrial glutamine- and NAG-dependent CPS III and a pyrimidine-related cytosolic glutamine-dependent CPS II activity, analogous to the only three other teleosts in which the distribution of these CPSs has been documented, i.e., largemouth bass (Cao *et al.*, 1991), marine toadfish (Anderson and Walsh, 1995) and singhi catfish (Saha *et al.*, 1997). The distribution of the activities of GDH and LDH, marker enzymes for mitochondrial and cytosolic fractions, respectively, indicates that although complete separation between the mitochondrial and cytosolic components was not attained, the subfractionation did provide significant enrichment of the two subcellular fractions, sufficient for the purposes described here.

CPS I-like activity in mitochondrial extracts of liver and kidney:

An unexpected finding was the presence, in the mitochondrial fractions of both liver and kidney, of a level of ammonia- and NAG-dependent CPS activity that was not affected by the presence of UTP, but almost at the level or little bit higher than the glutamine- and NAG-dependent activity (Tables 1 and 2). Similar observations were also made recently in another amphibious singhi catfish, *H. fossilis* (Saha *et al.*, 1997). Further, inclusion of both ammonia and glutamine along with NAG in the reaction mixture resulted in an increase in the level of activity showing some additive effect

(Tables 1 and 2). It should be noted here that this additive effect was not observed in the cytosolic fraction. (Tables 1 and 2). The observed additive effect might occur if either ammonia or glutamine can serve as nitrogen-donating substrate and the concentration of each is well below its Km value. This does not appear to be an explanation, however, because the measured Km values from ammonia and glutamine were found to be 2.2 and 0.46 mM, respectively.

Urea formation from ammonia and glutamine by perfused liver:

Additional support for the probable occurrence of both ammonia- and glutamine-dependent urea cycle-related CPS activity was obtained from the liver perfusion studies. When NH_4Cl and glutamine were infused separately into liver of walking catfish, a significant rate of urea formation was observed with either substrate (Table 3). When NH_4Cl and glutamine were infused together, the rate of urea formation was increased further showing some additive effect.

Subcellular localization of urea cycle enzymes in liver and kidney:

The subcellular localization of all the urea cycle enzymes (except CPS) both in liver and kidney of walking catfish are presented in tables 4 and 5. Most of the activity of OTC (86 and 87%) and ARG (76 and 77%) both in liver and kidney were found to be localized primarily in the mitochondrial fraction along with 83.5 and 92% of GDH activity in liver and kidney, respectively. ARG activity has been reported to be associated with the outer membrane of rat liver mitochondria due to non-specific binding, which could be solubilized by increasing the ionic (KCl) concentration in the homogenate (Rosenthal *et al.*, 1956; Soberon and Palcios, 1976). The homogenizing medium used in the present study contained 0.1 M KCl, suggesting that the mitochondrial localization of

ARG observed in liver and kidney of this fish is not an artifact due to non-specific binding to the outer membrane of the mitochondria. The activity of ASS (78 and 79%) and ASL (84 and 83%) were found to be localized in the cytosolic fraction along with 86 and 87% of LDH activity in liver and kidney, respectively.

Activity of urea cycle enzymes in extra-hepatic tissues:

All the urea cycle enzymes were also measured in the present study in some extra-hepatic tissues other than kidney, such as muscle, intestine and brain of walking catfish without separating out the different subcellular fractions (Table 6). As noted above for testing the different types of CPS activity in the mitochondrial and cytosolic fractions of liver and kidney, the same strategy was also used for measurement of different types of CPSs in all the three non-ureogenic tissues. When ammonia alone was taken in the reaction mixture, no significant activity of CPS activity could be detected in any of the tissues. However, when NAG was taken along with ammonia, significant level of activity could be detected in all the three tissues, which was not affected by the presence of UTP. This may indicate that CPS I-like activity is present in all these three tissues. Low levels of CPS activity could also be detected in all the three tissues when glutamine alone was taken in the reaction mixture, which was activated by 113, 185 and 187%, respectively, in presence of NAG, and partially inhibited by UTP in presence of glutamine and NAG by 36, 47 and 23% in muscle, intestine and brain, respectively. This may indicate that both CPS III and II activity are also present in all the three tissues with a relatively higher levels in brain. As noted above in case of liver and kidney, in all the three non-hepatic tissues also some additive effect of enzyme activity could be seen when both glutamine and ammonia were taken together in the reaction mixture along with NAG (Table 6). All the other urea cycle enzymes could also be detected in the three

different tissues except for ASS in brain and ASL in muscle, which could not be detected with the present assay method used.

NH_4Cl Exposure Experiment:

Survival of C. batrachus in NH_4Cl solution:

C. batrachus were exposed to different concentrations of NH_4Cl solution ranging from 10 mM to 100 mM concentrations. The fish did not show any behavioural changes in 10 mM NH_4Cl. The fishes became hyper-excitable at higher concentrations of NH_4Cl which increased with increasing concentration. At 100 mM NH_4Cl solution, they became very violent and did not survive beyond 1 to 2 days. Fishes survived up to 75 mM NH_4Cl for one month without any mortality. While in 25 mM solution fishes initially became hyper-excitable but calmed down within 24 hr and in natural habitat also these fishes do not face the problem of higher anbient ammonia beyond 25 mM concentration. Therefore, to study the effect of hyper-ammonia stress, fishes were treated with 25 mM NH_4Cl for 7 days, and the pattern of changes of ammonia and urea-N excretion, tissue levels of ammonia and urea, and the changes in the activity of urea cycle enzymes were studied in liver and kidney tissues of *C. batrachus* at different time intervals.

Excretion /absorption of ammonia and urea-N:

Ammonia: Absorption of ammonia by the fish kept in 25 mM NH_4Cl was calculated by subtracting the real amount of ammonia present in the medium at different time intervals from the initial concentration of NH_4Cl in the medium. Absorption of ammonia dominated over excretion of ammonia by the fish kept in 25 mM solution of NH_4Cl for

7 days (Table 7; Fig. 3). Significant amount of ammonia were absorbed by the fish on the 1st day itself, followed by further increase up to the day 5, but remained significantly high even on the day 7 of experiment. The initial rate of excretion of ammonia by the control fish was about 140 µmoles/kg body wt/hr, which reduced slightly to about 130 µmoles/kg body wt/hr from day 5 onwards.

Urea-N: The alteration in the rate of excretion of urea-N by *C. batrachus* kept in 25 mM NH_4Cl solution for 7 days have been presented in table 8 and figs. 3 and 4. There was an immediate increase in the rate of urea-N excretion from 32 ± 6.0 to 279 ± 21 µmoles/kg body wt/hr (771%) on the 1st day itself, followed by gradual increase to 590 µmoles/kg body wt/hr (1687%) on the day 5. No further increase in urea-N excretion rate was observed after the 5th day of treatment. The average rate of urea-N excretion by the control fish was about 34 µmoles/kg body wt/hr throughout the experimental periods.

Tissue ammonia and urea levels:

Ammonia: There was significant accumulation of ammonia in all tissues studied (except in brain) and in plasma of *C. batrachus* kept in 25 mM NH_4Cl solution for 7 days (Table 9; Figs. 5 and 7). Ammonia accumulated significantly after the 1st day of treatment in all the tissues (except in brain) and in plasma, followed by further increase after the 3rd and 7th day of treatment. Ammonia accumulated maximally in liver from 10.7 to 23.2 µmoles/g wet wt (116%) on the 7th day, in kidney from 10.1 to 22.6 µmoles/g wet wt (123%) on the 7th day, in muscle from 5.1 to 11.8 µmoles/g wet wt (131%) on the 3rd day, and in plasma from 0.65 to 2.47 µmoles/ml (280%) on the 7th day of treatment with 25 mM NH_4Cl solution. Brain being a highly sensitive tissue, there was no significant

increase of ammonia level even after 7th day of treatment.

Urea: There was significant increase in urea level in all the tissues including plasma of *C. batrachus* kept in 25 mM NH_4Cl solution for 7 days (Table 10; Figs. 6 and 7). Urea accumulated significantly in all the tissues studied and in plasma after the 1st day of treatment. In liver the urea level increased maximally from 3.6 to 10.2 µmoles/g wet wt (183%) on the 7th day, in kidney from 3.9 to 9.3 µmoles/g wet wt on the 3rd day, in muscle from 1.6 to 4.4 µmoles/g wet wt (175%) on the 3rd day, in brain from 2.2 to 2.9 µmoles/g wet wt (31%) on the 3rd day, and in plasma from 0.37 to 1.32 µmoles/ml (257%) on the 7th day of treatment.

Changes of urea cycle enzymes activity:

Liver: The activity of all the urea cycle enzymes (both tissue and specific) except OTC and ARG increased significantly in the liver of *C. batrachus* kept in 25 mM NH_4Cl solution (Tables 11 and 12; Figs. 8, 9 and 12). The tissue activity of CPS, ASS and ASL increased significantly after the 1st day of treatment having maximum increase of activity on the 3rd day in case of CPS (211%), and on the 7th day in case of ASS (59%) and ASL (60%). No significant increase of tissue activity of OTC and ARG could be observed all through the experimental periods. In control fishes the tissue activity of all the urea cycle enzymes in liver remained unaltered throughout the experimental periods.

The pattern of changes in the specific activity of urea cycle in liver was almost same as that of tissue activity (Table 12; Fig. 9 and 12). The CPS and ASS activity increased maximally on the 3rd day of treatment by 211% and 104%, respectively, whereas in case of ASL maximum increase (83%) was observed on the 7th day of treatment. No significant increase of specific activity was seen in case of OTC and ARG

all through the experimental periods. In control fishes, like the tissue activity, the specific activity of all the urea cycle enzyme in liiver remained unaltered all through the experimental periods.

Kidney: The activity of all the urea cycle enzymes (both tissue and specific) except OTC and ARG increased significantly in the kidney of *C. batrachus* kept in 25 mM NH_4Cl solution (Tables 13 and 14; Figs. 10, 11 and 12). The tissue activity of CPS, ASS and ASL increased significantly after the 1st day of treatment which increased maximally on the 3rd day in case of CPS (124%) and ASL (78%), and on the 7th day in case of ASS (79%). No significant increase of tissue activity of OTC and ARG could be observed in kidney throughout the experimental periods. In control fishes, the tissue activity of all the urea cycle enzymes remained unaltered for 7 days.

The pattern of changes in the specific activity of all the urea cycle enzymes in kidney was almost same as that of the tissue activity (Table 14; Figs. 11 and 12). There was significant increase of specific activity of CPS, ASS and ASL after 1st day of treatment, but increased maximally on the 3rd day of treatment by 146%, 64% and 83%, respectively. The specific activity of OTC and ARG did not show any significant change all through the experimental periods. In control fishes, the specific activity of all the urea cycle enzymes remained unaltered for 7 days.

Liver Perfusion Experiment:

Changes of ammonia level in the liver perfused with different concentrations of NH_4Cl:

Changes in ammonia level in the liver perfused with different concentration of NH_4Cl have been presented in table 16. No significant increase of ammonia level in perfused liver was observed until increased the addition of 1.25 μmoles/g liver/min NH_4Cl. Ammonia level in liver raised from 7.64 to about 28.5 μmoles/g wet wt of liver with the infusion of 5.08 μmoles/g liver/min NH_4Cl, followed by no further increase at a higher rate of infusion.

Formation of urea-N in the perfused liver:

The rate of urea-N released into the effluent by the perfused liver while infusing NH_4Cl at different rates was found to be a saturable process (Table 16; Fig. 13). Initially at a lower rate of infusion of NH_4Cl , that is, up to 1.25 μmoles/g liver/min, about 40-50% of the total ammonia taken up by liver was converted to urea-N. Hence, the rate of urea-N excreted increased with the increase of infusion of NH_4Cl until it reached a Vmax of 0.47 μmol/g liver/min with the infusion rate of 5.08 μmoles/g liver/min, followed by no further increase of urea-N excretion at a still higher rate of infusion.

Uptake of ammonia by the perfused liver:

Uptake of ammonia by the perfused liver, which was calculated by subtracting the amount of ammonia that was coming out into the effluent from the total ammonia infused in liver, was shown in table 16 and fig. 14. Initially up to the addition of 1.25 μmoles/g liver/min, about 50-70% of the total ammonia infused were taken up by liver. At higher rates of ammonia addition, the percentage uptake of ammonia gradually

decreased. The rate of uptake of ammonia also appeared to be a saturable process reaching a Vmax of 1.34μmoles/g liver/min with the infusion of 10.81 μmoles/g liver/min of NH_4Cl for 60 min (Fig. 14). However, the Vmax of urea-N excretion was only 0.47μmoles/g liver/min indicating that the rate of uptake of ammonia was higher (about 3 fold) than the rate of urea-N excretion.

Changes of urea cycle enzymes activity in the perfused liver:

Pattern of changes of tissue activity of urea cycle enzymes in liver infused with different concentration of NH_4Cl are shown in table 17 and fig. 15. At lower rates of NH_4Cl infusion, that is, up to 1.25 μmoles/g liver/min, no significant increase of tissue activity of any of the urea cycle enzymes were observed except for ASS, the activity of which increased significantly ($p<0.05$) even at 1.25 μmoles/g liver/min of NH_4Cl infusion. Significant stimulation of tissue activities of other urea cycle enzymes were seen from 2.32 μmoles/g liver/min of infusion. The activity of CPS was stimulated maximally from 3.92 ± 0.31 to 8.68 ± 0.71 U/g wet wt with the addition of 5.08 μmoles/g liver/min of NH_4Cl, and for ASS and ASL, from 65.8 ± 6.2 to 153.6 ± 6.4 and from 100.3 ± 7.2 to 151.3 ± 6.9 U/g wet wt, respectively, with the addition of 10.81 μmoles/g liver/min of NH_4Cl. Activities of CPS, ASS and ASL increased maximally by 121%, 133% and 51%, respectively. However, the tissue activities of OTC and ARG did not show any significant change in all the concentrations of NH_4Cl infused.

Pattern of changes of specific activity of urea cycle in the perfused liver infused with different concentration of NH_4Cl are shown in table 18 and fig.16. Significant stimulation of specific activity was also observed for ASS from 1.25μmoles/g liver/min of NH_4Cl addition, and for CPS and ASL, it was from 2.32 μmoles/g liver/min onwards Specific activities rose maximally from 0.048 ± 0.005 to 0.11 ± 0.02 for CPS, 0.81 ± 0.1

to 1.94 ± 0.12 for ASS and from 1.23 ± 0.14 to 1.88 ± 0.09 U/mg protein for ASL. Specific activities of CPS, ASS and ASL increased maximally by 129%, 140% and 53%, respectively. Like the tissue activity, specific activities of OTC and ARG also did not change in any of the concentration of NH_4Cl infused.

Aerial Exposure Experiment:

Excretion of ammonia and urea-N:

Ammonia: Alterations in the pattern of excretion of ammonia by *C. batrachus* kept outside water for different periods, followed by re-immersion in water have been presented in table 19 and fig. 17. There was immediate suppression in the excretion of ammonia by 98% by *C. batrachus* kept outside water for 12 hr, followed by further decrease to almost nil (99%) in later part of aerial exposure. There was immediate increase in the ammonia excretion rate when the fishes were re-immersed in water, followed by gradual decrease almost to the normal level after 12 hr of re-immersion. Maximum increase of ammonia excretion rate during 0 to 0.5 hr of re-immersion period was seen by the fish kept for 48 hr emersion (155%), followed by the fish kept for 24 hr (132%) and 12 hr (121%) of emersion in a decreasing order.

Urea-N: There was immediate suppression in urea-N excretion of about 95% by the fish kept outside water for 12 and 24 hr and by about 80% by the fish kept outside water for 48 hr (Table 20; Fig.17). However, the rate of urea-N excretion by all group of fishes increased tremendously, immediately after re-immersion in water, followed by a gradual decrease at later parts of re-immersion (Table 20; Fig.17). However, a higher rate of urea-N excretion was maintained by all group of fish even after 12 hr of re-immersion in

water unlike that of ammonia excretion.Maximum increase of urea-N excretion rate during 0 to 0.5 hr of re-immersion was seen by the fish kept outside water for 48 hr emersion (2200%), followed by the group of fish kept outside water for 24 hr (1453%) and 12 hr (693%) in a decreasing order.

Tissue ammonia and urea levels:

Ammonia: There was significant accumulation of ammonia in all the tissues including plasma of *C. batrachus* during exposure to air for various periods (Table 21; Figs.18 and 19) . In all the tissues studied (except brain) ammonia accumulated significantly within 12 hr of aerial exposure, followed by further increase after 24 and 48 hr of aerial exposure. Ammonia accumulated maximally after 48 hr of aerial exposure. The level of ammonia in liver increased from 10.7 to 24.17 µmoles/g wet wt, in kidney from 8.66 to 27.31 µmoles/g wet wt, in muscle from 7.97 to 16.56 µmoles/g wet wt, in brain from 3.6 to 4.9 µmoles/g wet wt, and in plasma from 0.6 to 2.17 µmoles/g ml after 48 hr of aerial exposure. Percentage wise maximum increase in ammonia accumulation was seen in plasma (261%), followed by kidney (215%), liver (126%), muscle (107%) and brain (36%) in a decreasing order.

Urea: There was significant accumulation of urea level in different tissues and in plasma of *C. batrachus* during exposure to air for different periods. (Table 22; Figs.18 and 19). In all the tissues including plasma, urea accumulated significantly within 12 hr of aerial exposure, followed by further increase after 24 and 48 hr of exposure. The level of urea in liver increased maximally from 3.67 to 17.3 µmoles/g wet wt, in kidney from 2.67 to 9.01 µmoles/g wet wt, in muscle from 0.95 to 1.95 µmoles/g wet wt, in brain from 1.9 to 3.8 µmoles/g wet wt, and in plasma from 0.35 to 1.79 µmoles/ml after 48 hr of aerial

exposure. Percentage wise maximum increase in urea accumulation was seen in plasma (411%), followed by liver (371%), kidney (237%), muscle (105%), and brain (100%) in a decreasing order.

The urea cycle enzymes activity:

Liver: There was significant increase of activity (both tissue and specific) of at least four enzymes of urea cycle such as CPS, OTC, ASS and ASL the liver of *C. batrachus* kept outside water for different periods (Tables 23 and 24; Figs. 20, 21 and 24). The tissue activity of CPS, ASL and OTC increased maximally by 100%, 58%, and 31%, respectively, after 48 hr of aerial exposure. Whereas, in the case of ASS the maximum increase of activity was seen after 12 hr of aerial exposure. The tissue activity of ARG did not show any significant change in any of the experimental periods.

The pattern of changes of specific activity of different enzymes of urea cycle was almost same as that of tissue activity. The specific activity of CPS and ASL increased maximally by 128% and 92%, respectively, after 48 hr of aerial exposure . Whereas, the specific activity of OTC and ASS increased maximally by 24% and 128%, respectively, after 24 hr of aerial exposure. The specific activity ARG in liver did not show any significant change in any of the experimental periods.

Kidney: There was significant increase of activity (both tissue and specific) of three enzymes of urea cycle such as CPS, ASS and ASL in the kidney of *C. batrachus* kept outside water for different periods (Tables 25 and 26; Figs. 22, 23 and 24). The tissue activity of CPS, ASS and ASL increased maximally by 118%, 84% and 76%, respectively, after 48 hr of aerial exposure. The tissue activity of OTC and ARG did not show any significant change in any of the experimental periods.

The pattern of changes of specific activity of different urea cycle enzymes in the kidney of *C. batrachus* kept outside water was almost same as that of tissue activity. The specific activity of CPS, ASS and ASL increased maximally by 123%, 104% and 75%, respectively, after 48 hr of aerial exposure. The specific activity of OTC and ARG did not show any significant change in any of the experimental periods.

Exposure to Alkaline Water:

Excretion of ammonia and urea-N:

Ammonia: The pattern of changes of excretion of ammonia by *C. batrachus* while exposed to alkaline water (pH 10) for 7 days with relation to control have been presented in table 27 and fig. 25. There was an immediate significant decrease of ammonia excretion rate to about 35 to 50% by the fish exposed to alkaline water, which gradually recovered to the normal level from the 5th day onwards. The rate of excretion of ammonia remained significantly lower than the control level till three and half days (84 hr) of exposure to alkaline water. The rate of excretion of ammonia by the control fish was initially about 210 µmoles/kg body wt/hr and slowly reduced to about 165 µmoles/kg body wt/hr on the 7th day.

Urea-N: The pattern of changes of urea-N excretion by *C. batrachus* exposed to alkaline water (pH 10) have been presented in table 28 and fig. 25. There was significant increase of about 50% of urea-N excretion by the fish within 12 hr of exposure to alkaline water, which increased further to about 250% on the 3rd day of exposure and maintained this highest rate of excretion till the 4th day, followed by a gradual decrease at later stages of exposure. However, the rate of urea-N excretion by the fish remained

significantly higher even on the 7th day of exposure to alkaline water. The rate of excretion of urea-N by the control fish was about 60 µmoles/kg body wt/hr all through the experimental periods.

Tissue ammonia and urea levels:

Ammonia: There was significant increase in the level of ammonia in all the tissues studied (except in brain) and in plasma of *C. batrachus* while exposed to alkaline water (Table 29; Figs. 26 and 28). Ammonia accumulated maximally on the 3rd day. which reduced to certain level on the 7th day of exposure to alkaline water but remained significantly higher than the control in most of the tissues. Percentage wise ammonia accumulated maximally in plasma (267%), followed by muscle (88%), kidney (70%) and liver (60%) in a decreasing order on the 3rd day of exposure to alkaline water.

Urea: There was significant accumulation of urea in all the tissues studied (except in brain) and in plasma of *C. batrachus*. on the 3rd day of exposure to alkaline water (pH 10) (Table 30; Figs. 27 and 28). However, the level of urea in all the tissues (except in liver) almost returned to the normal level on the 7th day of exposure to alkaline water. Percentage wise urea accumulated maximally in kidney (63%), followed by liver (50%), plasma (45%), and in muscle (32%), in a decreasing order on the 3rd day of exposure to alkaline water.

The Urea cycle enzymes activity:

Liver: There was significant initial increase of activity (tissue and specific) of at least three enzymes of urea cycle such as CPS, ASS and ASL in liver of *C. batrachus* exposed to alkaline water (Tables 31 and 32; figs. 29, 30 and 33). The tissue activity of CPS, ASS and ASL enzymes increased maximally by 82%, 59% and 52%, respectively, on the 3rd

day of exposure. The tissue activity of OTC and ARG enzymes in liver did not show any significant change during exposure to alkaline water.

The pattern of changes of specific activity of urea cycle enzymes in liver was almost same as that of tissue activity while exposed to alkaline water. The specific activity of CPS, ASS and ASL enzymes increased maximally by 153%, 121% and 111%, respectively, on the 3rd day of exposure to alkaline water. The specific activity of OTC and ARG enzymes did not show any significant change in the liver during exposure to alkaline water.

Kidney: There was significant initial increase of activity (tissue and specific) of at least three enzymes of the urea cycle such as CPS, ASS and ASL in kidney of *C. batrachus* after the 3rd day of exposure to alkaline water, followed by returning back the activity almost to the normal level on the 7th day of exposure (Table 33 and 34; Figs. 31, 32 and 33). The tissue activity of CPS, ASS and ASL increased maximally by 55%, 182% and 42%, respectively, on the 3rd day of exposure. The tissue activity of OTC and ARG remained unaltered throughout the experimental periods.

The specific activity of CPS, ASS and ASL enzymes in kidney of *C. batrachus* also increased maximally by 67%, 197% and 49%, respectively after the 3rd day of exposure to alkaline water, which later returned almost to the normal level on the 7th day of exposure. The specific activity of OTC and ARG enzymes in kidney did not show any significant change throughout the experimental periods.

TABLES

Table 1: CPS activity (units/g wet wt) in different subcellular fractions of *C. batrachus* liver in the presence of different nitrogen-donating substrates, NAG and/or UTP. Values are expressed as mean ± SEM (n = 3).

Components in the reaction mixture	Homogenate	Mitochondrial fraction	Cytosolic fraction
Ammonia		1.25 ± 0.10	0.55 ± 0.12
Ammonia + UTP		0.75 ± 0.14	0.34 ± 0.09
Ammonia + NAG		5.04 ± 0.37	0.64 ± 0.11
Ammonia + NAG + UTP		4.75 ± 0.45	0.45 ± 0.05
Glutamine		1.45 ± 0.14	3.87 ± 0.29
Glutamine + UTP		1.15 ± 0.16	1.25 ± 0.07
Glutamine + NAG		4.85 ± 0.85	3.96 ± 0.65
Glutamine + NAG + UTP		4.17 ± 0.80	1.45 ± 0.11
Ammonia + Glutamine + NAG		6.55 ± 0.82	4.07 ± 0.81
Ammonia + Glutamine + NAG + UTP		6.05 ± 1.15	0.95 ± 0.14
GDH	616 ± 56	490 ± 36 (80)	141 ± 22 (23)
LDH	1245 ± 122	205 ± 45 (16)	1047 ± 102 (84)

The reaction mixture for carbamyl phosphate synthetase (CPS) assay contained everything as mentioned in the Materials and Methods section, plus 5 mM ammonium chloride, 25 mM glutamine, 5 mM N-acetyl-L-glutamate (NAG) and/or 1 mM uridine-5'-triphosphate (UTP) wherever noted. One unit of enzyme activity was expressed as the amount that catalyzed 1 µmole of citrulline in case of CPS, and 1 µmole of NADH utilized for glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH) per hr at 30 °C

% of GDH and LDH activity out of the total are given in parentheses.

Table 2: CPS activity (units/g wet wt) in different subcellular fractions of *C. batrachus* kidney in the presence of different nitrogen-donating substrates, NAG and/or UTP. Values are expressed as mean ± SEM (n =3).

Components in the reaction mixture	Homogenate	Mitochondrial fraction	Cytosolic fraction
Ammonia		1.25 ± 0.18	0.45 ± 0.07
Ammonia + UTP		0.65 ± 0.09	0.30 ± 0.08
Ammonia + NAG		4.42 ± 0.52	0.55 ± 0.10
Ammonia + NAG + UTP		4.05 ± 0.37	0.45 ± 0.06
Glutamine		1.25 ± 0.16	2.53 ± 0.22
Glutamine + UTP		0.85 ± 0.11	0.45 ± 0.05
Glutamine + NAG		3.85 ± 0.46	3.12 ± 0.35
Glutamine + NAG + UTP		3.45 ± 0.44	0.75 ± 0.12
Ammonia + Glutamine + NAG		5.63 ± 0.85	3.51 ± 0.47
Ammonia + Glutamine + NAG + UTP		5.24 ± 0.74	0.81 ± 0.18
GDH	335 ± 31	290 ± 30 (86)	74 ± 15 (22)
LDH	445 ± 45	56 ± 11 (13)	376 ± 50 (85)

The concentrations of ammonium chloride, glutamine, NAG and/or UTP in the reaction mixture are the same as noted in table 1. Abbreviations are as in table 1.

% of GDH and LDH activity out of the total are given in parentheses.

Tabl e 3. Rate of urea synthesis from ammonia and glutamine separately, and from ammonia and glutamine together by the perfused liver of *C. batrachus*.

	Urea synthesis (μmole/g liver/hr)
Control	BLD
Ammonia (2 mM)	11.2 ± 1.24
Glutamine (5 mM)	6.4 ± 1.11
Ammonia (2 mM) + Glutamine (5 mM)	13.6 ± 1.57

Livers were perfused first with isotonic medium containing 5 mM glucose and 2 mM ornithine as mentioned in the Materials and Methods section for 20 min without ammonium chloride and glutamine, followed by the infusion of ammonium chloride (2 mM), glutamine (5 mM) or a combination of these two substrates. Values were obtained between 26 - 30 min of infusion and calculated as mean ± SEM (n = 4). BLD - below the level of detection.

Table 4: Activity (units/g wet) of different urea cycle enzymes except CPS and marker enzymes in different subcellular fractions of *Clarias batrachus* liver.

Enzyme	Homogenate	Nuclear fraction	Mitochondrial fraction	Cytosolic fraction	Recovered activity
OTC	203.5±11.2	10.4±1.5 (5.1)	175.4±13.6 (86.1)	27.8±4.2 (13.7)	213.6 (105)
ASS	53.3±7.9	2.9+0.7 (5.4)	14.2±2.2 (26.6)	41.5±8.1 (78)	58.6 (110)
ASL	42.4±10.2	2.4±0.4 (5.6)	7.8±1.1 (18.4)	35.7±3.9 (84.2)	45.9 (108)
ARG	4702±178	410±57 (8.7)	3615±177 (77)	1084±102 (23)	5109 (109)
GDH	562.5±52.4	87.5±8.9 (15.5)	470.0±48.6 (83.5)	140.0±15.1 (24.9)	697.5 (124)
LDH	1006±116	82.7±11.2 (8.2)	163.2±17.1 (16.2)	865.5±56.3 (86)	1111 (110)

1 unit of enzyme activity was defined as the amount of enzyme that catalysed 1 μmole of product formed or substrate used per hr at 30 ^{0}C for all the urea cycle related enzymes, and 1 μmole of NADH utilized per hr at 30 ^{0}C for GDH and LDH.
CPS- carbamyl phospate synthetase (urea cycle-related); OTC-ornithine transcarbamylase;
ASS- argininosuccinate synthetase; ASL-argininosuccinate lyase; ARG- arginase.

Table 5: Activity (units/g wet) of different urea cycle enzymes except CPS and marker enzymes in different subcellular fractions of *Clarias batrachus* kidney.

Enzyme	Homogenate	Nuclear fraction	Mitochondrial fraction	Cytosolic fraction	Recovered activity
OTC	112.3±12.1	8.8±1.2 (7.8)	97.4±12.8 (86.7)	18.4+2.8 (16.4)	124.6 (111)
ASS	51.4±6.8	5.5±0.8 (10.7)	9.2±1.6 (17.9)	40.7±7.1 (79.2)	55.4 (108)
ASL	38.6±6.62	3.8±0.74 (9.8)	6.4±1.1 (16.6)	31.9±5.4 (82.6)	42.1 (109)
ARG	1150±201	102±21 (8.9)	876±107 (76)	255±32 (22)	1203 (105)
GDH	312±37.5	39±5.9 (12.5)	287±24 (92)	88±10.7 (28)	414 (133)
LDH	420+62	39±8.21 (9.2)	48±6.6 (11.4)	366±58 (87)	451 (107)

Abbreviation are same as table 4
Unit defination of enzyme activity is same as table 4

Table 6: Activity of urea cycle enzymes (units/g wet wt) in muscle, intestine and brain of *C. batrachuus*. Values are expressed as mean ± SEM (n = 3).

Enzymes	Activity in		
	Muscle	Intestine	Brain
CPS			
Ammonia	0.35±0.06	0.45±0.08	0.85±10
Ammonia + NAG	0.65±0.08	1.07±0.11	2.04±0.25
Ammonia +NAG +UTP	0.59±0.07	0.88±0.10	1.96±0.22
Glutamine	0.55±0.07	0.65±0.08	0.88±0.10
Glutamine+ NAG	1.17±0.11	1.85±0.14	2.53±0.45
Glutamine + NAG + UTP	0.75±0.07	0.98±0.09	1.96±0.12
Ammonia + Glutamine + NAG	1.55±0.11	2.26±0.16	4.02±0.26
Ammonia+Glutamine+NAG+UTP	1.16±0.12	1.26±0.15	2.75±0.36
OTC	5.61±0.46	11.70±0.51	5.49±0.44
ASS	11.52±0.65	10.65±0.78	BLD
ASL	BLD	27.7±1.82	3.0±0.2
ARG	124.6±14.1	110.8±8.2	152.3±12.4

Abbreviation are same as in table 4

Unit defination of enzyme activity is same as table 4

BLD - below the level of detection

Table 7. Rate of uptake of ammonia (μmoles/kg body wt/hr) by *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).

	Days of treatment						
	1	2	3	4	5	6	7
Control	143± 8	144±20	140±24	143±18	130±15	131±12	128±6
Treated	-737±56	-1048±52	-1017±96	-1124±124	-1168±134	-909±107	-924±29

Table 8: Rate of excretion of urea-N (μmoles/kg body wt/hr) by *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).

	Days of treatment						
	1	2	3	4	5	6	7
Control	32±6.0	30±5	33±8	37±15	33 ±8	36±3	34±7
Treated	279±21 (+771)	337±19 (+1023)	508±36 (+1439)	560±32 (+1413)	590±32 (+1687)	568±19 (+1477)	545±10 (+1502)
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

% increase (+) in urea-N excretion with relation to control are given in parentheses.

Table 9: Changes in the levels of ammonia in different tissues (μmoles/g wet wt) and in plasma (μmoles/ml) of *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean $\pm$ SEM (n = 3).

Tissues		Days of exposure 1	3	7
Liver	Control	10.8±0.92	10.7±0.62	10.7±0.5
	Treated	14.4±1.09	19.7±1.15	23.2±2.02
		(+33)	(+84)	(+116)
	P	<0.05	<0.001	<0.01
Kidney	Control	10.6±0.54	9.6±0.74	10.1±0.67
	Treated	15.9±1.12	20.4±1.54	22.6±1.84
		(+50)	(+112)	(+123)
	P	<0.01	<0.001	<0.001
Muscle	Control	5.5±0.21	5.1±0.92	5.9±0.38
	Treated	8.2±0.54	11.8±0.88	12.2±0.84
		(+49)	(+131)	(+106)
	P	<0.05	<0.01	<0.001
Brain	Control	3.2±0.24	3.4±0.22	3.3±0.22
	Treated	3.8±0.32	4.1±0.27	3.9±0.34
		(+19)	(+20)	(+18)
	P	N.S	N.S.	N.S.
Plasma	Control	0.66±0.05	0.69±0.05	0.65±0.03
	Treated	1.01±0.14	2.21±0.18	2.47±0.25
		(+53)	(+220)	(+280)
	P	<0.05	<0.001	<0.001

% increase in ammonia level compared to control are given in parentheses.
N.S.- not significant

Table 10: Changes in the levels of urea in different tissues (μmoles/g wet wt) and in plasma (μmoles/ml) of *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 3).

		Days of exposure		
Tissues		1	3	7
Liver	Control	3.6±0.22	4.6±0.37	3.6±0.27
	Treated	8.1±0.57	10.7±0.87	10.2±0.59
		(+125)	(+132)	(+183)
	P	<0.001	<0.001	<0.001
Kidney	Control	3.6±0.27	3.9±0.24	4.2±0.28
	Treated	8.0±0.52	9.3±0.92	8.6±0.62
		(+122)	(+138)	(+104)
	P	<0.001	<0.001	<0.001
Muscle	Control	1.4±0.12	1.6±0.14	1.8±0.12
	Treated	2.9±0.23	4.4±0.34	3.6±0.34
		(+107)	(+175)	(+100)
	P	<0.001	<0.001	<0.01
Brain	Control	1.9±0.12	2.2±0.20	2.3±0.14
	Treated	2.4±0.15	2.9±0.18	2.8±0.32
		(+26)	(+31)	(+21)
	P	<0.05	<0.05	N.S.
Plasma	Control	0.38±0.04	0.40±0.05	0.37±0.04
	Treated	0.55±0.03	1.02±0.15	1.32±0.14
		(+44)	(+155)	(+257)
	P	<0.01	<0.01	<0.001

% increase in urea level compared to control are given in parentheses.
N.S.- not significant

Table 11: Changes of total (units/g wet wt) activity of urea cycle enzymes in the liver of *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes				
		CPS	*OTC*	*ASS*	*ASL*	*ARG*
1	Control	3.12±0.25	102.4±5.5	112.2±8.5	121.3±6.8	1024±65
	Treated	5.85±0.47	129.4±6.4	151.9±10.2	164.1±8.9	1117±100
		(+88)	(+26)	(+35)	(+35)	(+9)
	P	<0.01	N.S.	<0.05	<0.005	N.S.
3	Control	3.19±0.24	105.3±8.2	114.6±6.4	124.9±6.7	1100±71
	Treated	9.85±0.67	135±5.9	172.2±10.2	185±10.2	1107±105
		(+211)	(+28)	(+50)	(+48)	(+0.63)
	P	<0.001	N.S.	<0.05	<0.05	N.S.
7	Control	3.32±0.18	107.1±6.8	114.1±5.2	120.8±10.0	1112±52
	Treated	7.12±0.52	129.9±9.9	182.4±11.4	193.5±14.8	1144+98
		(+115)	(+21)	(+59)	(+60)	(+3)
	P	<0.01	N.S.	<0.05	<0.05	N.S.

% increase (+) in activity compared to control are given in parentheses.

CPS- carbamyl phospate synthetase (urea cycle-related); OTC-ornithine transcarbamylase; ASS- argininosuccinate synthetase; ASL-argininosuccinate lyase; ARG- arginase.

1 unit of enzyme was defined as that amount which catalyzed 1 μmole of product formed or substrate used per hr at 30 ^{0}C.

Table 12 : Changes of specific (units/mg protein) activity of urea cycle enzymes in the liver of *C. batrachus* while exposed to 25 mM NH_4CL. Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	0.035±0.002	1.18±0.11	1.29±0.08	1.31±0.09	11.7±0.47
	Treated	0.067±0.004	1.22±0.21	1.64±0.12	1.77±0.15	12.8±0.75
		(+91)	(+3)	(+27)	(+35)	(+9)
	P	<0.01	N.S.	<0.05	<0.05	N.S.
3	Control	0.036±0.003	1.18±0.11	1.32±0.01	1.42±0.11	12.2±0.39
	Treated	0.112±0.005	1.38±0.12	2.70±0.12	2.34±0.24	12.7±0.88
		(+211)	(+17)	(+104)	(+65)	(+4)
	P	<0.001	N.S.	<0.01	<0.01	N.S.
7	Control	0.038±0.004	1.17±0.21	1.31±0.12	1.32±0.11	12.8±0.45
	Treated	0.081±0.006	1.42±0.18	2.40±0.15	2.42±0.17	13.1±0.89
		(+113)	(+21)	(+82)	(+83)	(+2)
	P	<0.001	N.S.	<0.01	<0.01	N.S.

Abbreviations are same as table 11

% increase (+) in enzyme activity compared to control are given in parentheses.

Table 13: Changes of tissue (units/g wet wt) activity of urea cycle enzymes in the kidney of *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	2.53±0.12	59.3±2.62	84.1±4.2	82.2±2.7	918±48
	Treated	4.97±0.36	69.5±3.35	129.9±6.9	108.5±7.5	1024±57
		(+96)	(+17)	(+54)	(+32)	(+12)
	P	<0.01	N.S.	<0.01	<0.05	N.S.
3	Control	2.63±0.24	58.5±4.22	84.8±6.8	70.9±4.4	973±64
	Treated	5.92±0.52	70.7±4.45	147.8±9.1	126.5±7.9	1001±88
		(+124)	(+21)	(+74)	(+78)	(+3)
	P	<0.001	N.S.	<0.01	<0.01	N.S.
7	Control	2.63±0.24	58.3±5.23	84.8±9.3	79.1±4.2	904±58
	Treated	5.46±0.43	69.7±6.43	152.1±10.6	137.7±8.2	1024±65
		(+107)	(+20)	(+79)	(+74)	(+13)
	P	<0.001	N.S.	<0.01	<0.01	N.S.

Abbreviations are same as table 11

% increase (+) in enzyme activity compared to control are given in parentheses.
Enzyme unit defination is same as table 11

Table 14: Changes of specific (units/mg protein) activity of urea cycle enzymes in the kidney of *C. batrachus* while exposed to 25 mM NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	0.034±0.004	0.80±0.04	1.14±0.09	1.11±0.15	12.4±0.72
	Treated	0.066±0.005	0.93±0.05	1.74±0.13	1.42±0.18	13.5±0.52
		(+94)	(+16)	(+52)	(+28)	(+9)
	P	<0.01	N.S.	<0.01	N.S.	N.S.
3	Control	0.032±0.002	0.78±0.06	1.13±0.12	0.95±0.18	13.0±0.9
	Treated	0.079±0.005	0.95±0.05	1.86±0.14	1.74±0.21	13.5±1.32
		(+146)	(+22)	(+64)	(+83)	(+4)
	P	<0.001	N.S.	<0.01	<0.01	N.S.
7	Control	0.034±0.002	0.78±0.05	1.13±0.12	1.05±0.17	12.1±1.2
	Treated	0.072±0.004	0.93±0.06	1.83±0.15	1.80±0.19	13.7±1.4
		(+112)	(+20)	(+62)	(+71)	(+13)
	P	<0.001	N.S.	<0.01	<0.01.	N.S.

Abbreviations are same as table 11

% increase (+) in enzyme activity compared to control are given in parentheses.

Table 15 : Alteration of ammonia level (μmoles/g wet wt) in the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Ammonia infusion rate (μmoles/g liver/min)	Ammonia level in the perfused liver (μmoles/g wet wt)	*P*
0 (control)	7.64 ± 0.75	
0.4 ± 0.04	8.84 ± 0.66 (16)	N.S.
0.85 ± 0.09	10.45 ± 0.82 (37)	N.S.
1.25 ± 0.11	14.50 ± 1.02 (90)	<0.05
2.32 ± 0.26	20.05 ± 1.85 (162)	<0.001
5.08 ± 0.38	28.45 ± 2.24 (272)	<0.001
10.81± 0.81	28.65 ± 2.55 (275)	<0.001

% increase of ammonia level with relation to control are given in parentheses. NH_4Cl infusion at the rate of 5 μmoles/g liver/min corresponds to 1 mM concentration of NH_4Cl.
N.S - not significant.

Table 16: Rate of addition, efflux and uptake of ammonia, and efflux of urea-N (μmoles/g liver/min) by the perfused liver of *C. batrachus* when infused with different concentrations of NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Addition of ammonia	Efflux of ammonia	Uptake of ammonia	Efflux of urea-N
0.40 ± 0.04	0.11 ± 0.02	0.28 ± 0.025	0.124 ± 0.01
0.85 ± 0.09	0.31 ± 0.05	0.52 ± 0.03	0.207 ± 0.015
1.25 ± 0.11	0.62 ± 0.08	0.62 ± 0.045	0.310 ± 0.021
2.32 ± 0.26	1.55 ± 0.12	0.71 ± 0.06	0.360 ± 0.05
5.08 ± 0.38	4.12 ± 0.32	0.85 ± 0.07	0.450 ± 0.055
10.81 ± 0.81	9.47 ± 1.45	0.94 ± 0.085	0.440 ± 0.06

Table 17: Changes of tissue (units/g wet wt) activity of urea cycle enzymes in the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Ammonia infusion rate (μmoles/g liver/min)		CPS	OTC	ASS	ASL	ARG
0 (control)		3.92±0.31	190.4±11.1	65.8±6.2	100.3±7.2	4035±212
0.85±0.09		4.29±0.44 (+9)	187.6±15.2 (-1)	74.3±4.5 (+13)	97.3±6.5 (-3)	4132±224 (+2)
	P	N.S.	N.S.	N.S.	N.S.	N.S.
1.25±0.11		5.68±56 (+45)	218.2±12.2 (+15)	130.3±6.1 (+98)	122.4±11.4 (+22)	3945±191 (+8)
	P	N.S.	N.S.	<0.05	N.S.	N.S.
2.32±0.26		6.45±0.55 (+65)	212.6±14.5 (+7)	152.4±7.2 (+132)	147.6±8.2 (+47)	4345±191 (+8)
	P	<0.05	N.S.	<0.01	<0.05	N.S.
5.08±0.38		8.68±0.71 (+121)	212.6±14.5 (+12)	148.5±8.5 (+126)	140.8±6.2 (+40)	4402±224 (+9)
	P	<0.01	N.S.	<0.01	<0.05	N.S.
10.81±0.81		8.59±0.65 (+119)	219.3±11.2 (+15)	153.6±6.4 (+133)	151.3±6.9 (+51)	4378±237 (+9)
	P	<0.01	N.S.	<0.05	<0.05	N.S.

% increase (+) in enzyme activity compared to control are given in parentheses.

CPS- carbamyl phosphate synthetase (urea cycle-related); OTC-ornithine transcarbamylase; ASS- argininosuccinate synthetase; ASL-argininosuccinate lyase; ARG- arginase.

1 unit of enzyme was defined as that amount which catalyzed 1 μmole of product formed or substrate used per hr at 30 °C.

Table 18 : Changes of specific (units/mg protein) activity of urea cycle enzymes in the perfused liver of *C. batrachus* infused with different concentration of NH_4Cl. Values are expressed as mean ± SEM (n = 3).

Ammonia infusion rate (μmoles/g liver/min)		CPS	OTC	ASS	ASL	ARG
0 (control)		0.048±0.005	2.33±0.45	0.81±0.1	1.23±0.14	49.4±5.4
0.85±0.09		0.05±0.004 (+4)	2.16±0.28 (-7)	0.8±0.08 (+6)	1.12±0.08 (-9)	47.7±5.4 (-3)
	P	N.S.	N.S.	N.S.	N.S.	N.S.
1.25±0.11		0.067±0.004 (+40)	2.57±0.28 (+10)	1.53±0.07 (+89)	1.44±0.11 (+17)	46.47±5.7 (-6)
	P	N.S.	N.S.	<0.05	N.S.	N.S
2.32±0.26		0.08±0.006 (+67)	2.54±0.44 (+9)	1.91±0.11 (+136)	1.85±0.12 (+50)	54.38±6.1 (+10)
	P	<0.05	N.S.	<0.01	<0.05	N.S.
5.08±0.38		0.11±0.02 (+129)	2.78±0.34 (+19)	1.94±0.12 (+140)	1.84±0.10 (+50)	57.5±4.8 (+16)
	P	<0.01	N.S.	<0.005	<0.05	N.S.
10.81+0.81		0.11±0.06 (+129)	2.7±0.22 (+16)	1.91±0.11 (+136)	1.88±0.04 (+53)	54.5±4.2 (+10)
	P	<0.01	N.S.	<0.05	<0.05	N.S.

% increase (+) or decrease (-) in enzyme activity with relation to control are given in parentheses.

Abbreviations are same as table 17

Table 19 : Pattern of excretion of ammonia (μmoles/kg body wt/hr) by *C. batrachus* during emersion to air, followed by re-immersion in water. Values are expressed as mean ± SEM (n = 5).

Time of emersion		Control	Emersion	Re-immersion in water for the period of				
				0-0.5 hr	0.5-1 hr	1-2 hr	2-6 hr	6-12 hr
12 hr		200±16	3.03±0.4 (-98)	442±21 (+121)	366±24 (+83)	332±23 (+66)	264±16 (+33)	204±14 (+2)
	P		<0.001	<0.001	<0.01	<0.01	<0.05	N.S.
24 hr		210±14	2.7±0.4 (-99)	488±38 (+132)	434±20 (+106)	340±22 (+62)	280±12 (+33)	245±14 (+16)
	P		<0.001	<0.001	<0.001	<0.01	<0.05	<0.05
48 hr		220±16	2.44±0.3 (-99)	561±42 (+155)	472±32 (+114)	395±25 (+80)	299±22 (+36)	262±20 (+19)
	P		<0.001	<0.001	<0.001	<0.01	<0.05	<0.05

% increase (+) and decrease (-) in ammonia excretion rate with relation to control are given in parentheses.
N.S- not significant

Table 20 : Pattern of excretion of urea-N (μmoles/kg body wt/hr) by *C. batrachus* during exposure to air and on re-immersion in water. Values are expressed as mean ± SEM (n = 5).

Time of emersion		Control	Emersion	Re-immersion in water for the period of 0-0.5 hr	0.5-1 hr	1-2 hr	2-6 hr	6-12 hr
12 hr		28.2±3.8	1.06±0.08 (-96)	222±10 (+693)	206±11 (+636)	200±8 (+609)	198±12 (+602)	67±5 (+137)
	P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
24 hr		24.4±3.2	1.15±0.10 (-95)	379±18 (+1453)	379±18 (+1453)	220±14 (+802)	203±13 (+731)	57±4 (+133)
	P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
48 hr		21.6±2.4	4.18±0.41 (-80)	497±32 (+2200)	468±25 (+2066)	389±22 (+1700)	285±26 (+1219)	186±14 (+761)
	P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

% increase (+) and decrease (-) in urea-N excretion with relation to control are given in parentheses.

Table 21: Changes in ammonia levels in different tissues (μmoles/g wet wt) and in plasma (μmoles/ml) of *C. batrachus* while exposed to air for different time intervals. Values are expressed as mean ± SEM (n = 3).

Tissue	Hours of aerial exposure			
	0	12	24	48
Liver	10.70±0.08	16.42±1.22 (+53)	20.22±2.21 (+88)	24.17±1.82 (+126)
P		<0.01	<0.01	<0.001
Kidney	8.66±0.07	18.96±1.61 (+118)	25.20±2.24 (+190)	27.31±2.47 (+215)
P		<0.001	<0.001	<0.001
Muscle	7.97±0.05	9.52±0.96 (+19)	14.22±0.88 (+78)	16.56±1.66 (+107)
P		<0.05	<0.001	<0.01
Brain	3.6±0.02	4.2±0.22 (+16)	4.8±0.46 (+33)	4.9±0.56 (+36)
P		N.S.	<0.05	<0.05
Plasma	0.60±0.04	1.15±0.007 (+91)	1.44±0.15 (+140)	2.17±0.23 (+261)
P		<0.001	<0.01	<0.001

% increase in the levels of ammonia compared to control are given in parentheses.

Table 22: Changes in urea levels in different tissues (μmoles/g wet wt) and in plasma (μmoles/ml) of *C. batrachus* while exposed to air for different time intervals. Values are expressed as mean ± SEM (n = 3).

Tissue		Hours of aerial exposure			
		0	12	24	48
Liver		3.67±0.18	8.09±0.64 (+120)	14.33±1.4 (+290)	17.30±1.65 (+371)
	P		<0.001	<0.001	<0.001
Kidney		2.67±0.13	4.82±0.34 (+80)	6.95±0.8 (+160)	9.01±1.22 (+237)
	P		<0.01	<0.001	<0.001
Muscle		0.95±0.04	1.27±0.12 (+33)	1.56±0.16 (+64)	1.95±0.19 (+105)
	P		<0.05	<0.01	<0.01
Brain		1.9±0.12	2.6±0.11 (+36)	3.4±0.12 (+78)	3.8±0.41 (+100)
	P		<0.05	<0.01	<0.01
Plasma		0.35±0.03	0.84±0.05 (+140)	1.17±0.18 (+234)	1.79±0.12 (+411)
	P		<0.001	<0.001	<0.001

% increase in urea levels compared to control are given in parentheses.

Table 23: Changes of tissue (units/g wet wt) activity of urea cycle enzymes in the liver of *C. batrachus* while exposed to air for different time intervals. Values are expressed as mean ± SEM (n = 3).

Time of aerial exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
0 hr		3.31±0.008	100.5±6.1	68.6±7	114.2+5.6	1033±29
12 hr		5.53±0.16 (+77)	103.8±4.5 (+3)	198±9 (+188)	128.3±5.4 (+12)	1066±28 (+3)
	P	<0.001	N.S	<0.001	<0.05	N.S
24 hr		5.72±0.07 (+83)	126.2±7.2 (+25)	194.1±9 (+182)	171.3±6.3 (+50)	904±39 (-12)
	P	<0.001	0.05	<0.001	<0.01	N.S
48 hr		6.24±0.04 (+100)	131.5±8.4 (+31)	192±10 (+179)	180.7±5.8 (+58)	940±40 (-9)
	P	<0.001	<0.05	<0.001	<0.01	N.S

% increase (+) or decrease (-) in enzyme activity with relation to control are given in parentheses.

CPS-carbamyl phosphate synthetase (urea cycle-related); OTC-ornithine transcarbamylase; ASS-argininosuccinate synthetase; ASL-argininosuccinate lyase; ARG-arginase.

N.S.-not significant

1 unit of enzyme activity was defined as that amount which catalyzed 1 μmole of product formed or substrate used per hr at 30 ^{0}C.

Table 24: Changes of specific (units/mg protein) activity of urea cycle enzymesin the liver of *C. batrachus* while exposed to air for different time intervals.Values are expressed as mean ± SEM (n = 3)

Time of aerial exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
0 hr (control)		0.035±0.007	1.16±0.12	1.17±0.17	1.30±0.18	11.9±0.5
12 hr		0.063±0.008 (+80)	1.19±0.15 (+3)	2.27±0.29 (+94)	1.40±0.16 (+8)	12.2±1.3 (+3)
	P	<0.01	N.S.	<0.01	N.S.	N.S.
24 hr		0.078±0.006 (+122)	1.44±0.15 (+24)	2.67 ±0.32 (+128)	2.37±0.22 (+82)	12.4±1.2 (+4)
	P	<0.001	N.S	<0.001	<0.01	N.S.
48 hr		0.08±0.009 (+128)	1.36±0.15 (+17)	2.55±0.24 (+117)	2.50±0.26 (+92)	12.8±1.4 (+8)
	P	<0.001	N.S	<0.001	<0.01	N.S.

Abbreviations are same as table 23

% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

Table 25: Changes of tissue (units/g wet wt) activity of urea cycle enzymes in the kidney of *C. batrachus* while exposed to air for different time intervals. Values are expressed as mean ± SEM (n = 3).

Time of aerial exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
0 hr		2.63±0.14	60.9±5.5	80.9±4.4	94.6±4.2	729±34
12 hr		4.06±0.41 (+54)	64.8±5.1 (+6)	132.4±5.6 (+64)	98.3±6.1 (+4)	803±43 (+10)
	P	<0.01	N.S.	<0.01	N.S.	N.S.
24 hr		5.52±0.22 (+109)	68.2±4.2 (+12)	144.8±7.9 (+78)	142.4±13 (+50)	821±49 (+13)
	P	<0.001	N.S.	<0.001	<0.01	N.S.
48 hr		5.75±0.24 (+118)	71.7±5.8 (+17)	149.4±8.0 (+84)	166.5±14 (+76)	886±62 (+21)
	P	<0.001	N.S.	<0.001	<0.01	N.S.

Abbrevations are same as table 23
Unit defination of enzyme activity are same as table 23
% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

Table 26 : Changes in the specific (units/mg protein) activity of urea cycle enzymes in the kidney of *C. batrachus* while exposed to air for different time intervals. Values are expressed as mean ± SEM (n = 3).

Time of aerial exposure		Enzyme				
		CPS	OTC	ASS	ASL	ARG
0 hr		0.034±0.007	0.81±0.13	1.08±0.09	1.26±0.04	9.7±0.54
12 hr		0.055±0.005 (+61)	0.90±0.14 (+11)	1.85±0.12 (+71)	1.35±0.06 (+8)	11.1±1.1 (+13)
	P	<0.05	N S.	<0.01	N.S.	N.S.
24 hr		0.075±0.003 (+120)	0.94±0.09 (+16)	1.99±0.12 (+84)	1.87±0.11 (+48)	11.3±0.95 (+16)
	P	<0.001	N.S.	<0.001	<0.01	N.S.
48 hr		0.076±0.01 (+123)	0.95±0.07 (+17)	2.21±0.16 (+104)	2 21±0.3 (+75)	11.7±1.2 (+20)
	P	<0.001	N.S.	<0.001	<0.01	N.S.

Abbreviations are same as table 23

% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

Table 27: Pattern of of excretion of ammonia (μmoles/kg body wt/hr) by *C. batrachus* while exposed to alkaline (pH 10) water. Values are expressed as mean ± SEM (n = 3).

	Hours of exposure												
	12	24	36	48	60	72	84	96	108	120	132	144	156	168
Control	178±14	213±28	219±25	203±27	198±20	213±22	210±13	195±18	189±15	185±14	183±14	178±14	171+15	166+10
pH 10	115±6	104±12	142±7	147±12	152±18	160±21	161±19	167±21	175±18	180±14	209±20	190±15	180+16	185+17
	(-35)	(-51)	(-35)	(-28)	(-23)	(-25)	(-23)	(-14)	(-7)	(-3)	(+14)	(+7)	(+6)	(+11)
P	<0.01	<0.01	<0.01	<0.05	<0.05	<0.05	<0.05	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

% increase (+) and decrease (-) in ammonia excretion with relation to control are given in parentheses.

N.S. - not significant

Table 28: Pattern of excretion of urea-N (µmoles/kg body wt/hr) by *C. batrachus* while exposed to alkaline (pH 10) water. Values are expressed as mean ±SEM (n = 3).

	Hours of exposure													
	12	24	36	48	60	72	84	96	108	120	132	144	156	168
Control	59±3	56±10	60±3	60±12	67±14	63±14	64±11	67±8	61±19	66±10	55±10	58±3	52±3	59±5
pH 10	90±6	102±12	152±10	193+24	210±22	219±27	223±27	217+30	151±15	107±9	98±14	90±3	82±4	78±5
	(+53)	(+82)	(+130)	(+222)	(+213)	(+248)	(+248)	(+224)	(+148)	(+62)	(+78)	(+55)	(+57)	(+32)

% increase (+) or decrease in the rate of urea-N excretion compared to control are given in parentheses.

Table 29: Changes in the levels of ammonia in different tisssues (μmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* while exposed to alkaline water (pH 10). Values are expressed as mean ± SEM (n = 3)..

		Days of treatment	
		3	7
Liver	Control	9.6±0.32	9.7±0.37
	pH 10	15.4±0.47	12.4±0.67
		(+60)	(+38)
	P	<0.001	<0.05
Kidney	Control	8.1±0.41	8.4±0.44
	pH 10	13.8±0.34	12.4±0.36
		(+70)	(+48)
	P	<0.001	<0.01
Muscle	Control	4.3±0.32	4.5±0.22
	pH 10	8.1±0.41	6.4±0.27
		(+88)	(+42)
	P	<0.001	<0.01
Brain	Control	2.3±0.15	2.4±0.11
	pH 10	2.8±0.12	2.7±0.13
		(+21)	(+12)
	P	N S.	N.S.
Plasma	Control	0.42±0.05	0.44±0.06
	pH 10	1.54±0.14	0.96±0.05
		(+267)	(+118)
	P	<0.001	<0.001

% increase in ammonia levels compared to control are given in parentheses.

N.S. - not significant

Table 30: Changes in the levels of urea in different tissues (μmoles/g wet wt and in plasma (μmoles/ml), of *C. batrachus* while exposed to alkaline water (pH 10). Values are expressed as mean ± SEM (n = 3).

Tissues		Days of treatment	
		3	7
Liver	Control	6.4 ± 42	6.7 ± 0.36
	pH 10	9.6 ± 0.45	8.5 ± 0.59
		(+50)	(+27)
	P	<0.01	<0.05
Kidney	Control	5.4 ± 0.37	5.9 ± 0.25
	pH 10	8.8±0.51	6.8 ± 0.32
		(+63)	(+15)
	P	<0.001	N.S
Muscle	Control	1.9 ± 0.11	2.2 ± 0.12
	pH 10	2.5 ± 0.14	2.4 ± 0.14
		(+32)	(+3)
	P	<0.01	N.S.
Brain	Control	2.3 ± 0.12	2.4 ± 0.18
	pH 10	2.6 ± 0.16	2.7 ± 0.12
		(+13)	(+12)
	P	N.S.	N.S.
Plasma	Control	0.47 ± 0.07	0.50 ± 0.03
	pH 10	0.68 ± 0.09	0.58 ± 0.04
		(+45)	(+16)
	P	<0.05	N.S.

% incresae in urea levels with relation to control are given in parentheses.
N.S. - *not significant*

Table 31: Changes of tissue activity (units/ g wet wt) of urea cycle enzymes in the liver of *C. batrachus* while exposed to alkaline water (pH 10). Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	5.36±0.14	107.5±3.4	49.1±2.0	60.4±3.3	1066±117
	Treated	9.76±1.2	125.2±10	78.3+5.0	92.1±4.0	1076±55
		(+82)	(+17)	(+59)	(+52)	(+0)
	P	<0.01	N.S.	<0.01	<0.001	N.S
7	Control	5.18±0.37	104.0±3.9	48.2+3.0	59.5±2.5	969±49
	Treated	7.96+1.23	112.7±11	53.1±4.0	72.1±4.0	987±42
		(+34)	(+8)	(+10)	(+21)	(+2)
	P	<0.05	N.S	N.S	<0.05	N.S

% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

CPS- carbamyl phospate synthetase (urea cycle-related); OTC-ornithine transcarbamylase; ASS- argininosuccinate synthetase; ASL-argininosuccinate lyase; ARG- arginase;

N.S - Not significant

1 unit of enzyme activity was defined as that amount which catalyzed 1 μmole of product formed or substrate used per hr at 30 ^{0}C.

Table 32 : Changes of specific activity (units/ mg protein) of urea cycle enzymes in the liver of *C. batrachus* while exposed to alkaline water(pH 10).Values are expressed as mean ± SEM (n = 3).

Days of exposure		Enzymes CPS	OTC	ASS	ASL	ARG
3	Control	0.047±0.005	0.95±0.04	0.43±0.05	0.53±0.06	9.39±1.94
	Treated	0.119±0.021	1.17±0.05	0.95±0.03	1.12±0.11	10.12±0.6
		(+153)	(+23)	(+121)	(+111)	(+8)
	P	<0.01	N.S.	<0.001	<0.01	N.S.
7	Control	0.079+0.004	0.91±0.02	0.43±0.05	0.59±0.06	9.59±0.50
	Treated	0.084±0.012	1.07±0.14	0.65±0.04	0.87±0.08	10.04±1.30
		(+6)	(+17)	(+51)	(+47)	(+5)
	P	N.S	N.S.	<0.01	<0.01	N.S.

Abbreviations are same as table 31

% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

Table 33: Changes of tissue (units/ g wet wt) activity of urea cycle enzymes in the kidney of C. *batrachus* while exposed to alkaline water (pH 10). Values are expressed as mean ± SEM (n=3).

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	3.76±0.28	32.32±0.9	26.34±2.5	53.3±2.0	1059±32
	Treated	5.84±0.4	40.71±6.0	74.15±5.5	75.5±3.4	1163±55
		(+55)	(+26)	(+182)	(+42)	(+10)
	P	<0.01	N.S.	<0.001	<0.01	N.S.
7	Control	3.50±0.13	32.19±2.7	22.28±3.2	55.2±9.4	1059±71
	Treated	4.24±0.17	34.66±3.5	28.54±4.2	57.1±5.6	1034±98
		(+21)	(+8)	(+3)	(+3)	(-2)
	P	<0.05	N.S.	N.S.	N.S.	N.S.

% increase (+) or decrease (-) of enzyme activity compared to control are given in parentheses.

Abbreviation are same as table 31

Unit defination of enzyme activity are same as table 31

Table 34 : Changes of specific (units/ mg protein) activity of urea cycle enzymes in the kidney of *C. batrachus* while exposed to alkaline water (pH 10). Values are expressed as mean ± SEM (n = 3)

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	0.051±0.004	0.44±0.06	0.36±0.05	0.73±0.07	14.51+2.1
	Treated	0.085±0.006	0.50±0.06	1.07±0.07	1.09±0.11	16.86±2.4
		(+67)	(+14)	(+197)	(+49)	(+16)
	P	<0.01	N.S	<0.001	<0.01	N.S.
7	Control	0.045±0.007	0.42±0.05	0.29±0.09	0.71±0.05	13.68±1.8
	Treated	0.058±0.003	0.47±0.007	0.39±0.06	0.78±0.08	14.16±1.7
		(+29)	(+12)	(+34)	(+10)	(+4)
	P	<0.05	N.S.	<0.05	N.S.	N.S.

Abbreviations are same as table 31

% increase (+) or decrease (-) in enzyme activity compared to control are given in parentheses.

FIGURES

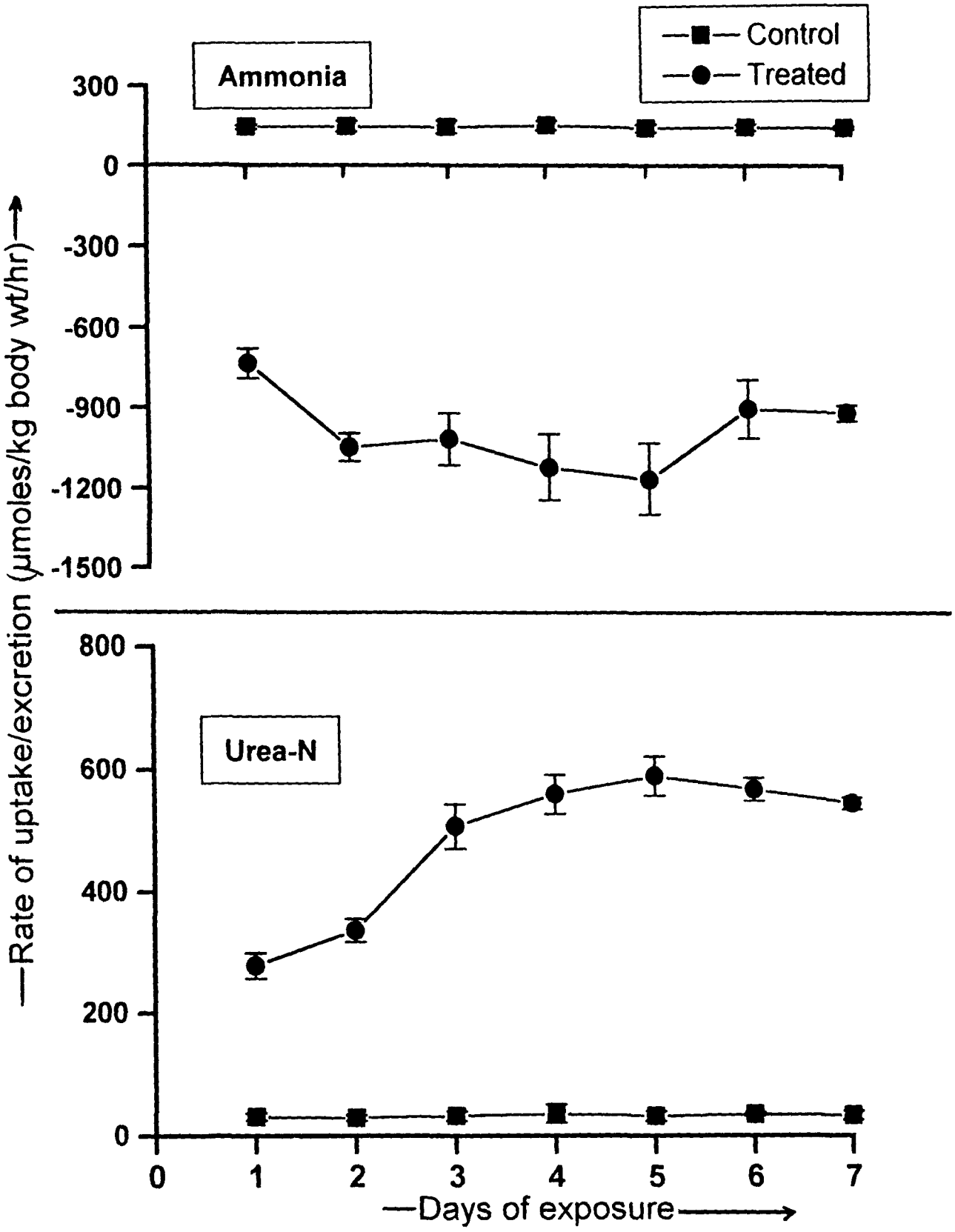


Fig. 3 Changes in the rate of uptake/excretion of ammonia and urea-N (µmoles/kg body wt/hr) by *C. batrachus* while exposed to 25 mM NH_4Cl.

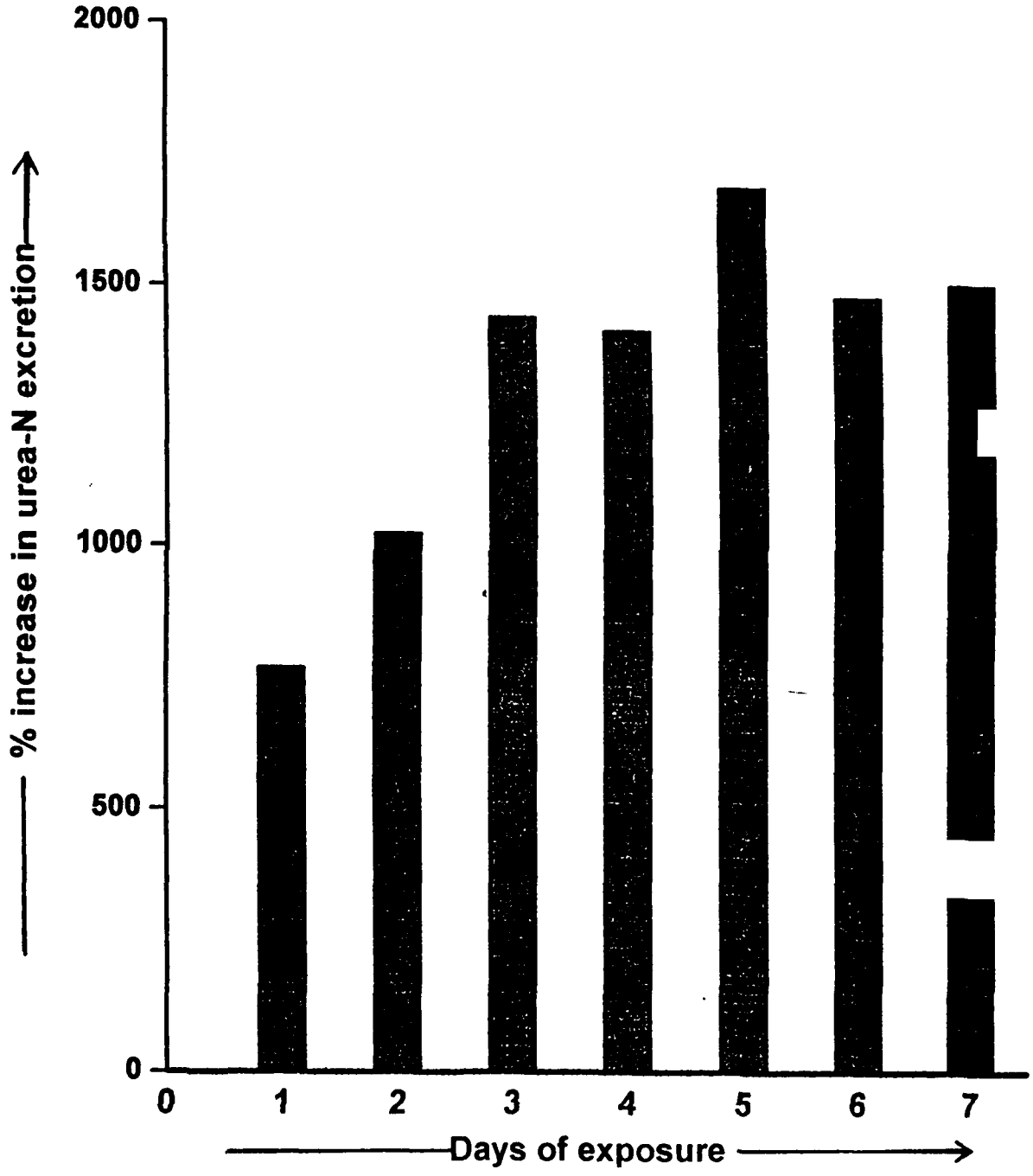


Fig. 4 Percentage increase in the rate of excretion of urea-N by *C. batrachus* while exposed to 25 mM NH_4Cl

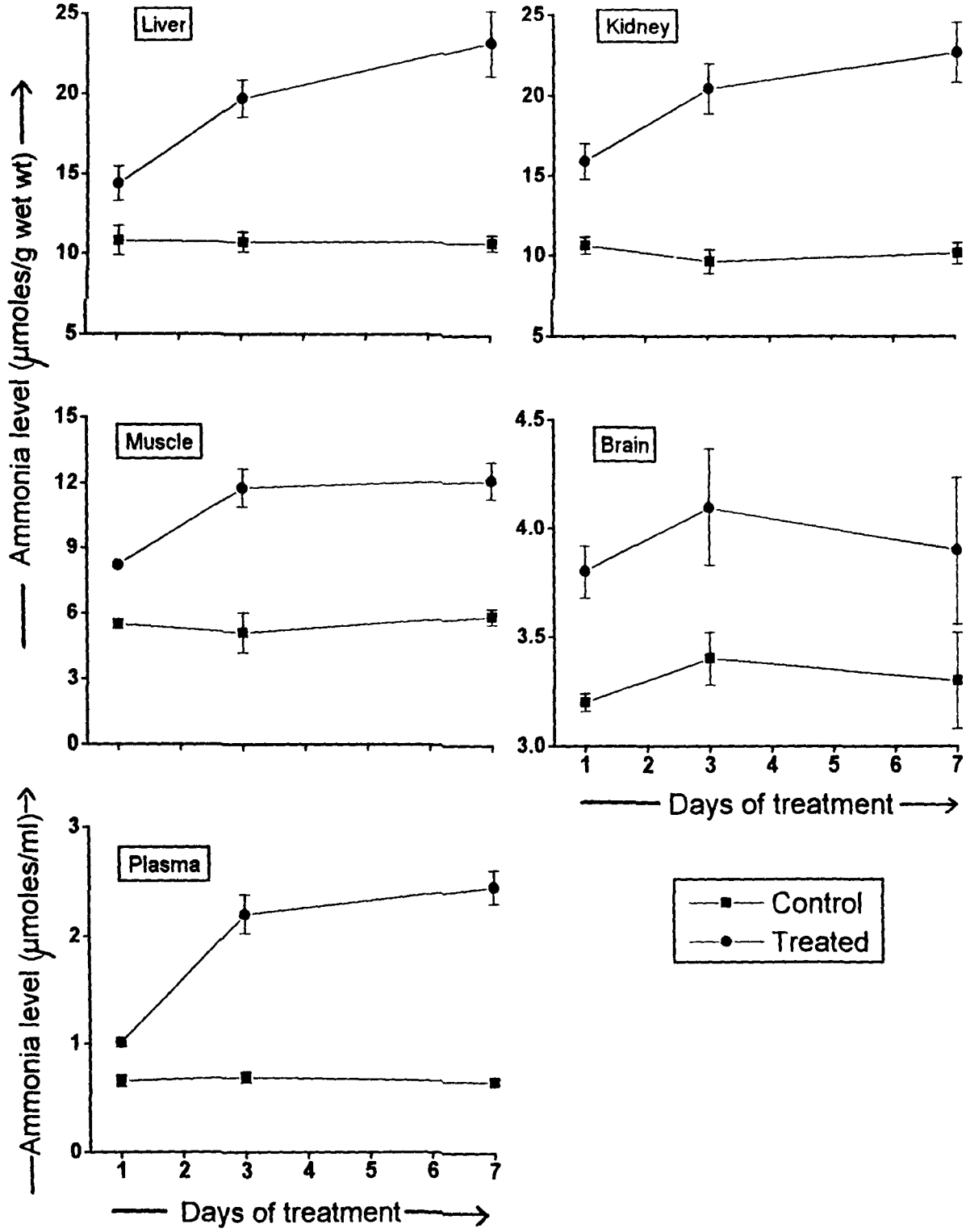


Fig. 5 Changes in the levels of ammonia in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* while exposed to 25 mM NH_4Cl.

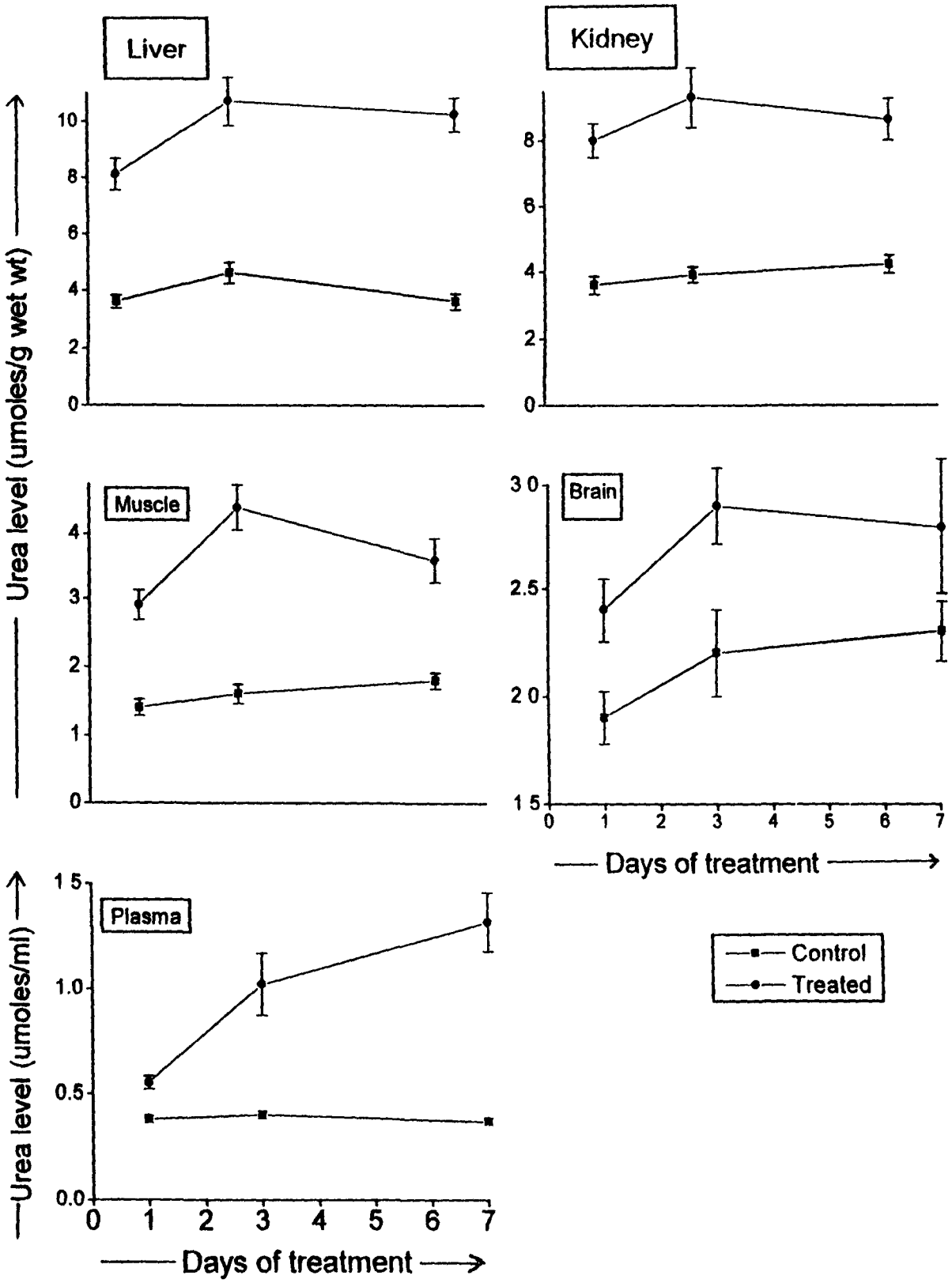


Fig. 6 Changes in the levels of urea in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* while exposed to 25 mM NH_4Cl.

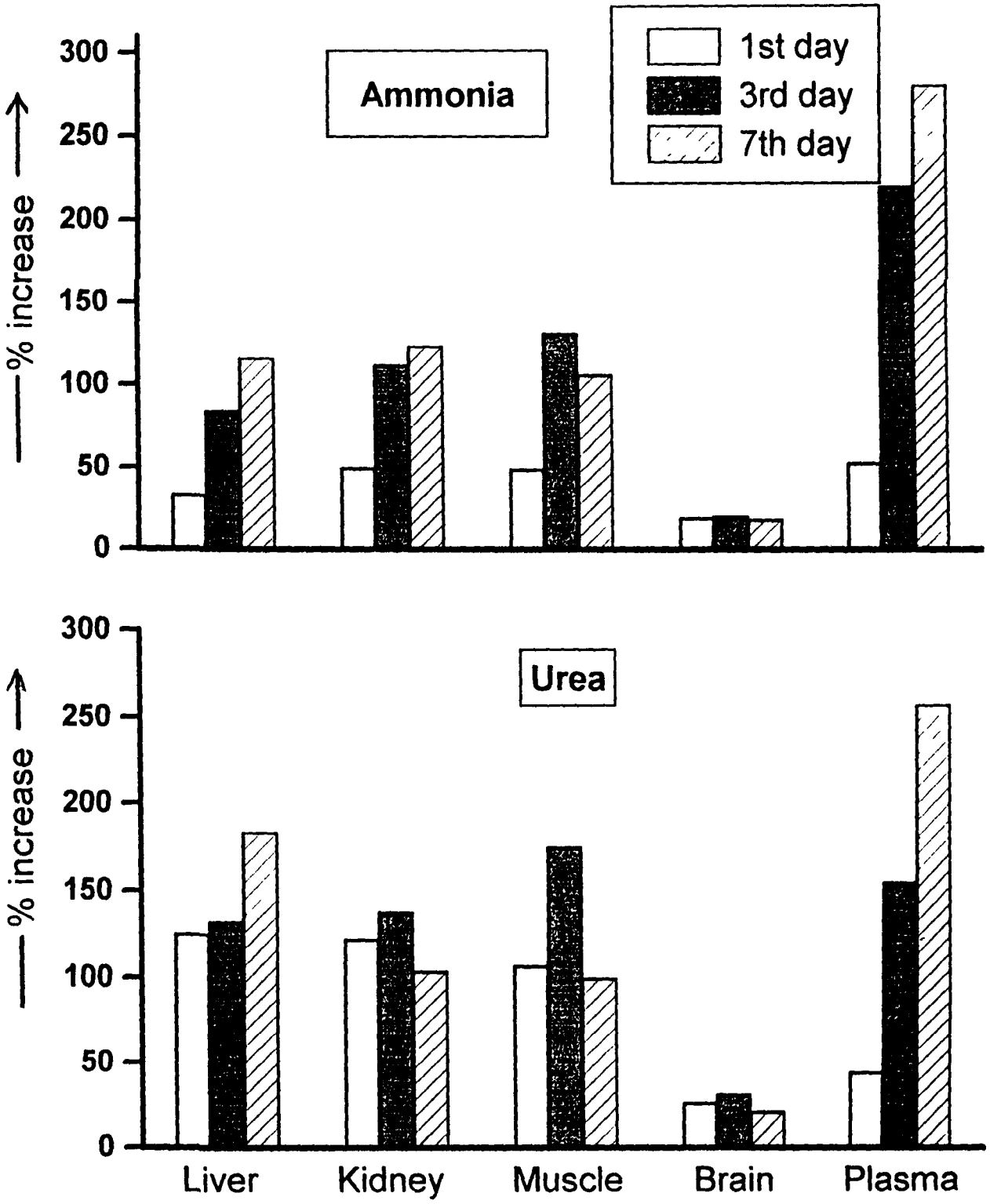


Fig. 7 **Percentage changes in the levels of ammonia and urea in different tissues and in plasma of *C. batrachus* while exposed to 25 mM NH_4Cl.**

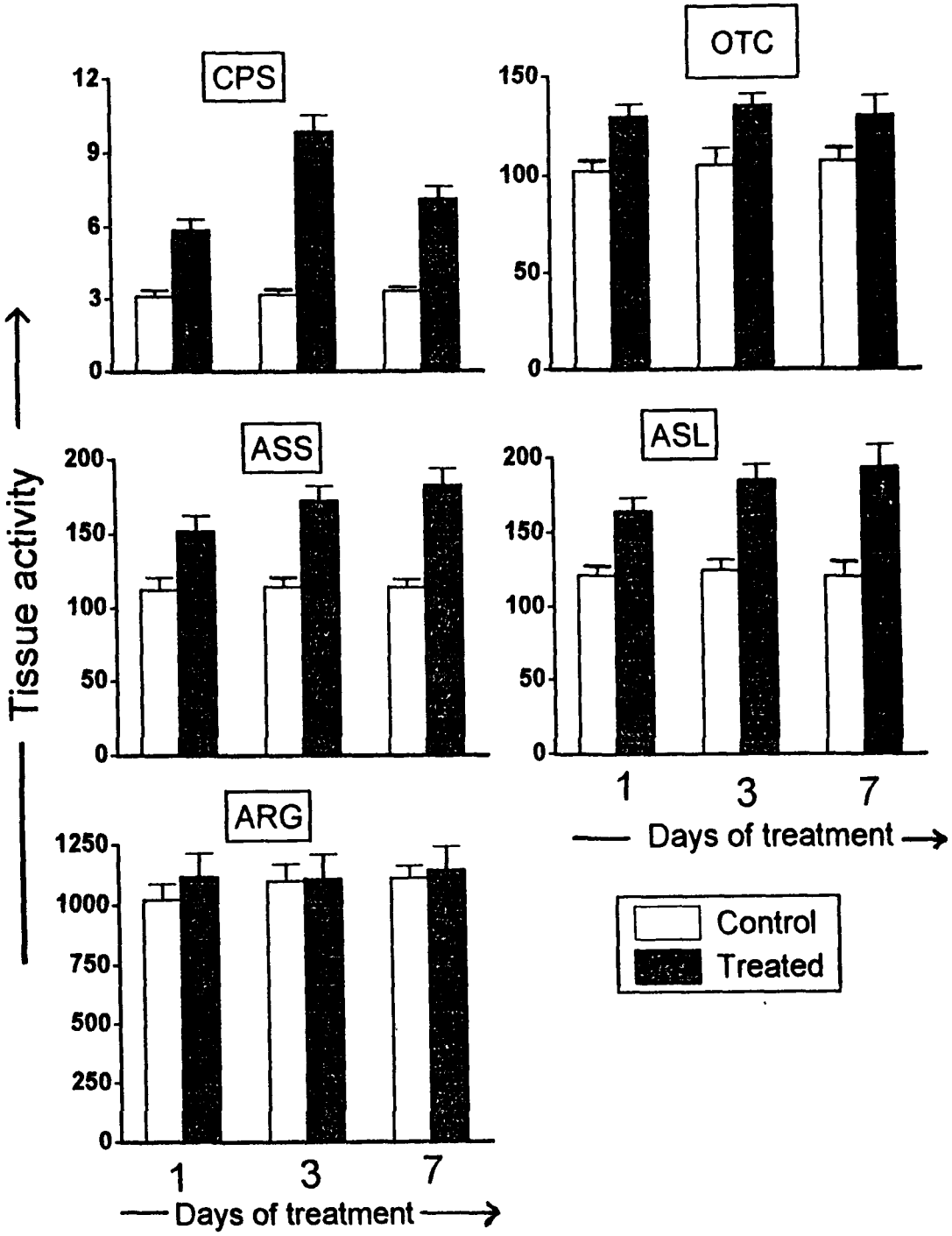


Fig. 8 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to 25 mM NH_4Cl.

CPS-carbamyl phosphate synthetase, OTC- ornithine transcarbamylase, ASS- argininosuccinate synthetase, ASL- argininosuccinate lyase, ARG- arginase.

1 unit of enzyme activity is expressed as that amount which catalyzed 1 µmole of product formed or substrate used per hr at 30°C.

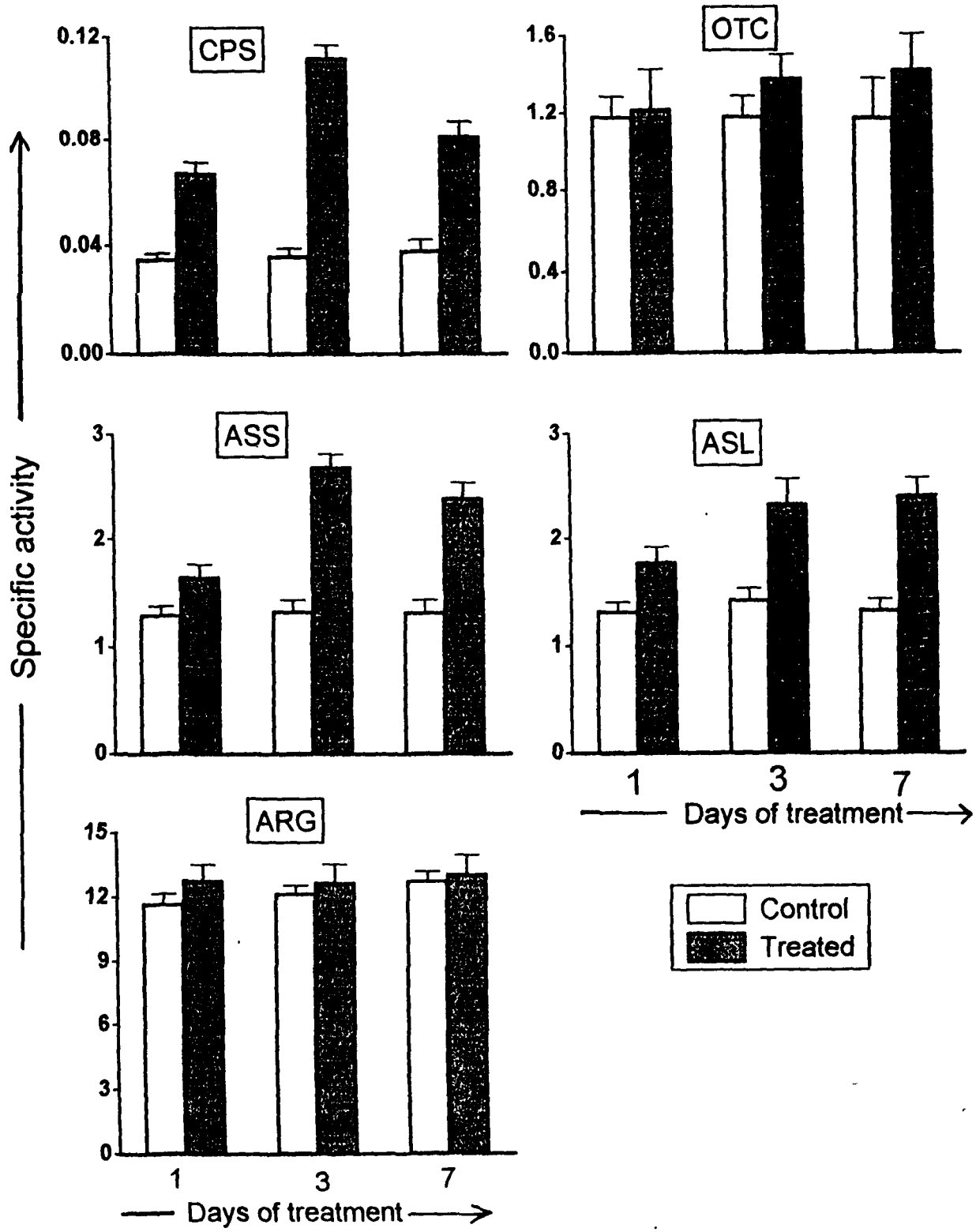


Fig. 9 Changes in specific activity (units/mg protein) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to 25 mM NH_4Cl.

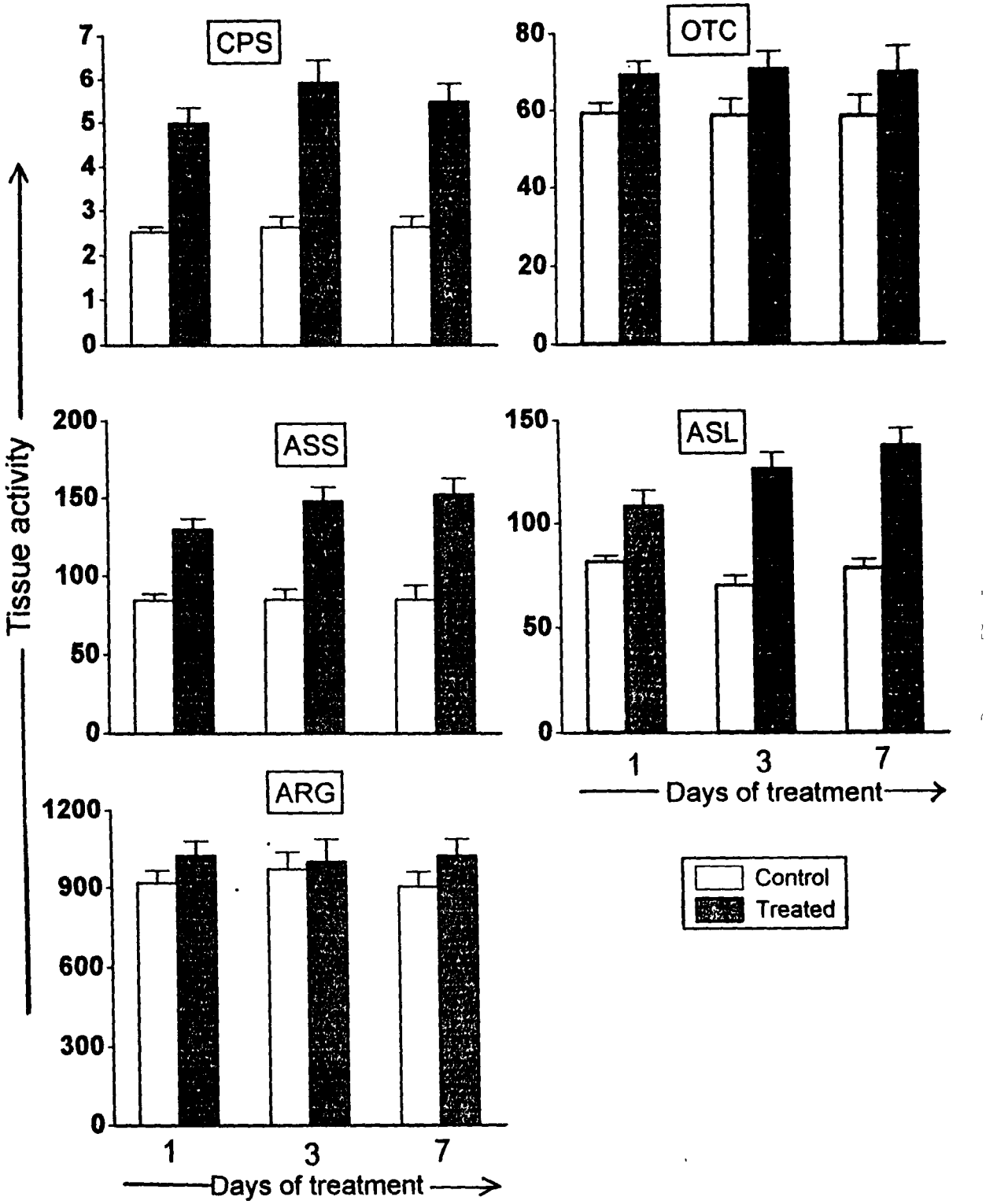


Fig. 10 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the kidney of *C. batrachus* while exposed to 25 mM NH_4Cl.

Abreviations and unit defination are same as Fig. 8

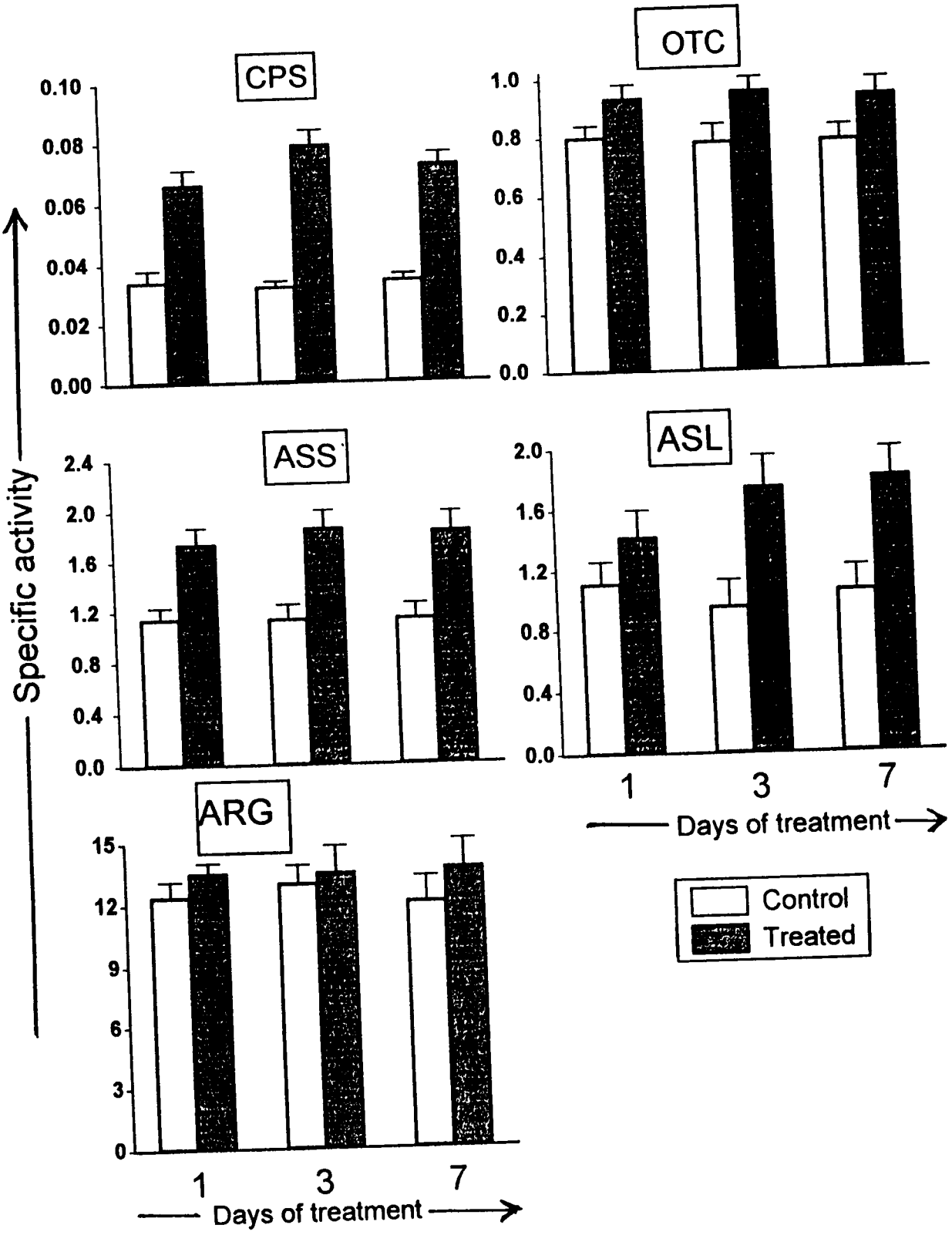


Fig. 11 Changes in specific activity (units/mg protein) of different urea enzymes in the kidney of *C. batrachus* while exposed to 25 mM NH_4Cl.

Abreviations are same as Fig. 8

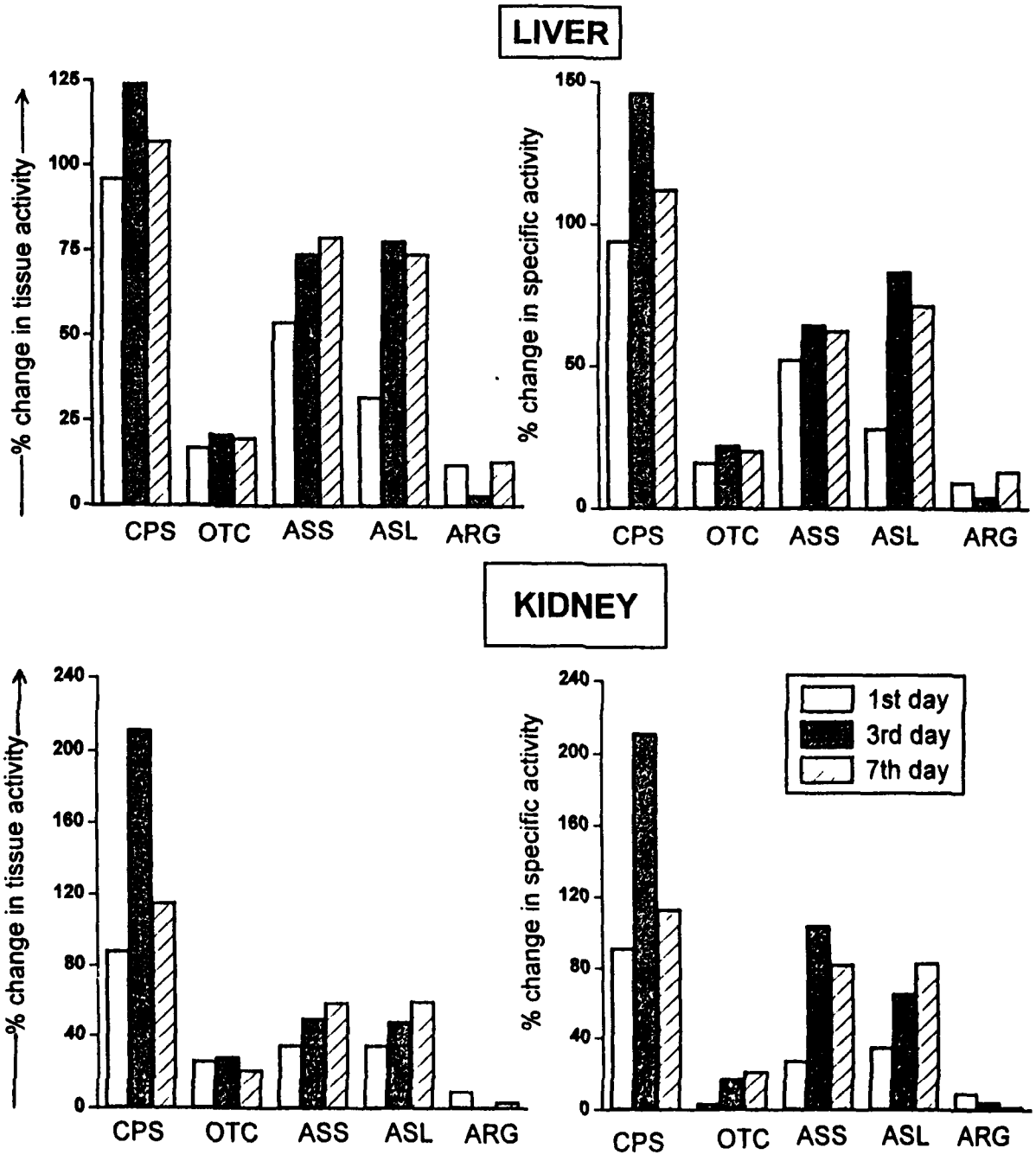


Fig. 12 Percentage changes in tissue and specific activity of urea cycle enzymes in the liver and kidney of *C. batrachus* while exposed to 25 mM NH_4Cl.

Abreviations are same as Fig. 8

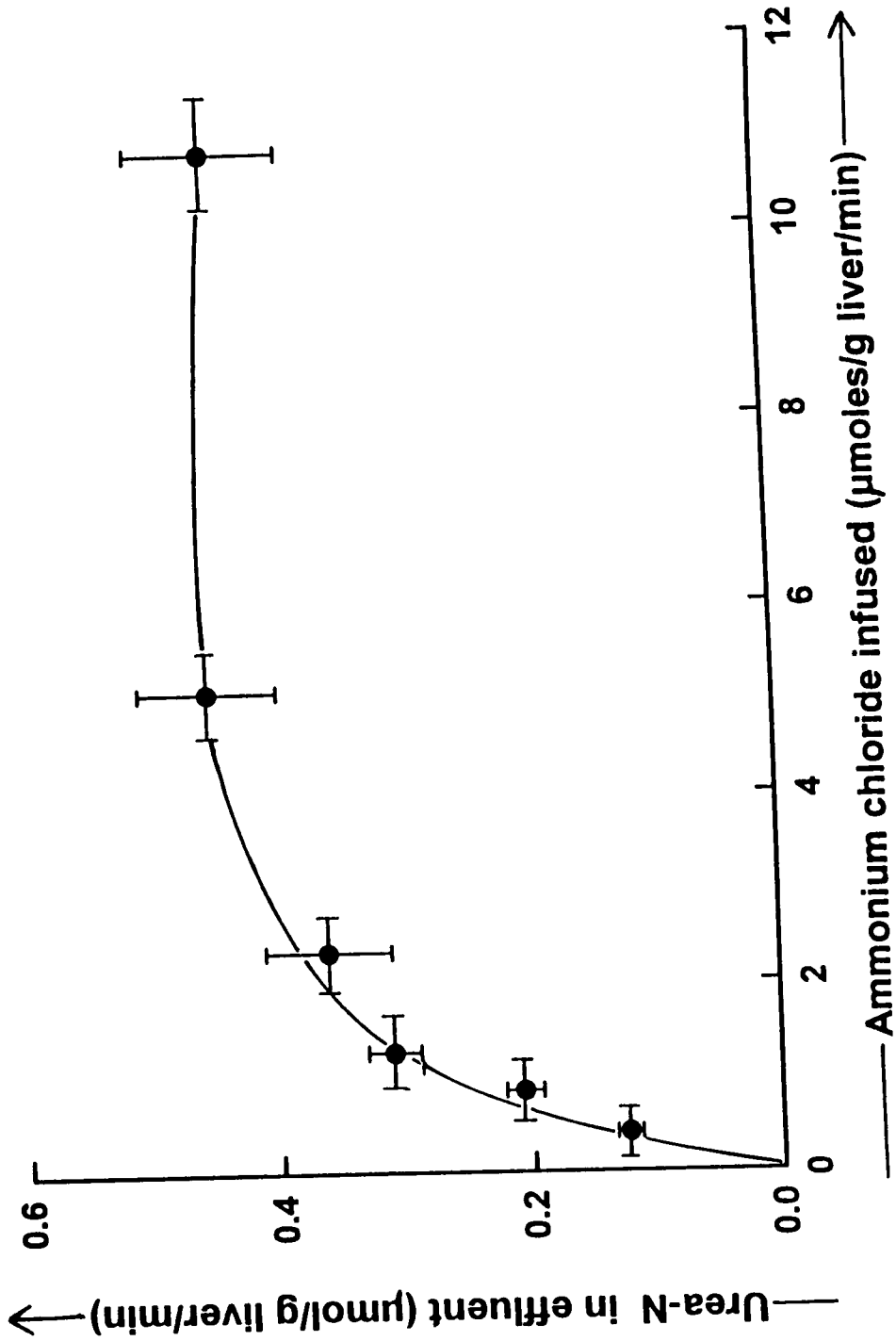


Fig. 13 Urea-N excretion (µmoles/g liver/min) in the effluent by the perfused liver of *C. batrachus* infused with diferrent concentrations of NH_4Cl.

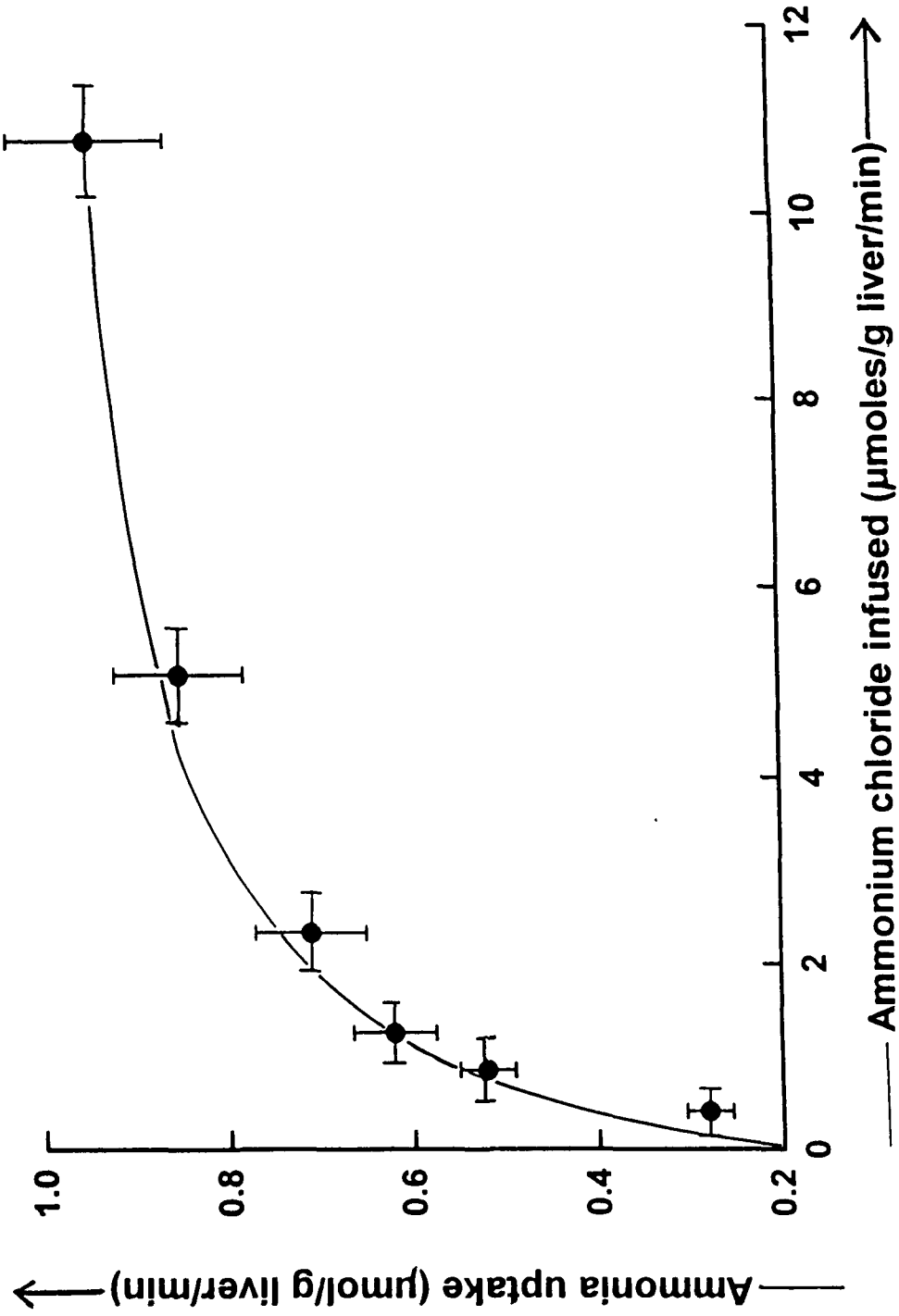


Fig. 14 Ammonia uptake (µmoles/g liver/min) by the perfused liver of *C. batrachus* infused with different concentrarins of NH_4Cl.

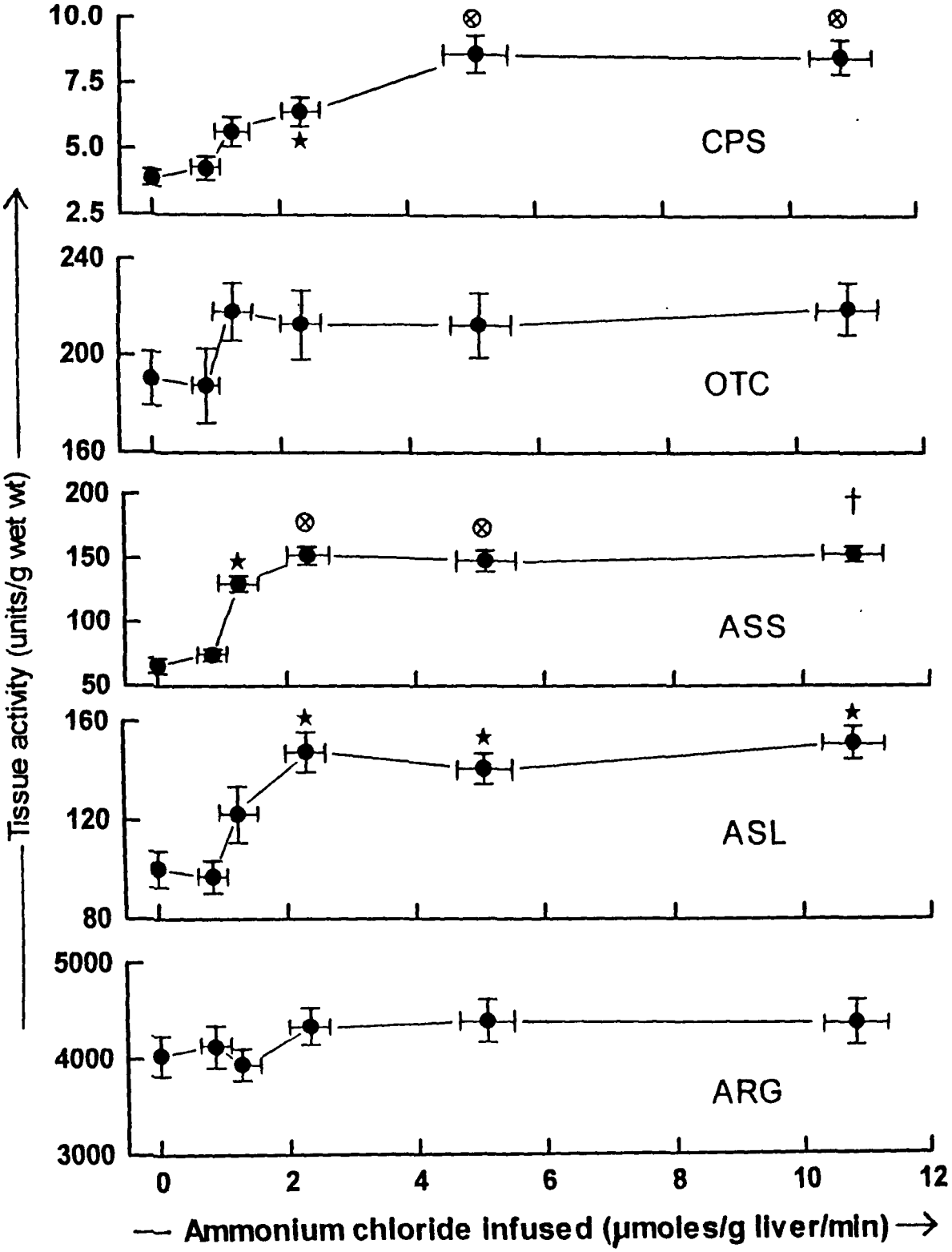


Fig. 15 Changes in tissue activity (units/g wet wt) of urea cycle enzymes in the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl.

Abreviations and unit defination are same as Fig. 8
★, ⊗, †: p values significant at <0.05, <0.01, <0.001 level, respectively.

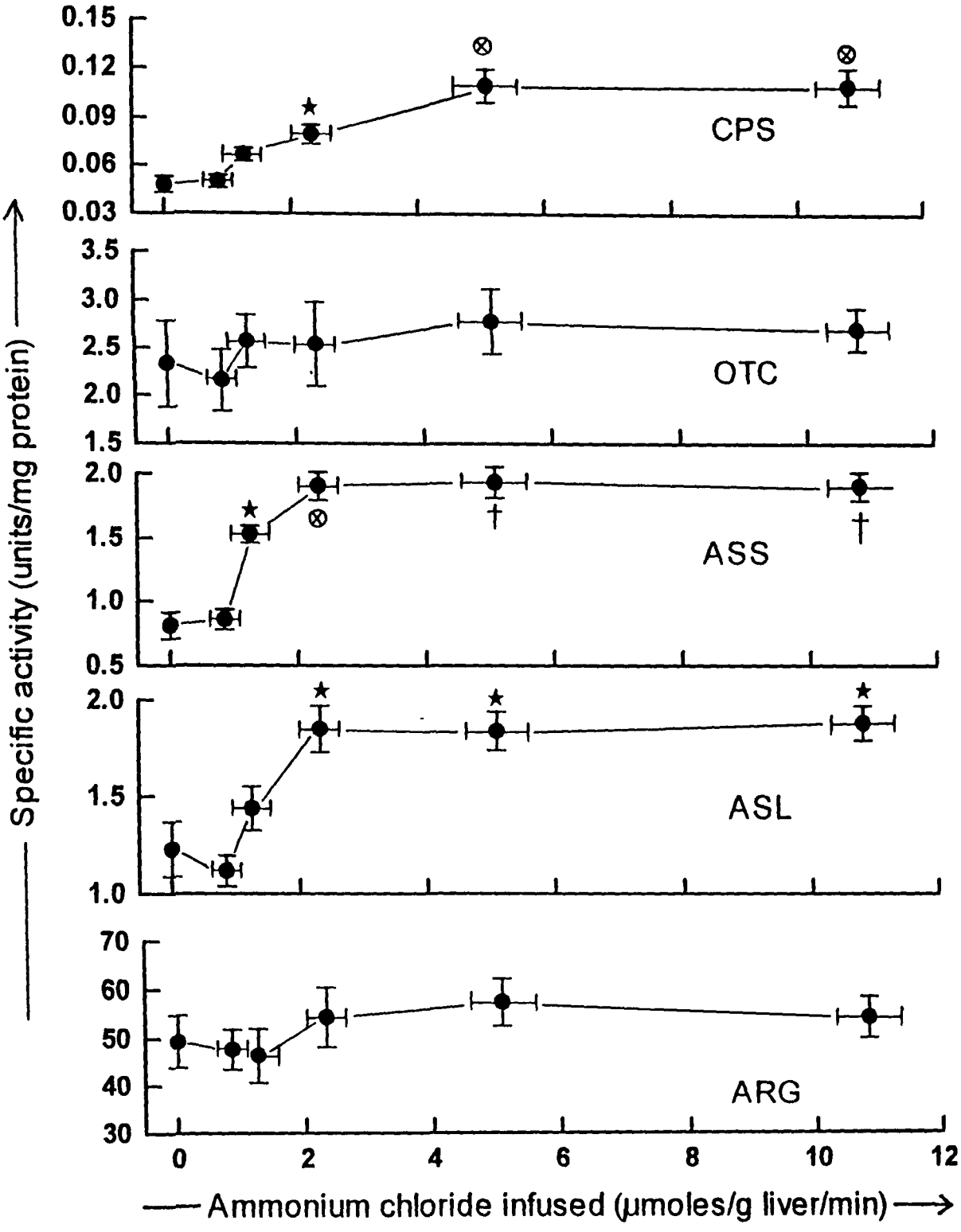


Fig. 16 Changes in specific activity (units/mg protein) of urea cycle enzymes in the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl.

Abreviations are same as Fig. 8

★, ⊗, †: p values significant at <0.05, <0.01, <0.001 level, respectively.

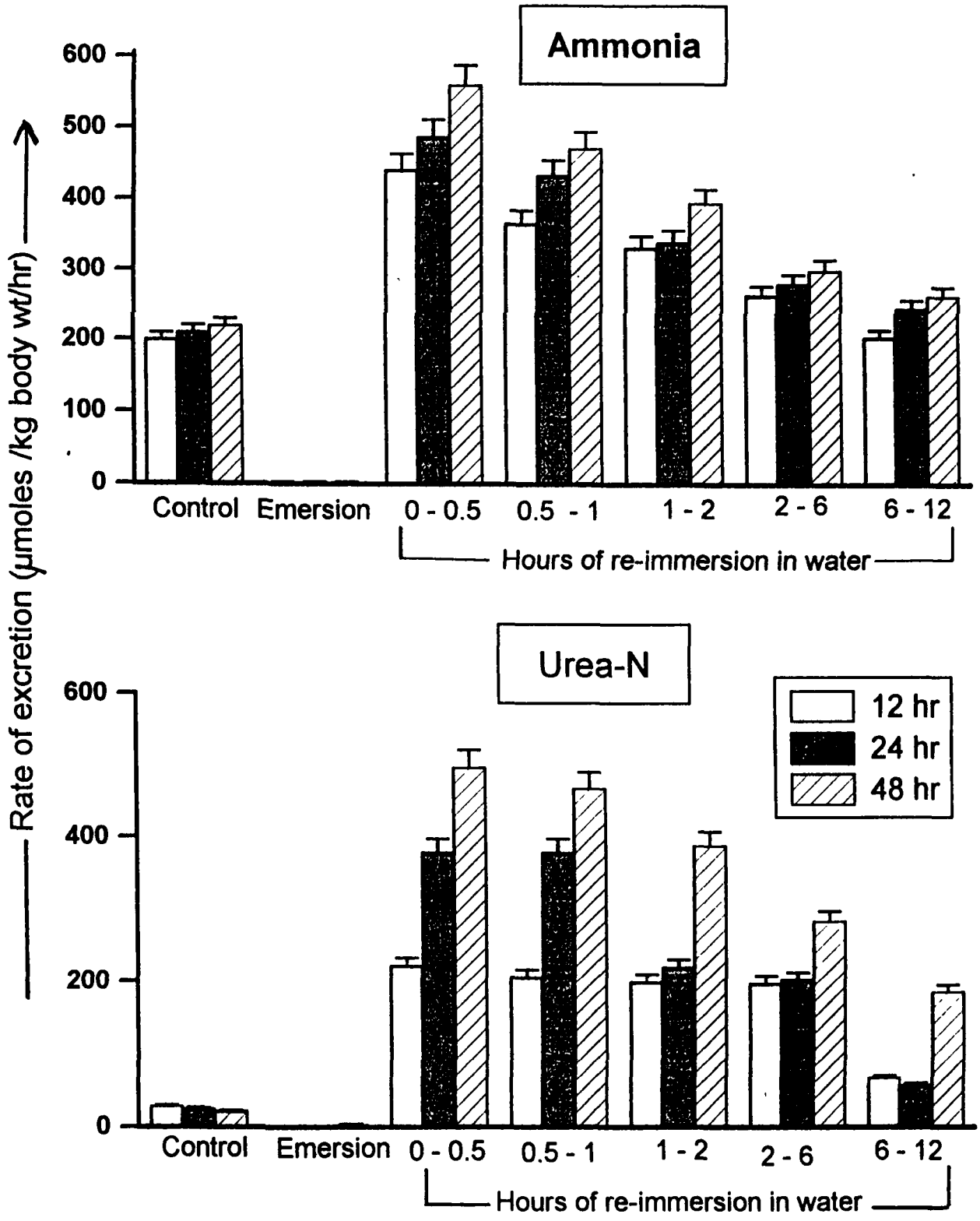


Fig. 17 Rate of excretion of ammonia and urea-N (µmoles/kg body wt/hr) by *C. batrachus* during emersion to air,followed by re-immersion in water.

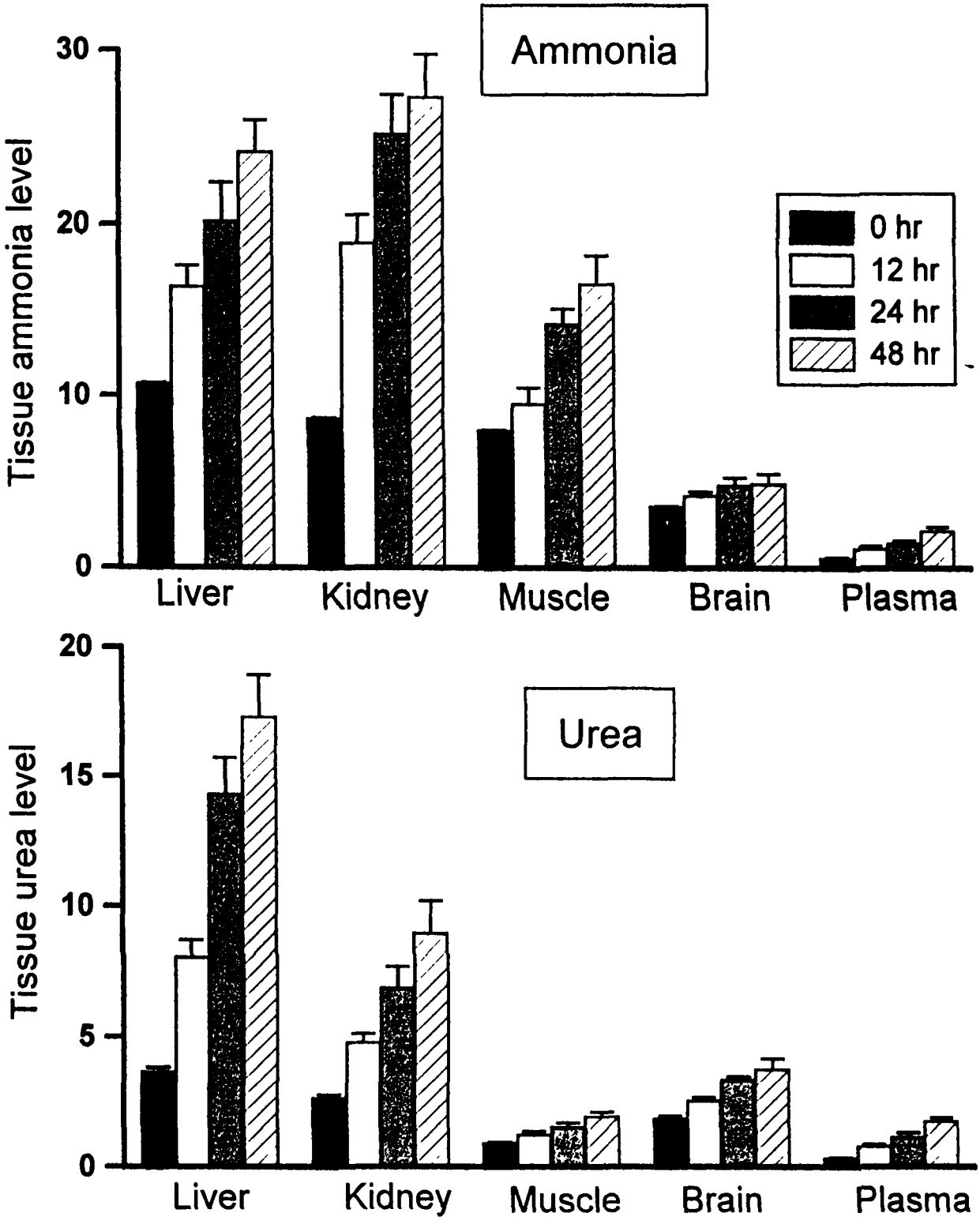


Fig. 18 Changes in the levels of ammonia and urea in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* while exposed to air.

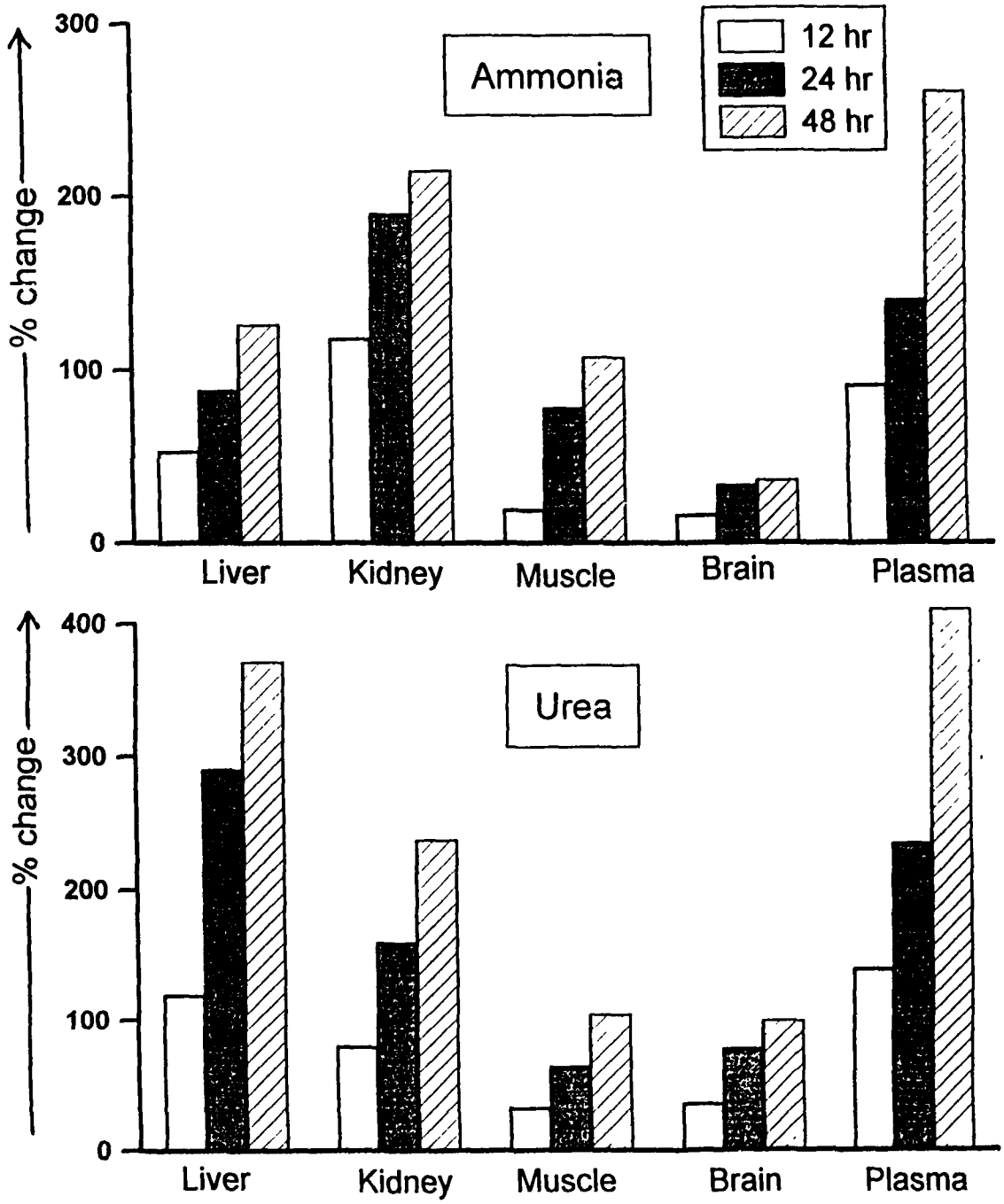


Fig. 19 Percentage changes in the levels of ammonia and urea in different tissues and in plasma of *C. batrachus* while exposed to air.

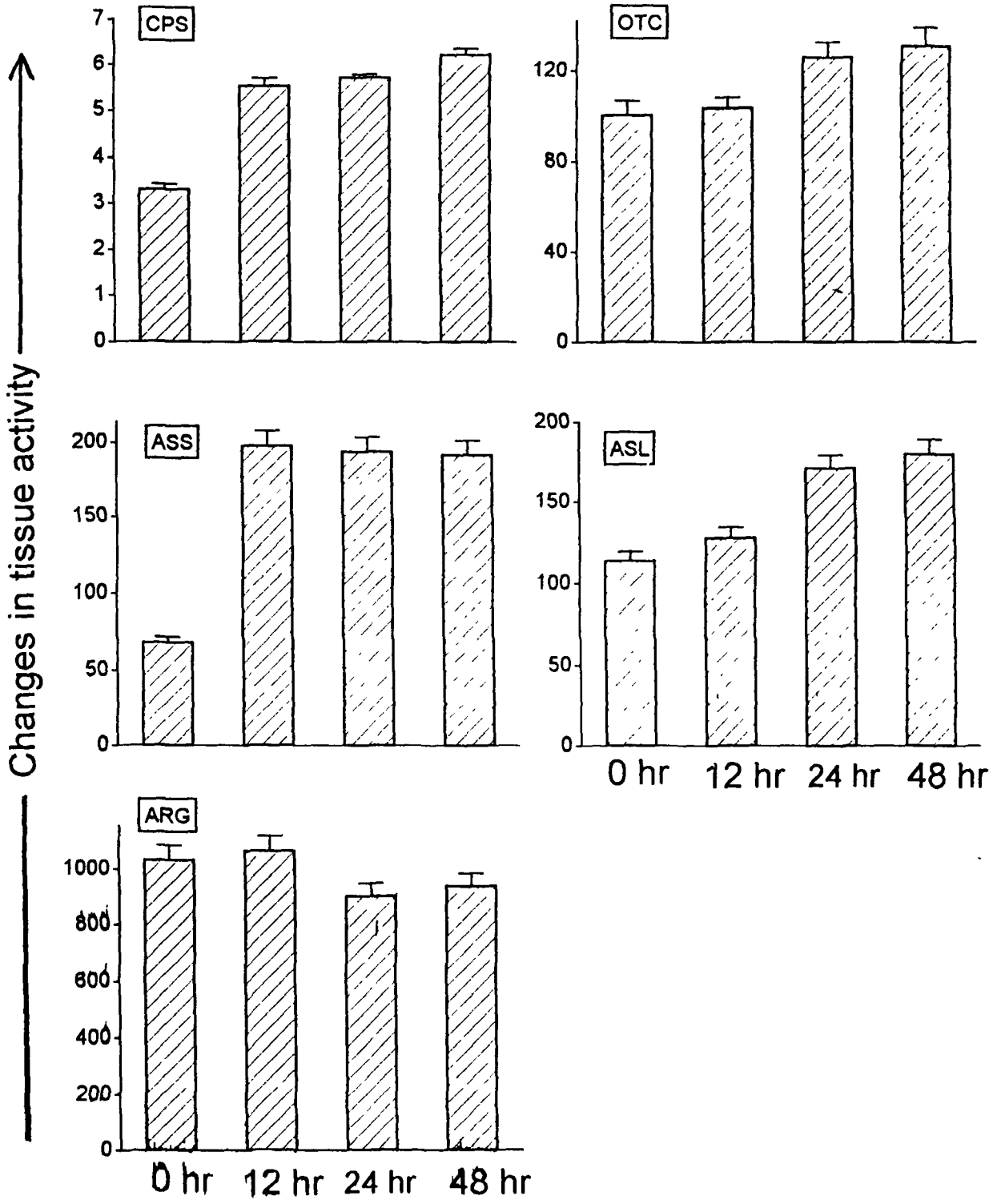


Fig. 20 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to air.

Abreviations and unit defination are same as Fig. 8

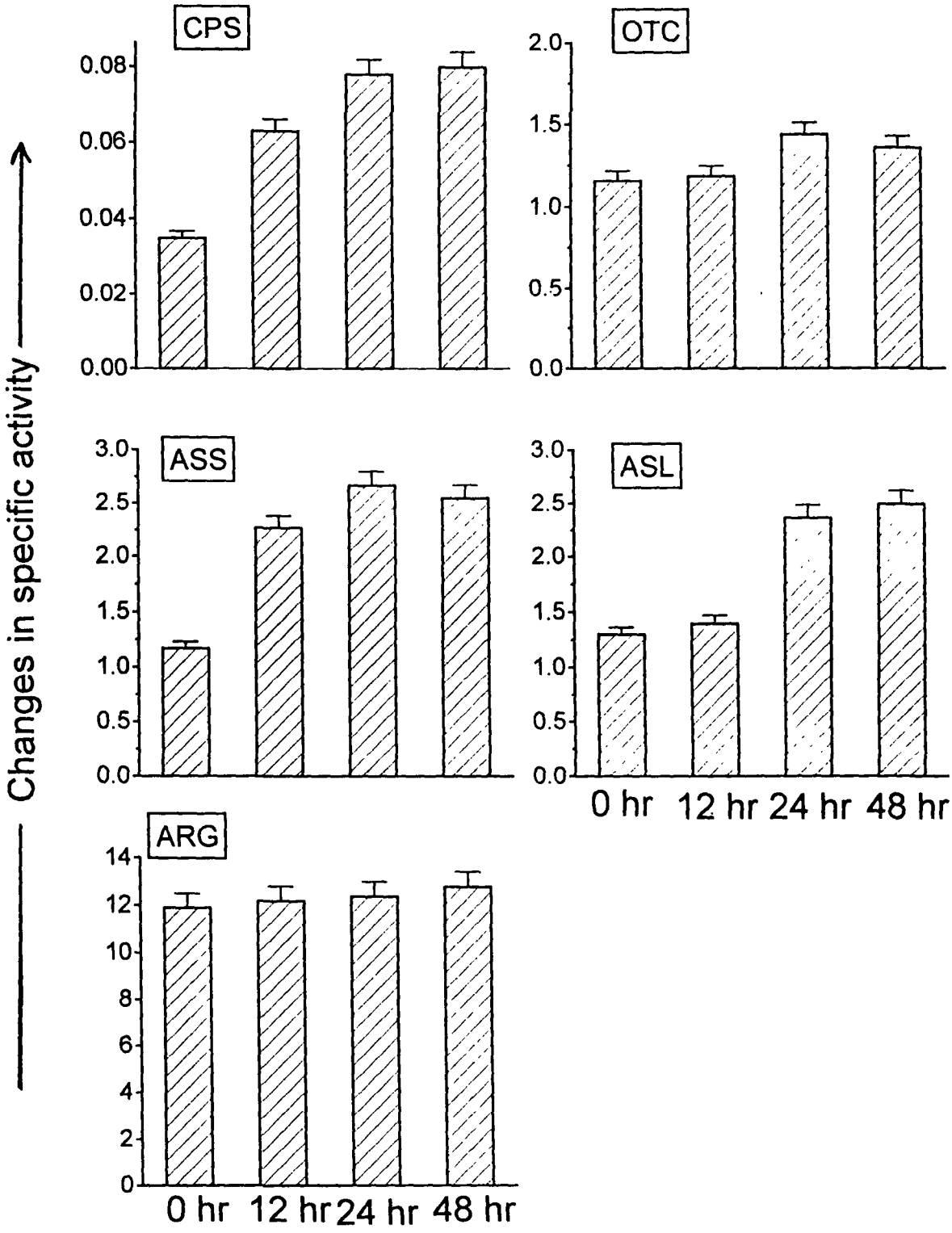


Fig. 21 Changes in specific activity (units/mg protein) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to air.

Abreviations are same as Fig. 8

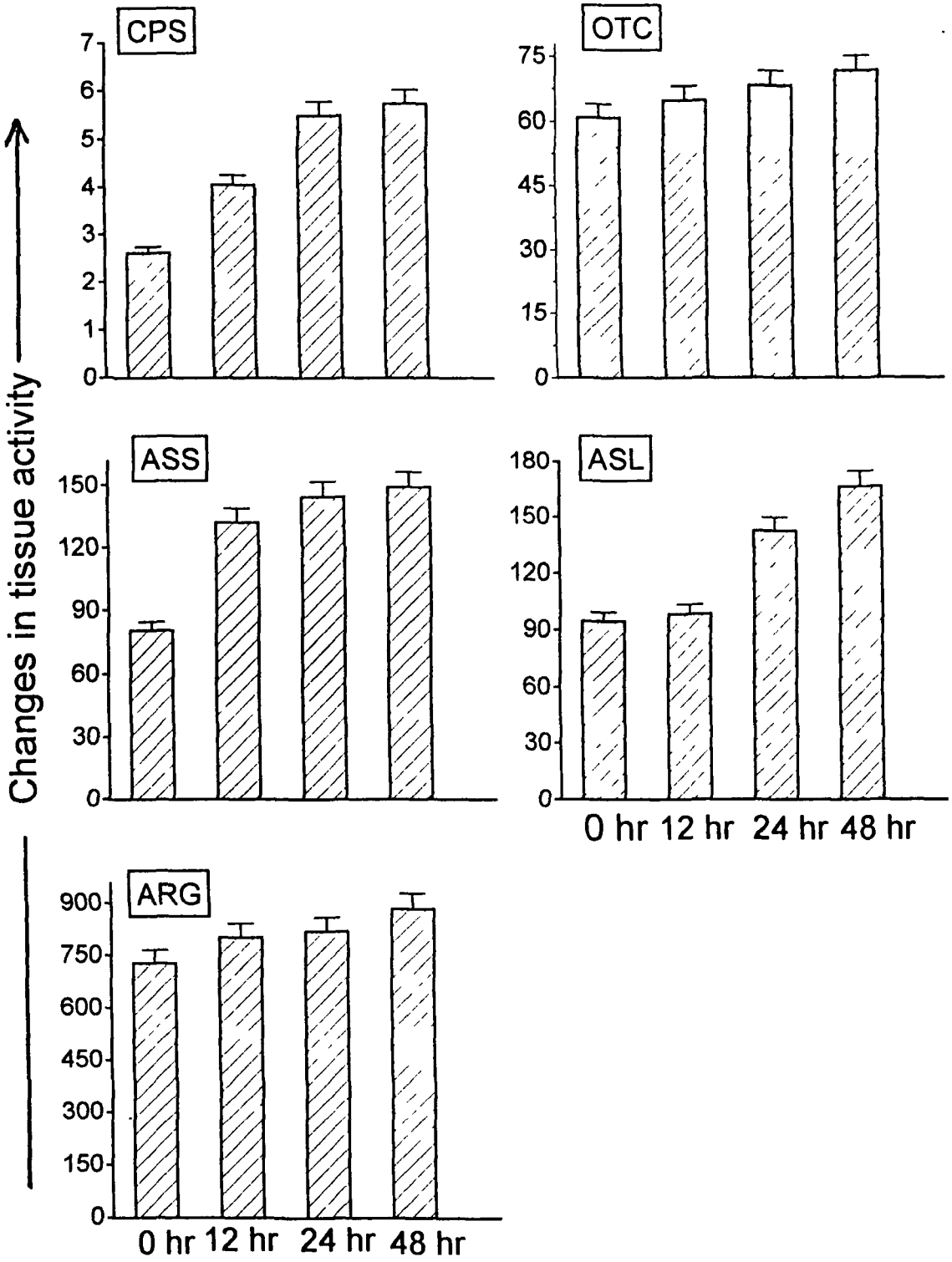


Fig. 22 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the kidney of *C. batrachus* while exposed to air.

Abreviations and unit defination are same as Fig. 8

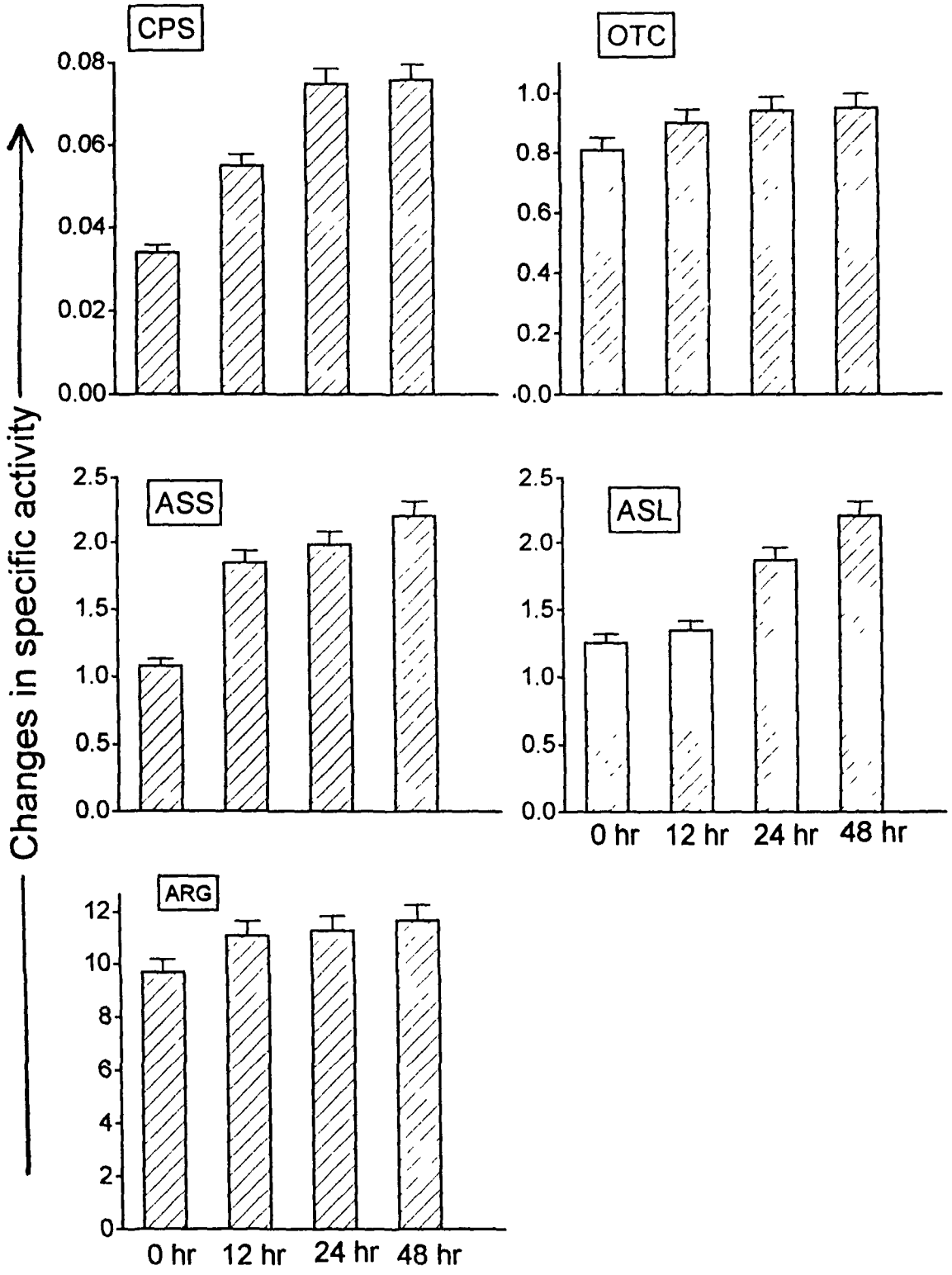


Fig. 23 Changes in specific activity (units/mg protein) of different urea cycle enzymes in the kidney of *C. batrachus* while exposed to air.

Abreviations are same as Fig. 8

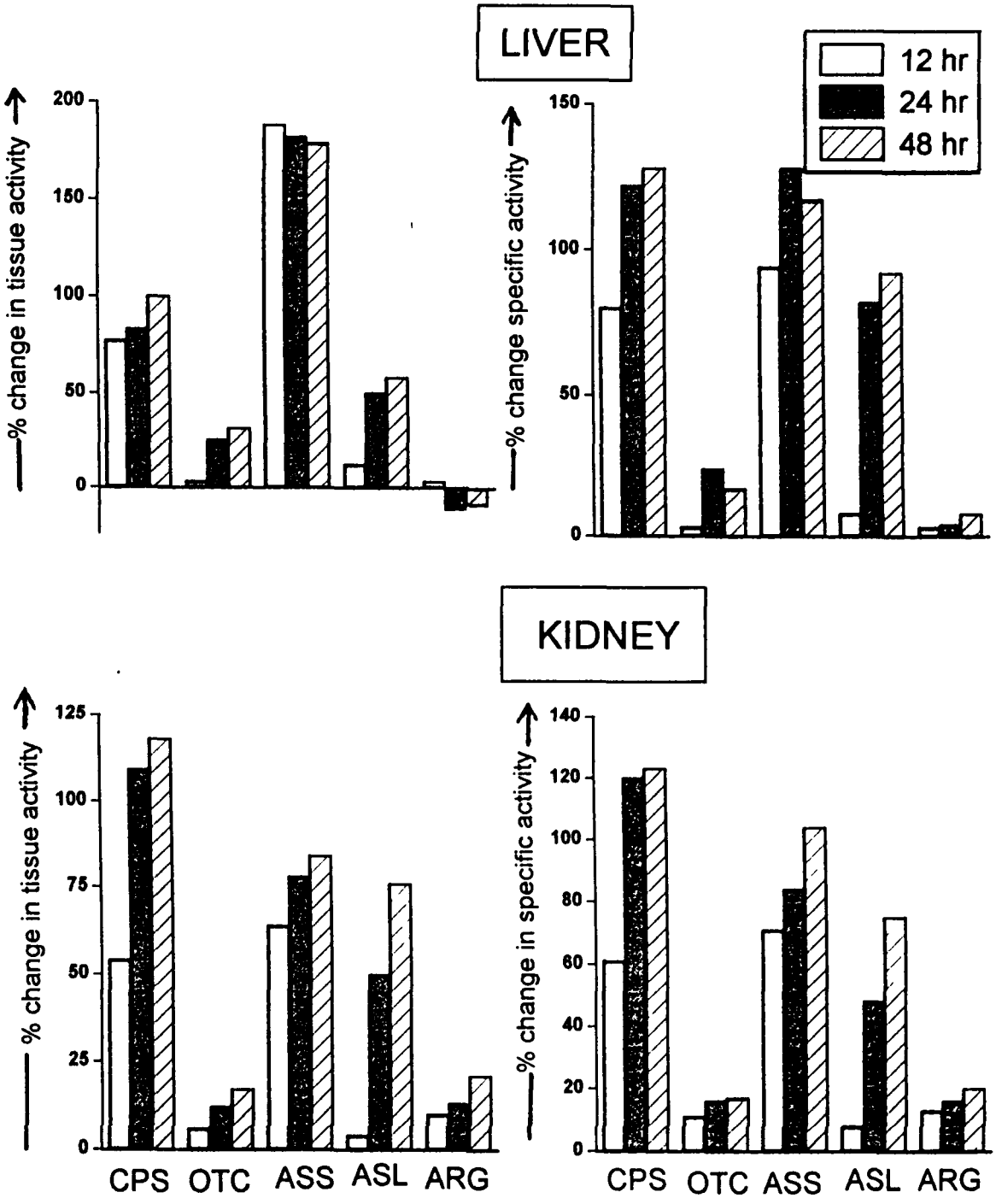


Fig. 24 Percentage changes in tissue and specific activity of different urea cycle enzymes in the liver and kidney of *C. batrachus* while exposed to air.

Abreviations are same as Fig. 8

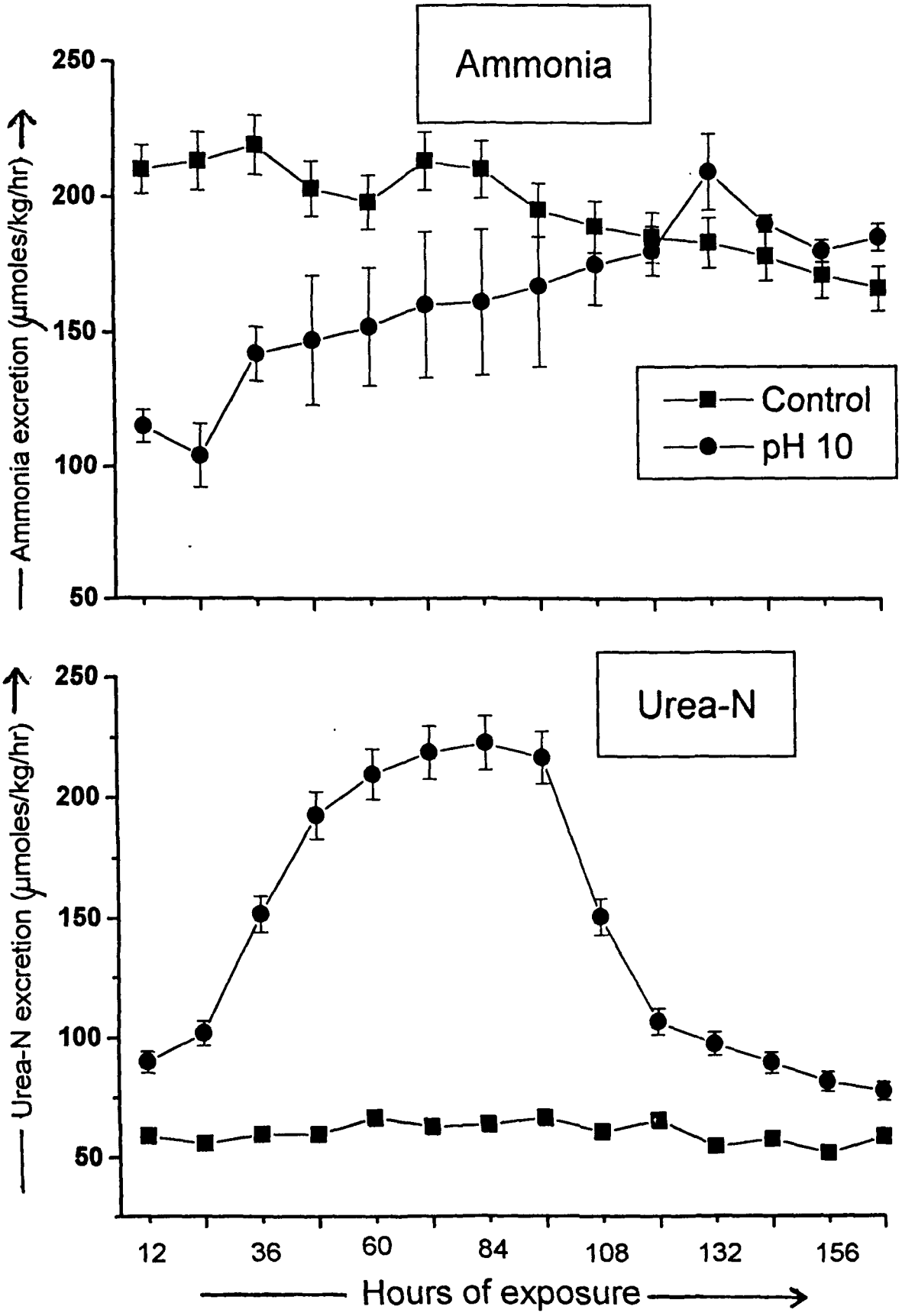


Fig. 25 Pattern of excretion of ammonia and urea-N (µmoles/kg body wt/hr) by *C. batrachus* while exposed to alkaline (pH 10) water.

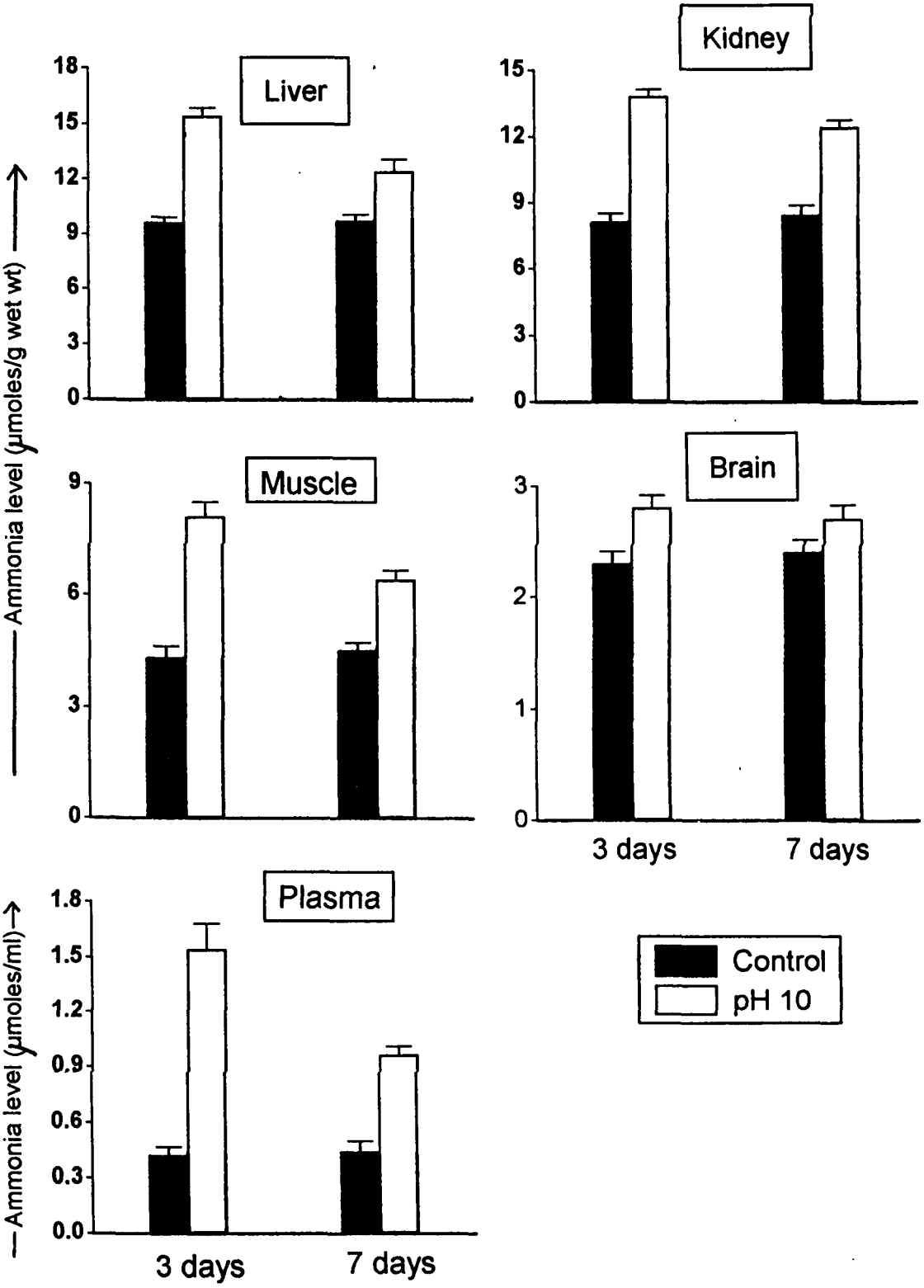


Fig. 26 Changes in the levels of ammonia in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* exposed to alkaline (pH 10) water.

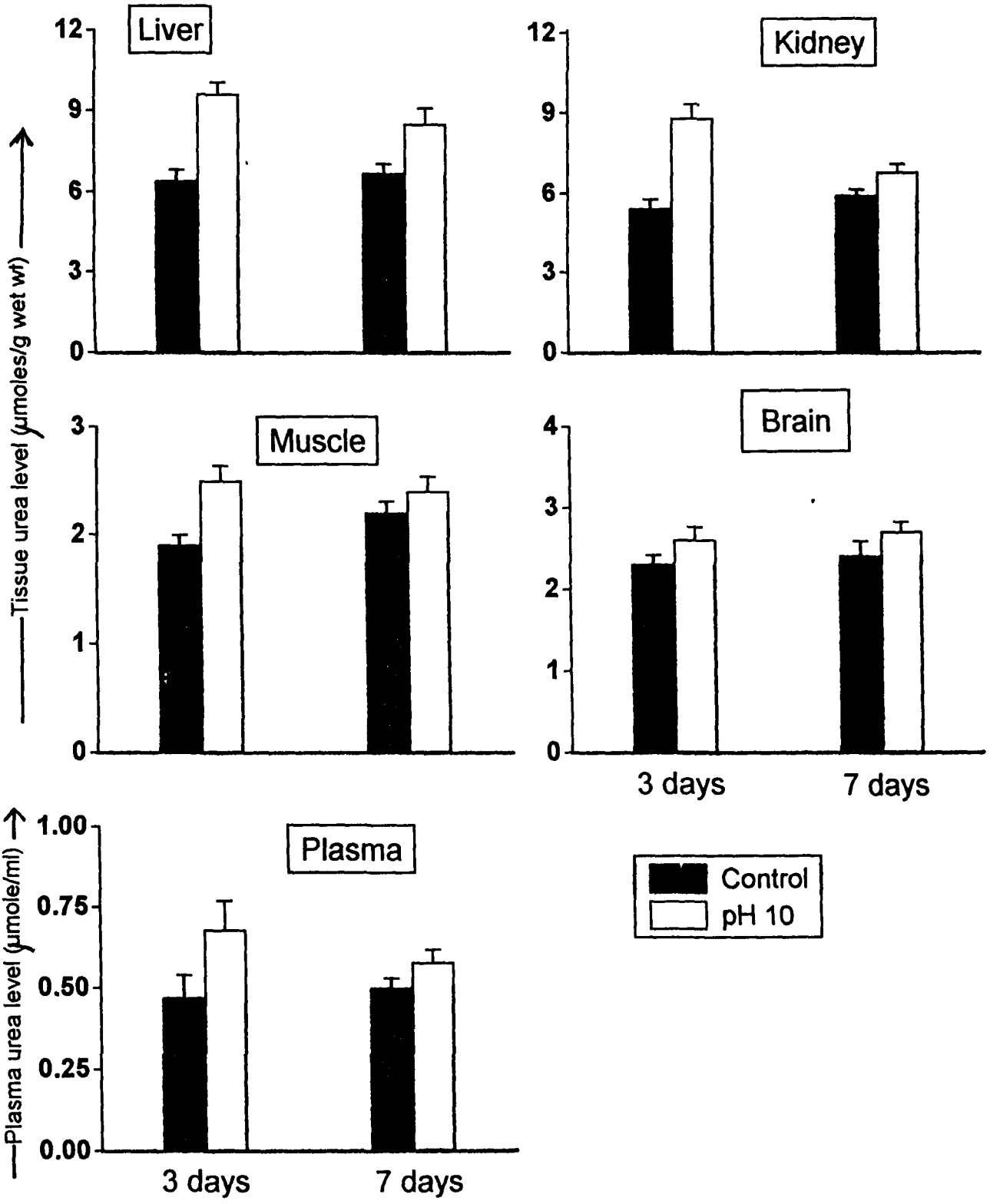


Fig. 27 Changes in the levels of urea in different tissues(µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* while exposed to alkaline (pH 10) water.

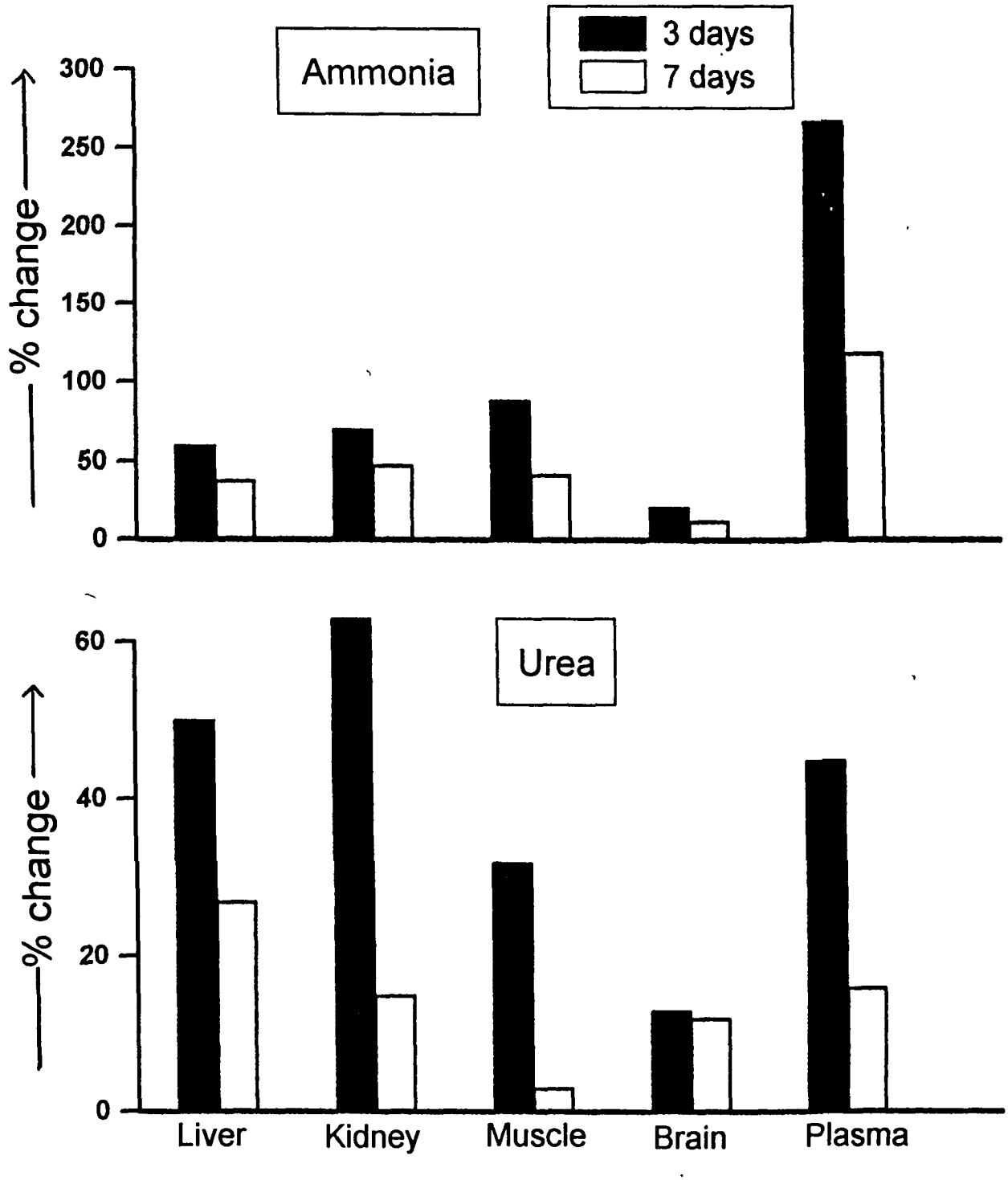


Fig. 28 Percentage changes in ammonia and urea levels in different tissues and in plasma of *C. batrachus* while exposed to alkaline (pH 10) water.

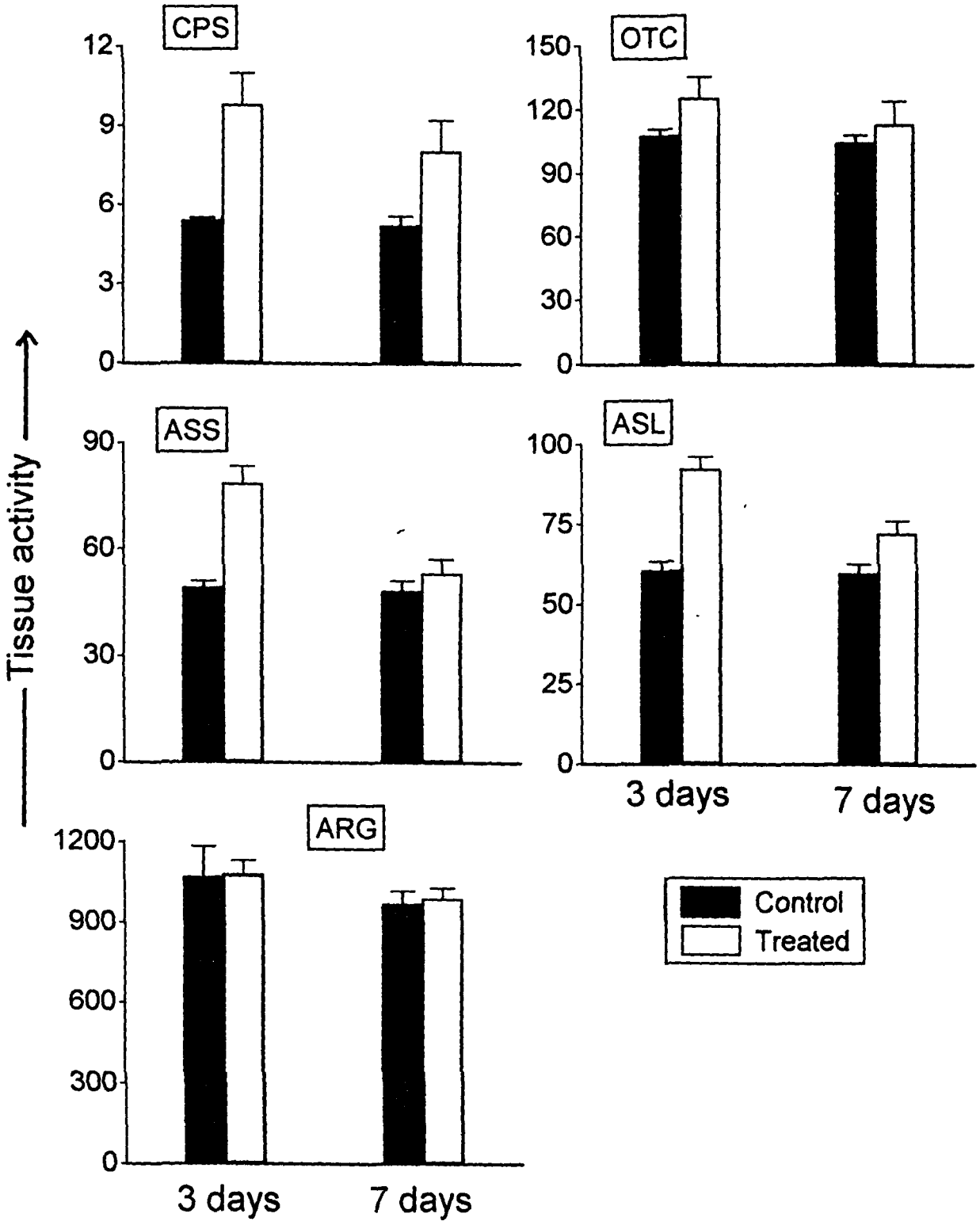


Fig. 29 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to alkaline (pH 10) water.

Abreviations and unit defination are same as Fig. 8

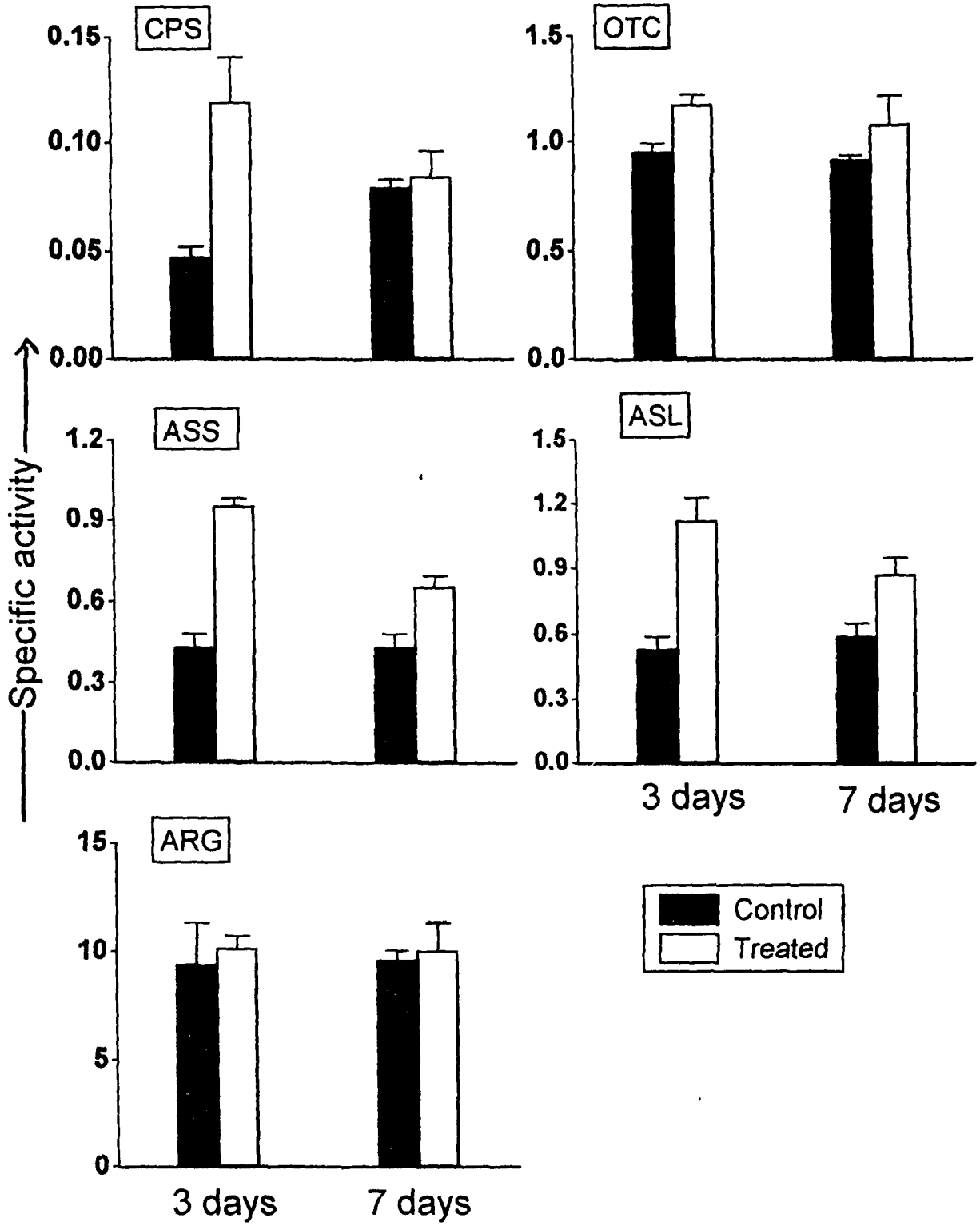


Fig. 30 **Changes in specific activity (units/mg protein) of different urea cycle enzymes in the liver of *C. batrachus* while exposed to alkaline (pH 10) water.**

Abreviations are same as Fig. 8

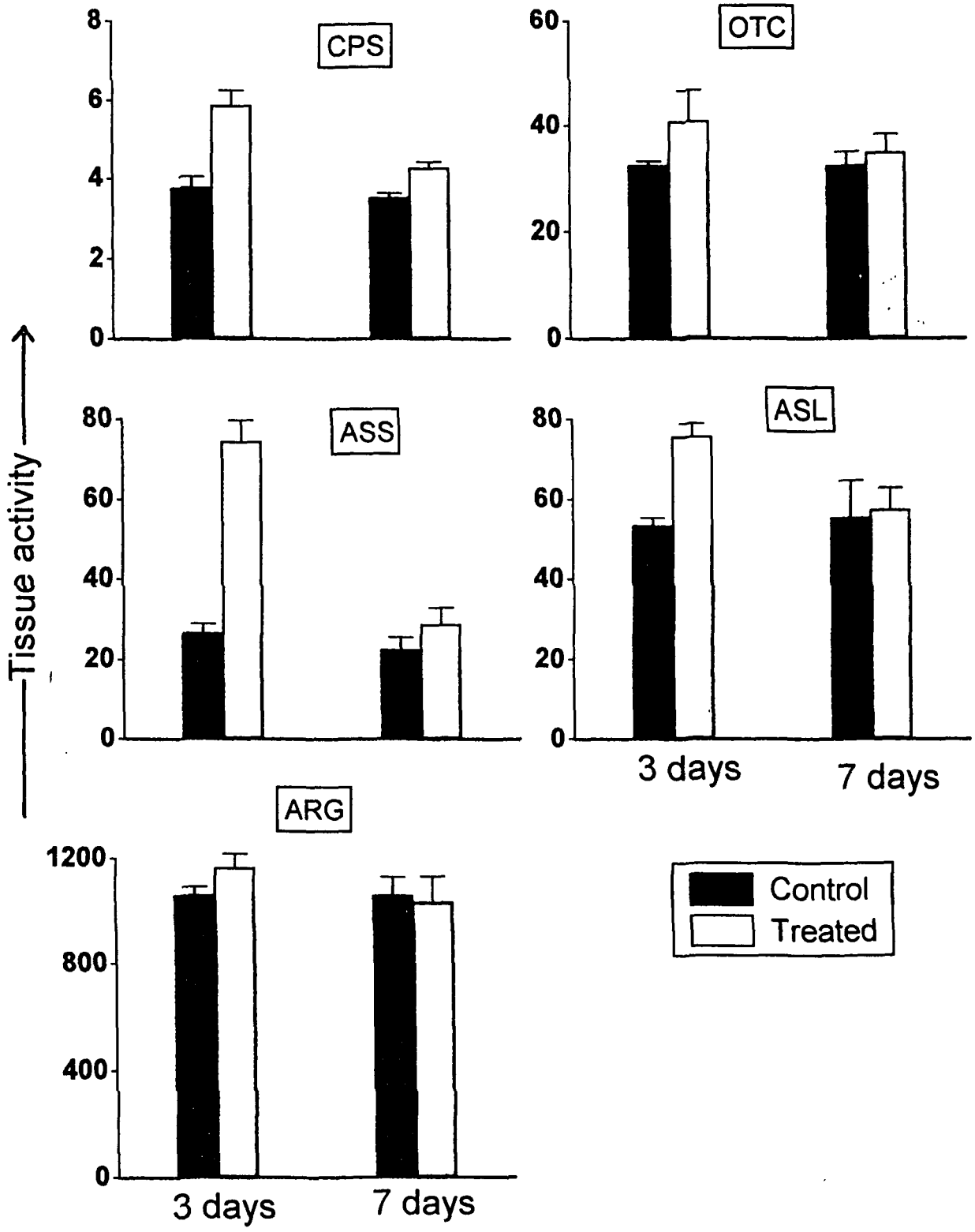


Fig. 31 Changes in tissue activity (units/g wet wt) of different urea cycle enzymes in the kidney of *C. batrachus* while exposed to alkaline (pH 10) water.

Abreviations and unit defination are same as Fig. 8

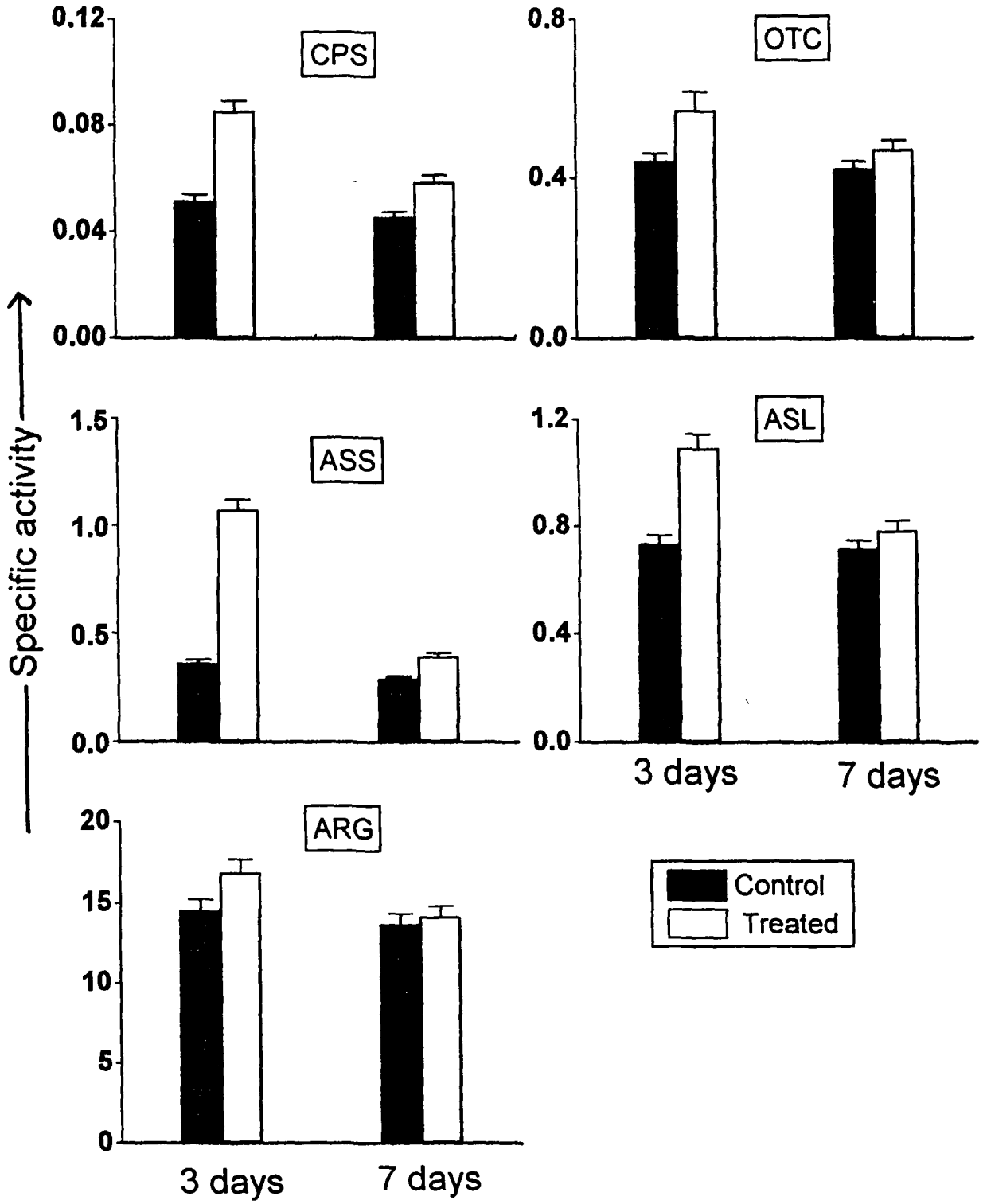


Fig. 32 Changes in specific activity (units/mg protein) of different urea cycle enzymes in the kidney of *C. batrachus* while exposed to alkaline (pH 10) water.

Abreviations are same as Fig. 8

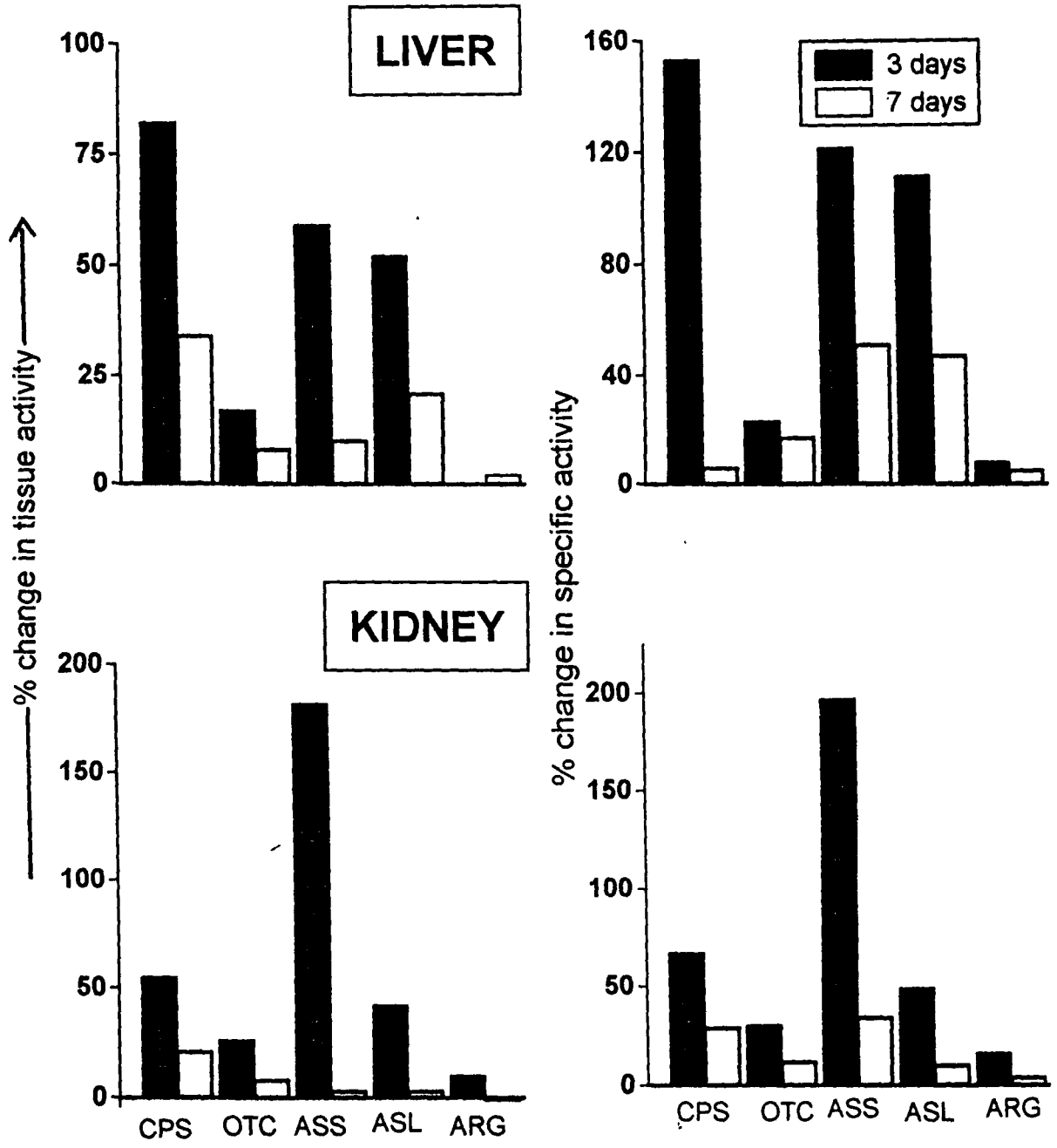


Fig. 33 Percentage changes in tissue and specific activity of urea cycle enzymes in the liver and kidney of *C. batrachus* while exposed to alkaline (pH 10) water.

Abreviations are same as Fig. 8

DISCUSSION

Types of CPSs, the Subcellular Localization of the Urea Cycle Enzymes and the Urea Cycle Enzymes Activity in Extra-hepatic Tissues:

The urea cycle-related CPS activity, which has been reported to date in a limited number of fish species, have been suggested to be CPS III type (glutamine- and NAG-dependent) with the involvement of glutamine synthesis by glutamine synthetase as a first step of urea formation via the urea cycle. This has been directly confirmed in liver of *S. acanthias* (a representative marine elasmobranch), where the GS is localized exclusively in the mitochondria and mitochondrial formation of citrulline from ammonia has been shown to involve obligatory intermediate formation of glutamine (Anderson and Casey, 1984). This was accompanied by an absence of the pyrimidine-related CPS II in the liver, presumably because glutamine is utilized exclusively for urea formation (Hong *et al.*, 1995). The presence of CPS III activity has recently been documented in one Indian air-breathing singhi catfish (*H. fossilis*) both in liver and kidney (Saha *et al.*, 1997) along with the reports of localization of GS (Chakravorty *et al.*, 1989) and arginase (Dkhar *et al.*, 1991) primarily in the mitochondria resembling that of elasmobranchs. In largemouth bass (Cao *et al.*, 1991), in *O. tau* and *P. notatus* the liver GS is localized primarily in the cytosol; in *O. beta*, however, a significant level of the GS activity is present in the mitochondria as well as in the cytosol, and the level of mitochondrial GS may fluctuate in relation to the ureotelic nature of the fish's nitrogen metabolism (Walsh and Milligan, 1995). Also, unlike elasmobranchs, cytosolic pyrimidine-related CPS II is present in the liver of those teleost species, where mitochondrial CPS III activity has been reported and

the presence of CPS II activity has been documented (Anderson, 1980; Cao *et al.*, 1991; Anderson and Walsh, 1995; Saha *et al.*, 1997). In the walking catfish, high levels of GS activity were reported both in liver and kidney, and were mainly localized in the mitochondria (Tables 3 and 4). In this respect, walking catfish resembles elasmobranchs and singhi catfish, and like other teleosts (including singhi catfish) cytosolic CPS II is present in liver and kidney.

The presence of CPS III was not unusual in walking catfish with its known ureotelic capacity. However, the presence of significant ammonia- and NAG-dependent CPS (CPS I-like) activity in liver and kidney mitochondria of walking catfish, resembling that of singhi catfish (Saha *et al.*, 1997), is unique from that observed in other teleosts. The presence of a gene for both a CPS III and CPS I in walking catfish would not seem to be a likely explanation for these two activities within the context of the current understanding of the structural relationships between these two enzymes, their currently understood species distribution (CPS I is present only in ureotelic mammalian and amphibian species, CPS III is present only in invertebrates and fishes) and the prevailing view that CPS I evolved from CPS III (Mommsen and Walsh, 1989; Campbell and Anderson, 1991; Helbig and Atkinson, 1994; Hong *et al.*, 1994; Anderson, 1995a,b). Perhaps adaptation in walking and singhi catfish and closely related species was achieved as a separate event in which the CPS III gene underwent duplication and one gene subsequently lost the structural requirements for utilization of glutamine as substrate. Alternatively, perhaps the observed CPS I-like activity represents an adapted form of CPS III with separate ammonia and glutamine binding sites. It is perhaps difficult to say with the present study whether the glutamine-dependent CPS III described here is similar to the CPS III activities of dogfish and largemouth bass. Elucidation of the nature of the two

types of activities will require purification and characterization of the enzyme(s) responsible for these activities and/or the mRNA(s) coding for the enzyme(s).

The subcellular localization of urea cycle enzymes in liver and kidney of walking catfish was found to be analogous to that of singhi catfish (Dkhar *et al.*, 1991) and elasmobranchs (Casey and Anderson, 1982) , i.e., CPS III, OTC and ARG are localized in the mitochondria, and ASS and ASL are localized in the cytosol (Tables 3, 4; Fig. 34).GS is also reported to be localized in the mitochondria (Saha *et al.*, 1999) of both liver and kidney of walking catfish. The co-localization of GS along with CPS III in mitochondria probably helps in the assimilation of ammonia more efficiently. Unlike that of elasmobranchs, CPS I-like activity has also been documented in the mitochondria of liver and kidney of this walking catfish. Pyrimidine synthesis via the CPS II probably needs glutamine to be transported out from the mitochondria to the cytosol. The presence of significant levels of both kinds of urea cycle-related CPS (CPS III and CPS I-like) activity, enabling this walking catfish to utilize ammonia, either directly or indirectly as glutamine, as substrate for carbamyl phosphate formation in mitochondria, may be an adaptive physiological feature characteristic of these species that is related to their remarkable tolerance to high levels of ambient NH_4Cl and their ability to switch from ammoniotelism to ureotelism when exposed to high levels of ammonia and during exposure to air (present study). The physiological significance of mitochondrial localization of ARG in this walking catfish and many other teleosts reported earlier is not very clear. One consequence of the localization of ARG in the mitochondria could be to supply ornithine directly for carbamyl phosphate synthesis inside the mitochondria and also could be due to replacement of transporter for ornithine by arginine in the mitochondrial membrane as suggested by Mommsen and Walsh (1989). Another consequence could be, as suggested in

elasmobranchs (Anderson, 1995a), that urea formed in the mitochondria serves as a feedback inhibitor for CPS III and also for GS.

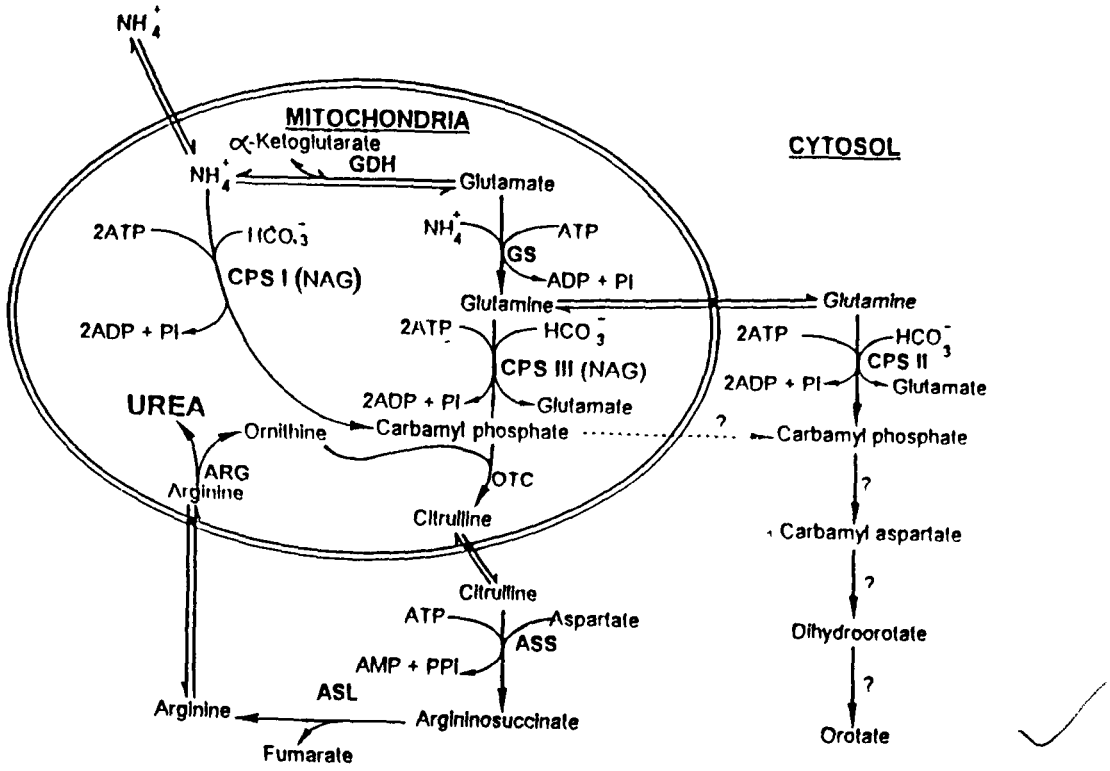


Fig. 34. Illustration of urea cycle in liver and kidney of *C. batrachus*.

CPS I, II and III - carbamyl phosphate synthetase I, II and III; OTC - ornithine transcarbamylase; ASS - argininosuccinate synthetase; ASL - argininosuccinate lyase; ARG - arginase; GS - glutamine synthetase; GDH - glutamate dehydrogenase; ? - not confirmed.

In addition to two potential ureogenic tissues (liver and kidney), whether other tissues are involved in urea synthesis via the urea cycle, we surveyed the activities and urea cycle enzymes in muscle, intestine and brain of this walking catfish. Clearly, all the enzymes appear to be present in all the three tissues except ASS in brain and ASL in muscle (Table 6). Growing evidence suggests that extra-hepatic tissues could also participate in urea synthesis in fish as has been reported in several species of Indian air-breathing teleosts (Saha and Ratha, 1987,1989), toadfish (Wood *et al.*, 1995), rainbow trout (Korte *et al.*, 1997), common carp and bowfin (Felskie *et al.*, 1997). Lindley *et al.* (1999) suggested that the muscle is the primary site of urea synthesis in the alkaline lake-adapted tilapia (*O. a. grahami*) due to the presence of all the urea cycle enzymes activity at high levels in muscle. There could also be the involvement of more than one tissue in the same species responsible for urea synthesis especially when one or two enzymes of urea cycle are lacking in specific tissues. For example, in our walking catfish the synthesis of citrulline or argininosuccinate may take place in brain and muscle, respectively, followed by the transportation of these intermediary products via the blood to either liver and kidney for ultimately converting to urea. Although the activity of different urea cycle enzymes in muscle were comparatively low, but the muscle represents a high percentage of the body mass. Therefore, the total units of different enzymes could be physiologically significant in this fish.

Exposure to Higher Ambient Ammonia Solution:

Higher levels of ammonia in water has been a common toxicological problem for freshwater fishes (Randall and Wright, 1987; Campbell, 1991; Wood, 1993). As mentioned in the Introduction section, ammonia toxicity in various ammoniotelic teleosts

has been studied extensively and also the LC_{50} value for unionized ammonia for various fishes were also determined. The walking catfish (*C. batrachus)* was found to tolerate very high ambient ammonia (up to 75 mM NH_4Cl) which is much higher compared to any other teleosts and even aquatic amphibians (present study).This indicates the presence of efficient physiological and biochemical mechanisms for detoxification of excess ammonia in this fish. One such adaptation in this fish is the presence of a functional urea cycle in liver and kidney of walking catfish (Saha and Ratha, 1989).

Ammonia is a neurotoxin. It interferes with energy metabolism, electrophysiological properties and level of neurotransmitter in brain (Copper and Plum, 1987). Walking catfish, on exposure to NH_4Cl at ambient concentrations from 10 to 75 mM, became initially hyper-excitable. Acclimatization of walking catfish to the changed environment gradually calmed down the irritational symptoms. A similar response was reported in goldfish (Olson and Fromm, 1971). All the fishes exposed to 100 mM NH_4Cl solution died within 1 to 2 days. Accumulating of acidic metabolites from glycolysis and krebs cycle at higher ambient ammonia was suggested to cause anoxia and deaths due to a decrease in blood pH in coho salmon (Sousa and Meade, 1977). However, Hillaby and Randall (1979) could not observe any decrease in blood pH when the total ammonia load in the blood of rainbow trout (*S. gairdneri*) was elevated to a toxic level. Therefore, the decreasing pH of the ambient medium from 6.85 to 6.46 with an increasing concentration of NH_4Cl from 75 to 100 mM, respectively, might not be the main cause of death of walking catfish. The death could be due to enhanced accumulation of ammonia *in vivo.*

Increased level of ambient ammonia has been reported to alter nitrogen excretion pattern in aquatic vertebrates. Decrease in excretion of total nitrogen and ammonia under elevated ammonia stress has been reported in rainbow trout, *S. gairdneri* (Fromm and

Gillette, 1968; Olson and Fromm, 1971). The walking catfish (*C. batrachus*) treated with 25 mM NH_4Cl also showed an immediate suppression in ammonia excretion (Table 7). In the singhi catfish *(H. fossilis)* is reported to excrete primarily (99%) through the extra-renal sources (gills and skin) (Saha *et al.*, 1988), which could also be true in this walking catfish. The same surfaces (gills and skin) might have been used for absorption of ammonia into the body in favour of concentration gradient from the higher external ammonia concentration. Initial suppression of ammonia excretion was also reported in two other air-breathing teleosts, *C. punctatus* (Arya, 1979), and in singhi catfish (Saha and Ratha, 1990) exposed to high ammonia solution. However, there was immediate increase of urea-N excretion by walking catfish by about 9-fold on the first day of exposure to NH_4Cl, followed by further increase to about 18-fold on the 5th day of exposure (Table 8; Figs. 3, 4). A six fold increase of urea-N excretion was observed in singhi catfish exposed to different concentrations of NH_4Cl for 28 days (Saha and Ratha, 1990). A similar 10-fold increase of urea-N excretion was reported in *X. laevis* immersed in 5 mM NH_4Cl (Janssens, 1972). Ammonia accumulated significantly in all tissues of walking catfish after first day of treatment with 25 mM NH_4Cl, followed by a further increase at later part of treatment (Table 9; Figs.5, 7). Suppression of excretion and absorption of ammonia from the ambient medium by the walking catfish probably resulted in this accumulation. The accumulation was tissue specific. Brain, being highly susceptible to ammonia toxicity (Copper and Plum, 1987), showed no significant accumulation of ammonia. Effective enzymatic detoxification of ammonia to glutamate and then to glutamine by the enzymes GDH and GS, respectively, has been reported in fish brain (Levi *et al.*, 1974; Walton and Cowey, 1977; Iwata *et al.*, 1981; Chakravorty *et al.*, 1989; Das *et al.*, 1991). In this walking catfish also very high levels of GDH (ammonia utilizing direction) and GS in brain were found (Saha

et al., 1999; unpublished observation by Dutta and Saha). Liver and kidney, which are the main organs for ammonia metabolism in fish (Pequin and Serfaty, 1963; Vellas and Serfaty, 1974; Casey *et al.*, 1983) accumulated at a very high level (about 25 μmoles/g wet wt). Blood being the carrier of metabolites, the concentration of ammonia increased to about 4-fold in plasma compared to the control fish. Accumulation of ammonia at different levels in higher ambient ammonia concentration indicative of the fact that different tissues of walking catfish probably have different maximum loading capacities as suggested in singhi catfish (Saha and Ratha, 1994). Ammonia transport in freshwater teleosts via the gill membrane primarily takes by diffusion as NH_4^+ and partly by Na^+-related carrier transport of NH_4^+ (Hillaby and Randall, 1979; Wilkie, 1997). The distribution of ammonia in different tissue compartments has been reported to be influenced by both the pH and electrochemical gradient (Wright *et al.*, 1988). However, the real mechanism of ammonia transport in his fish has not yet been studied. It is interesting to note that the maximum concentration of ammonia in plasma did not exceed 2.5 mM when the ambient concentration was 25 mM. This is quite a feat which might have contributed to the survival of the fish at that high ambient concentration of NH_4Cl.

The control fish, which were fasting in freshwater, did not show significant change in ammonia levels in various tissues (Table 9). However, they showed gradual reduction in the rate of excretion of ammonia during starvation (Table 7; Fig. 3).

Metabolic detoxification of ammonia to urea could not be observed in most freshwater teleosts due to having a non-functional or incomplete urea cycle (for review, see Saha and Ratha, 1998). However, the presence of a functional urea cycle enzymes activity at relatively high levels in liver and kidney tissues of walking catfish have already been reported (Saha and Ratha, 1989). The accumulation of ammonia *in vivo* was

associated with the stimulation of activity of most of the key enzymes of urea cycle both in liver and kidney of walking catfish when exposed to 25 mM NH_4Cl within one day, followed by further increase on the 3rd day of exposure and remained high till the end of the experiment (Tables 11-14; Figs. 8-12). Even though ARG activity was assayed at a high unphysiological pH (9.5), its physiological level of activity would also be very high, which could be the reason of not further stimulating the activity at high ambient ammonia. Likewise, the normal physiological activity of OTC was also high in this fish causing no further stimulation of activity at high ambient ammonia.

Stimulation of urea cycle activity in walking catfish was accompanied with the significant accumulation of urea in different tissues and in plasma (Table 10; Figs. 6, 7). Accumulation of urea was also tissue specific even though it is a highly permeant solute. Liver accumulated highest concentration of urea (10.2 µmoles/g wet wt), followed by kidney (8.6 µmoles/g wet wt). Muscle, which constitutes the major bulk of fish tissue, retained comparatively low concentration of urea (3.6 µmoles/g wet wt). Either urea was rapidly diffused from muscle through skin, or muscle had some special physiological barrier to protect it from excess urea accumulation. Again, blood being the carrier of different metabolites, there was significant increase of urea level in plasma possibly coming from different ureogenic tissues after synthesis when the fish was exposed to higher ambient ammonia. The presence of a specific urea transporter protein has been reported in mammalian erythrocytes and kidney tubules (Sands *et al.*, 1992; Gillian and Sands, 1992), and more recently hepatocytes and red blood cells of elasmobranchs and teleost fishes (Walsh *et al.*, 1994a).

Accumulation of ammonia seemed to induce the activity of urea cycle enzymes for enhanced conversion of toxic ammonia to less toxic urea. Such ammonia-induced

ureogenesis has been reported in ureotelic amphibians (Balinsky *et al.*, 1961; McBean and Goldstein, 1967; Janssens and Cohen, 1968; McBean and Goldstein, 1970; Janssens, 1972). Significant increase in the rate of urea excretion was also reported in the ureogenic toadfish, *O. beta*, with high levels of external ammonia or exposure to air (Walsh *et al.*, 1990).

It is difficult to explain the real mechanism(s) of stimulation of ureogenesis under hyper-ammonia stress in this walking catfish. However, one can hypothesize that higher accumulation of tissue ammonia probably serves as a modulator to induce ureogenesis in this catfish. This hypothesis was further supported from the results of perfusion experiments, where the liver was loaded with ammonia by infusing NH_4Cl at different rates (mentioned below).

In addition to ammonia, certain hormones such as glucocorticoids, glucagon and thyroxine also may play some role in the regulation of ureogenesis in this walking catfish as reported in mammals (for review, see Takiguchi and Mori, 1995) and in the amphibian, *X. laevis* (Balinsky *et al.*, 1972). Little information is available about the hormonal regulation of ureogenesis in fish. Mommsen *et al.* (1991) studied the effects of several hormones including glucagon, glucagon-like peptide, epinephrine and vasoactive peptides, on the regulation of urea synthesis in isolated hepatocytes of gulf toadfish, *O. beta*, but could not find any response to any of the hormones. However, GS, which appears to be an important enzyme related to urea synthesis in fish for supplying ammonia nitrogen to CPS III, was found to be stimulated by cortisol released at high levels during confinement/crowding in the same toadfish (Hopkins *et al.*, 1995). Mommsen *et al.* (1991) suggested that a cortisol related stress response may be only one of several mechanisms by which ureogenesis is activated in gulf toadfish rather than higher ammonia load. However,

in this walking catfish higher ammonia load appears to be an important modulator for regulation of ureogenesis as suggested also in another catfish, the singhi catfish (Saha and Ratha, 1994; Saha *et al.*, 1995). However, elucidation of the role of hormones in ureogenesis under different stresses in different Indian air-breathing teleosts including the walking catfish are probably necessary to draw any definite conclusion. Further, there could be other possible mechanism(s) of controlling ureogenesis in walking catfish such as more abundance or synthesis of N-acetyl glutamate (NAG) (allosteric regulator) under hyper-ammonia stress, which can regulate at least the two urea cycle-related CPSs, CPS I and III, as has been shown in rat liver (Lund and Wiggins, 1984), and more recently in the gulf toadfish, *O. beta* during confined (stress) conditon (Julsrud *et al.*, 1998). The presence of both the urea cycle-related CPSs, CPSI-like and CPS III (most predominated in teleost species) have been reported (present study). But the assay method used here for CPS activity does not ditinguish between these two different types of CPSs. Therefore, it is not possible at present to identify whether both the CPSs or only one type was induced under hyper-ammonia stress. Another possible mechanism could be the activation of pre-existing inactive enzymes serving ammonia as a regulatory molecule. Another possibility could be the increase in synthesis of urea cycle enzyme proteins under hyper-ammonia stress. However, to conclude definetely one has to look into the abundance of mRNAs of various urea cycle enzymes in this fish under hyper-ammonia stress.

However, the results from the NH_4Cl exposure experiment clearly suggest that the exposure of the walking catfish, *C. batrachus* to higher ambient NH_4Cl solution resulted in accumulation *in vivo* of ammonia and associated induction of activities of urea cycle enzymes in liver and kidney which accelerated the rate of ureogenesis. The conversion of excess ammonia to urea and the increase in excretion of urea-N reported here controlled

the *in vivo* concentration of ammonia and urea. Thus, ammoniotelic walking catfish turned towards ureotelism to survive at high (25 mM) concentration of ambient NH_4Cl.

Liver Perfusion Experiment with NH_4Cl:

It is evident from the results of NH_4Cl exposure experiment that the higher accumulation of ammonia *in vivo* which took place in different tissues is one of the major reasons of stimulation of ureogenesis in the walking catfish as a physiological adaptation to survive under hyper-ammonia stress. However, the threshold concentration and optimal concentration of ammonia *in vivo* needed for initiation and to cause maximum induction of urea cycle enzymes activity could not be determined from that experiment. This could be known by infusing different concentrations of NH_4Cl in the perfused liver (most ureogenic tissue) of walking catfish and monitoring the level of activity of different urea cycle enzymes and the efflux of urea-N from the perfused liver.

In this perfusion experiment, the liver, which has been reported to be the major ureogenic tissue for conversion of ammonia to urea (Saha and Ratha, 1989; Saha *et al.*, 1999) like all other ureotelic mammals and amphibia, was infused with different concentrations of NH_4Cl for 60 min by the perfusion technique to find out the role of urea cycle in detoxification of ammonia and the threshold level of ammonia loading needed to cause maximum stimulation of ureogenesis.

The results obtained in the present study clearly indicated that the higher accumulation of ammonia could be one of the major factors for the stimulation of ureogenesis at least in the liver of this catfish. No significant increase in ammonia level was observed in the perfused liver at a lower rate of NH_4Cl infusion (up to 0.85 μmol/g

liver/min) for 60 min (Table 15). The excretion of ammonia into the effluent while infusing NH_4Cl at a lower rate was also found to be less (about 30% out of the total infused), indicating that most of the ammonia infused into the liver was either converted to urea-N and/or to some non-essential amino acids (not determined in the present study). Further, out of the total ammonia taken up by the perfused liver at a lower rate (up to 1.25 μmol/g liver/min) only about 40 to 50% was converted to urea-N causing any significant change of activity of urea cycle enzymes except for ASS (Tables 17,18). The normal physiological level of activity of the urea cycle enzymes in the liver of this ureogenic teleost, possibly along with other detoxification pathways, might be sufficient to detoxify most of the ammonia that was taken up by the perfused liver causing any change of ammonia level *in vivo*. Recently, the conversion of some part of accumulated ammonia to various non-essential amino acids under high ammonia load in the perfused liver has also been reported in this walking catfish (Saha *et al.*, 2000). The maximum accumulation of ammonia of about 28 μmoles/g wet wt of tissue was observed with 5.08 μmoles/g liver/min of NH_4Cl infusion with no further increase in ammonia level in liver at a higher rate of ammonia addition (Table 15). It is, therefore, evident from this perfusion experiment that the accumulation of ammonia in the liver is a saturable process.

Both the urea-N excretion rate (Fig. 13) and the rate of uptake of ammonia (Fig. 14) by the perfused liver were also found to be a saturable process. The Vmax of urea-N excretion (0.47 μmole/g liver/min) was obtained with the addition of 5.08 μmoles/g liver/min of NH_4Cl. At a still higher rate of ammonia addition the urea-N excretion rate became independent of ammonia (substrate) load, while the Vmax of ammonia uptake (1.34 μmole/g liver/min) was obtained at a still higher rate of NH_4Cl addition (10.81 μmoles/g liver/min). It may so happen that initially at a lower ammonia load, it is the

induced urea cycle which is mainly responsible for the detoxification of ammonia, whereas, at a higher ammonia load other detoxification pathways are also actively involved in the detoxification process in addition to the induced urea cycle. It has been shown that some extra-hepatic tissues of this fish are also known to possess the activity of all or some of the urea cycle enzymes (Saha and Ratha, 1989; Saha *et al.*, 1999). It is, therefore, not possible to predict from this experiment what would be the Vmax of urea-N excretion by the whole animal under higher ammonia load except the Vmax of urea-N excretion by the perfused liver.

Significant stimulation of activity (both tissue and specific) of urea cycle enzymes except for OTC and ARG was seen with the addition of NH_4Cl at the rate of 2.32 μmoles/g liver/min, which was accompanied by the rise of tissue ammonia level from 7.64 $\pm$ 0.75 to 20.05 $\pm$ 1.85 μmoles/g wet wt within 60 min of infusion (Tables 15, 17, 18; and Figs. 15, 16). Although the level of ammonia in liver increased further at a still higher rate of NH_4Cl infusion, it did not cause any further change of activity of urea cycle enzymes except for CPS, whose activity increased till the addition of 5.08 μmoles/g liver/min of NH_4Cl. Therefore, these results indicated that an increase of 10-15 μmoles/g wet wt of hepatic ammonia from its physiological level is possibly necessary to achieve any significant and/or maximum stimulation of activity of urea cycle enzymes in this walking catfish. Whereas, in the case of singhi catfish an increase of 3-5 μmoles/g wet wt in the hepatic tissue was sufficient to cause any significant change of activity of urea cycle enzymes (Saha *et al.*, 1995). In the case of ureotelic alkaline-lake Magadi tilapia, a 3-fold increase of urea excretion was reported on exposure to 0.5 mM NH_4Cl, without causing any significant induction of urea cycle and some other ammonia detoxifying enzymes (Wood *et al.*, 1989; Walsh *et al.*, 1993). In this case, possibly the accumulation of ammonia *in vivo*

might not have reached the threshold level to cause any significant induction of urea cycle enzymes activity, as has been noticed in the liver of *C. batrachus* infused with a lower rate of NH_4Cl. The increase in the rate of urea-N by the perfused liver of walking catfish at a lower rate of NH_4Cl infusion and by the tilapia (whole fish) exposed to NH_4Cl were mainly because of high physiological activity of urea cycle enzymes. In contrast, when a purely ammoniotelic teleost, the largemouth bass (*M. salmoides*), where the levels of activity of various urea cycle enzymes are very low, was exposed to 0.25 and 1 mM of NH_4Cl, neither the change of urea-N excretion nor any change of urea cycle enzymes activity was observed (Kong *et al.*, 1998).

Thus, from the results of this perfusion experiment, it is evident once again that the higher accumulation of ammonia *in vivo* under hyper-ammonia stress is one of the major reasons for stimulation of ureogenesis by inducing the activity of some key enzymes of urea cycle in this walking catfish. Other possible mechanism(s) of stimulation of ureogenesis under hyper-ammonia stress have already been mentioned above in the NH_4Cl exposure experiment. The physiological level of activity of OTC and ARG as such is high in this fish and even the highest ammonia load in the perfused liver possibly did not have any stimulatory effect on these two enzymes of the urea cycle for enhanced ureogenesis.

Increase of pH in the external water was reported to stimulate the ureogenesis in Lahontan cutthroat, *O. c. henshawi* (Wilkie *et al*, 1993), in tilapia, *Oreochromis nilotica* (Wright, 1993), and in rainbow trout, *O. mykiss* (Wilkie and Wood, 1991). However, in the present study, while perfusing the liver of walking catfish, the pH of the media containing different concentrations of NH_4Cl were kept constant (pH 7.5). Therefore, the stimulation of ureogenesis under higher ammonia load noticed herein cannot be considered the effect of pH change of the media. But, the intracellular pH (pHi) rising because of

intracellular ammonia penetration was not taken into account in the present study and this might be another or an additional cause of induction of ureogenesis. In ureogenic toadfish, *O. beta* stimulation of ureogenesis was also reported while exposing to higher ambient ammonia and also to exposure to air for extended periods of time (Walsh *et al.*, 1994b), and the reason for this stimulation was concluded later to be due to stress related secretion of cortisol (Hopkins *et al.*, 1995). In our walking catfish, however, it is evident from the perfusion experiment that higher ammonia load in liver beyond a threshold level can cause stimulation of ureogenesis via the induced urea cycle. Presence of such physiological adaptive strategy is probably necessary in this unique group of air-breathing walking catfish to survive under hyper-ammonia stress in their normal habitat or while living outside water or while burrowing inside mud.

Adaptation to Dehydration Stress:

One unique adaptation in Indian air-breathing walking catfish is the presence of accessory air-breathing structures which helps them to obtain sufficient oxygen supply while living in semidry conditions, possibly by increasing the ventilation of the air-breathing organs (Boutilier, 1990). These air-breathing structures are thought to have evolved as an adaptation to hypoxic conditions during severe periodic droughts (Johansen, 1970; Randall *et al.*, 1981). In addition, another major problem faced while living in a water restricted environment especially in summer, is to detoxify ammonia, since ammonia excretion by the gills is not possible without the availability of sufficient water in the surrounding habitat (Campbell, 1991). However, the possibility of excretion of ammonia via other routes, albeit at a reduced rate, like by ammonia volatilization, through the skin or through renal sources

still remains open in fishes living in water restricted condition (for review, see Saha and Ratha, 1998). Another unique characteristics of this air-breathing fish with respect to nitrogen metabolism is the presence of a functional urea cycle (Saha and Ratha, 1989). Further, this catfish is found to tolerate a very high ambient ammonia concentration (present study). Also, it has been found in the present study that this fish turns towards ureotelism from ammoniotelism under hyper-ammonia stress. Similar observations were also made in another air-breathing fish, the singhi catfish (*H. fossilis*) under hyper-ammonia stress (Saha and Ratha, 1990, 1994; Saha *et al.*, 1995). Various workers have investigated the role of ureogenesis in avoiding the accumulation of endogenously produced ammonia to lethal concentrations in some amphibious fishes, such as in mudskippers, *P. cantonensis*, *B. pectinirostris* (Morii *et al.*, 1978, 1979), amphibious teleosts, *B. pholis* (Davenport and Sayer, 1986), and recently in marble goby, *O. marmoratus* (Jow *et al.*, 1999) while exposed to air for short periods of time. Ureogenesis does not appear to play any significant role in detoxification of ammonia in any of these species. However, a shift towards ureotelism in three Indian air-breathing teleosts, *H. fossilis* (Ratha *et al.*, 1995), *Anabas scandens* and *C. gachua* (Ramaswamy and Reddy, 1983), and in two mudskippers, *P. sobrinus* and *P. cantonensis* (Gordon *et al.*, 1969, 1978) while exposed to air for a shorter period have been reported.

Complete suppression of nitrogen excretion was reported during aerial exposure of mudskippers (Gordon *et al.*, 1978; Morii *et al.*, 1978). Excretion of small quantity of ammonia along with secreted mucous has been reported in a marine air-breathing fish, *B. pholis* during aerial exposure (Davenport and Sayer, 1986). Copious amount of mucous was also secreted by the walking catfish, which might had helped in the excretion of small quantity of ammonia and urea-N by this fish during water deprivation (Tables 19, 20; Fig

17). When the fish was re-immersed back in water after various periods of aerial exposure, there was sharp increase in both ammonia and urea-N excretion, followed by a gradual decrease.The rate of ammonia excretion returned almost to the normal level after 12 hr of re-immersion. Whereas the rate of urea-N excretion remained significantly higher (about 2.5-fold higher than the control), which initialy increased by about 8.5-fold, even after 12 hr of re- immersion. In control fishes the ratio of ammonia : urea-N was about 8.5. When re-immersed in water, the ratio changed to about 2, 0.5 and 0.4 within 0 to 0.5 hr of re-immersion after keeping the fish outside water for 12, 24 and 48 hr, respectively. Even after 12 hr of re-immersion, the ratio of ammonia : Urea-N excretion remained lower (about 1.4 to 3.75 -fold) than the control fishes. This results indicated that ureogenesis was stimulated in the walking catfish kept outside water for different periods. This shift in nitrogen excretion pattern was accompanied by stimulation of activity of three key enzymes of urea cycle such as CPS, ASS and ASL both in liver and kidney by about 2 to 3-fold (Tables 23-26; Figs.20-24). The activity of OTC and ARG enzymes are as such very high in this fish. Therefore, possibly there was no need of stimulation of activity of these two enzymes during aerial exposure. Another important enzyme related to urea synthesis in fish is GS, whose activity was also found to stimulate by about 3-fold both in liver and kidney of this catfish when exposed to air (Dutta and Saha, unpublished observation). Increase in GS activity, the enzyme believed to be responsible for trapping of ammonia for urea synthesis in fish via the urea cycle (Campbell and Anderson, 1991; Mommsen and Walsh, 1991; Anderson, 1995a) could be a critical step in this fish in the transition from ammoniotely to ureotely while confined to water restricted condition

The south African aquatic toad, *X. laevis,* also faces a similar problem of periodic droughts and survive by aestivating in the mud peat (Alexander and Bellerby, 1938; Rose,

1950). A transition from ammoniotelism to ureotelism in *X. laevis* with a very high accumulation of urea during aestivation or while living in the moist peat has been reported by various workers (Balinsky, 1981; Janssens and Cohen, 1968). This transition to ureotelism was also accompanied by 5-fold increase in the activity of CPS in the liver of *X. laevis* (Janssens and Cohen, 1968). The aestivating lungfish, *Protopterus* has also been reported to accumulate large quantities of urea by converting the accumulated ammonia to urea via the urea cycle during aestivation (Forster and Goldstein, 1966; Janssens and Cohen, 1968). However, these workers could not find any elevation of CPS and other urea cycle enzymes activity in *Protopterus* liver and concluded that the urea cycle probably continues to operate at the normal rate during aestivation, since the physiological level of activity of urea cycle enzymes as such is high.

There was significant accumulation of both ammonia and urea in all tissues (except ammonia in brain) and in plasma of walking catfish having maximum accumulation in the fish kept outside water for 48 hr (Tables 21, 22; Figs.18, 19). Brain, being a highly sensitive tissue, is apparently adapted to maintaining low ammonia concentrations due to having the high activity of GDH (ammonia utilizing direction) and GS in this tissue (Saha *et al.*, 1999, unpublished observations by Dutta and Saha).

The higher accumulation of urea in different tissues in walking catfish living outside water could represent a means to remain hydrated in an other wise dehydrating environment, causing only a little change in muscle water content from 80 ± 0.95% to 78 ± 0.82% (n = 5), since increased urea concentrations in the body would unable the fish to prevent excessive water loss as suggested in various amphibians (for review, see Jorgensen, 1997). However, there was little increase of blood osmolarity (increased form 265 ±3 to 276 ± 5 mOsmol/l, (n = 5), determined by the freezing point depression method with a

Camlab osmometer) in the walking catfish kept outside water. Therefore, it appears that the enhanced production of urea under water restricted conditions in this catfish served primarily to avoid the accumulation of ammonia to a toxic level.

The hypothesis has been put toward explaining the regulation of ureogenesis during exposure to higher ambient ammonia in this walking catfish (mentioned above) (i.e., higher accumulation of ammonia, which results from reduced excretion due to lack of water, acts as a modulator for stimulation of ureogenesis) probably also holds good during exposure to air or while they are living in water restricted conditions during the drought season. However, with the available data the real mechanism(s) of stimulation of ureogenesis in these air-breathing fish under hyper-ammonia stress is not clear and should be studied in details at the molecular level including the purification and characterization of all the urea cycle enzymes, and the abundance of mRNA level of individual enzymes under hyper-ammonia stress

In addition to the stimulation of ureogenesis, there could be other means of detoxification of ammonia by the walking catfish living in water restricted condition, such as the conversion of endogenously produced ammonia to various non-essential free amino acids (FAAs). Enhanced synthesis and accumulation of non-essential FAAs have recently been observed in the perfused liver of this walking catfish under higher ammonia load (Saha *et al.*, 2000). Such an adaptation has been reported in the mudskipper (*P. cantonensis*) (Iwata *et al.*, 1981) and in marble goby (*O. marmoratus*) during exposure to air (Jow *et al.*, 1999). Thus, one has also to look into the role of amino acid metabolism in this walking catfish under condition of water shortage.

In conclusion, it appears that this air-breathing walking catfish is able to manage the problem of accumulation of ammonia to a toxic level while living in water restricted

conditions especially during summer. This it may do so by stimulating the synthesis of urea via the induced urea cycle from the accumulated ammonia *in vivo* as one of the physiological adaptations enabling them to survive for months inside the moist peat in a water restricted condition.

Exposure to Alkaline Water :

The walking catfish survived well at alkaline water of pH 10 for several days without any mortality except for initial violent escape attempt. This was an indication that this catfish has the capacity to adapt in this stressful condition by tuning certain key metabolic pathways. One of the important pathways which may be affected by alkalinity is the nitrogen metabolic pathway , which would also result in certain changes in nitrogen excretion pattern. When the walking catfish was exposed to alkaline water (pH 10), there was an initial suppression of ammonia excretion, followed by gradual recovery to the control level from 5th day onwards (Table 27; Fig. 25). Similar observations were also made in rainbow trout *(O. mykiss)* (Wilkie and Wood, 1991; Wilson *et al.*, 1998) and in Lahontan cutthroat trout (*O. c. henshawi*) (Wilkie *et al.*, 1994) when exposed to water above pH 9 and many other freshwater teleosts (for review, see Wilkie and Wood, 1996) In most fish it seems that ammonia is excreted by passive diffusion of NH_3 across the gills according to its partial pressure gradient (Cameron and Heisler, 1983; Wilson *et al* , 1994, Wilkie, 1997). The gradient may be enhanced by an acid boundary layer at the water interface which traps NH_4^+ (Wright *et al.*, 1989; Wilson *et al.*, 1994)

Initial decrease of ammonia excretion rate by walking catfish upon exposure to alkaline water was possibly due to elevation of gill water resulting to decrease of H^+

concentration which shifted the $NH_3^+ \rightleftharpoons NH_4^+$ equilibrium towards NH_3 formation The resultant increase in gill water P_{NH_3} reduced ΔP_{NH_3} leading to decrease in ammonia excretion as suggested by Wilkie and Wood (1996) in their hypothetical model (Fig 2) The decrease in ammonia excretion was accompanied by significant increase of ammonia level in plasma and various tissues of walking catfish on the third day of exposure (Table 29, Figs. 26, 28). But, when the ammonia excretion almost returned to the normal level from 5th day onwards, tissue level of ammonia also decreased to some extent on the 7th day of exposure although it remained significantly higher than the control In plasma, of course, the level was quite high even on the 7th day favouring better clearance of ammonia through gill in favour of ΔNH_3.

In contrast to ammonia excretion, there was significant increase in urea-N excretion by the walking catfish immediately after exposure to alkaline water, having a maximum increase on the 3rd day (Table 28, Fig 25), followed by a gradual decrease at later stages of exposure. This was also accompanied by significant induction of activity of key enzymes of urea cycle on the 3rd day of exposure both in liver and kidney (Table 31-34, Figs 29-33) indicating that the urea synthesis rate was stimulated initially when the fish was exposed to alkaline water possibly due to higher accumulation of ammonia *in vivo* This also led to significant accumulation of urea in different tissues and in plasma (Table 30, Figs. 27, 28). Similar increase in urea-N excretion was also observed in Lahontan cutthroat (Wilkie *et al.*, 1993) and in rainbow trout (Wilkie and Wood, 1991) when exposed to alkaline water (above pH 9) Restoration of normal rate of ammonia excretion after the 5th day coincided with the returning of urea-N excretion to the normal level, indicating possibly there was no need of extra synthesis of urea-N at later stages, since the fish

already did overcome the danger of extra accumulation of ammonia to a toxic level. Therefore, no further accumulation of ammonia in different tissues was observed at later stages of exposure. The initial stimulation of activity of different key enzymes of urea cycle tackled the problem of ammonia toxicity, followed by returning back to its original level of activity at later stages. In Lahontan cutthroat trout and rainbow trout, the initial increase of urea-N excretion, which was observed during exposure to alkaline water, was suggested to be mainly contributed by the uricolytic pathway, since in both the fishes the presence of significant level of activity of all the three uricolytic enzymes- uricase, allantoinase and allantoicase were reported with a barely detectable level of activity of CPS III, the key regulatory enzyme of urea cycle (Wilkie and Wood, 1991; Wilkie *et al.*, 1993, 1994). Most interesting case was of lake Magadi tilapia (*O. a. grahami*), which lives successfully throughout its life cycle in highly alkaline lake, excretes most of its nitrogenous wastes as urea-N and has a full complement of urea cycle (Randall *et al.*, 1989; Wood *et al.*, 1989). The occurrence of uricolytic pathway in walking catfish has not yet been studied, but it appears that like the lake Magadi tilapia active urea cycle plays a vital role in this fish for detoxification of accumulated ammonia under alkaline stress.

Clearly, another key adaptation that would normally benefit fish living at high pH would be unusually high tolerance to ammonia. This walking catfish has been found to tolerate an exceptionally high ambient ammonia and also can allow accumulation of high ammonia at a level much higher than many other teleosts. (see NH_4Cl exposure experiment in the present study). Therefore, no mortality of fish was observed when the walking catfish was exposed to highly alkaline water (pH 10).

The mechanism of initial stimulation of ureogenesis in this walking catfish while exposed to alkaline water appears to be same as explained for hyper-ammonia stress (i.e.,

decrease in ammonia excretion resulted to higher accumulation of ammonia *in vivo* resulting to stimulation of certain key enzymes of urea cycle serving ammonia as a regulatory molecule). Increase in blood pH, which might have occurred in alkaline water, could be another factor for stimulation of ureogenesis. However, one has to study in detail the real mechanism of stimulation of ureogenesis in alkaline water before drawing any definite conclusion.

CONCLUSION

Conclusion:

The ureogenic air-breathing walking catfish (*Clarias batrachus)* possesses both types of urea cycle-related carbamyl phosphate synthetases (CPSs), CPS III (teleost type) and CPS I-like (amphibian and mammalian type) in various tissues, which is unique since these two types of CPSs together is normally not present in a single vertebrate species Due to having both types of urea cycle-related CPS activities in this ureogenic walking catfish, conversion of toxic ammonia to urea efficiently via the urea cycle might have become possible. This may also have some evolutionary significance with relation to evolution of urea cycle in vertebrate species. The pyrimidine synthesis-related CPS II was also found to be present in various tissues of the walking catfish. Like all other vertebrates, the urea cycle- related CPS (CPS III and CPS I-like) and OTC enzymes are located inside the mitochondria ,and unlike other ureotelic vertebrates ARG is also located inside the mitochondria. The two other enzymes of urea cycle , ASS and ASL are located in the cytosol. The walking catfish is found to tolerate very high ambient ammonia (up to 75 mM NH_4Cl), which is much higher than any other teleosts. A transition from ammoniotelism to ureotelism is possible in this fish under hyper-ammonia stress and also when exposed to aerial habitat by stimulating the activity of certain key enzymes of urea cycle as a sort of physiological adaptation. Initial stimulation of ureogenesis, the period when it needs to set up a new equilibrium for normal excretion of ammonia, also takes place in this fish to avoid the ammonia toxicity when exposed to alkaline water (pH 10). It is hypothesized that the higher accumulation of ammonia *in vivo*, which takes place in this walking catfish under various environmental stresses, is one of the major factors for stimulation of ureogenesis mainly to avoid the ammonia toxicity. However, elucidation of the roles of other factors

in ureogenesis under various environmental stresses in this walking catfish along with the purification and characterization of all the urea cycle enzymes, and also studies on the abundance of mRNAs for all the urea cycle enzymes are probably necessary to draw a definite conclusion about the real mechanism(s) of stimulation of ureogenesis.

However, it is evident from the present study that the Indian air-breathing freshwater teleosts possess various physiological adaptations related to nitrogen metabolism, in addition to other physiological and morphological modifications which might have taken place in the process of evolution under various environmental constraints faced by them in their normal habitat.

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BIO-DATA

1. **Name** : **Ms.Lipika Das**

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(i) **Saha, N., Das, L. and Dutta, S. (1999) Types of carbamyl phosphate synthetases and subcellular localization of urea cycle and related enzymes in air-breathing walking catfish, *Clarias batrachus*. *Journal of Experimental Zoology*, 283:121-130.**

(ii) 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 Physiolology and Biochemistry* 21: 303-311.

(iii) Saha N. , Das L., Dutta, S. and Goswami, U.C. (2000) Role of ureogenesis in the mud-dwelled air-breathing singhi catfish (*Heteropneustes fossilis*) under conditions of water shortage. *Comparative Biochemistry and Physiology* (in press).

10. Papers presented in Seminars/ Symposia

(i) Effect of alkalinity on ureogenesis in a freshwater air-breathing teleost, *Clarias batrachus.*
National Seminar on Environmental Biology and Fish Biology, Visva Bharati University, Santiniketan, April, 1999.

(ii) Stimulation of ureogenesis in the perfused liver of an Indian air-breathing catfish, *Clarias batrachus* infused with different concentrations of ammonium chloride.
National Seminar on Trends in Environmental Biology, North-Eastern Hill University, Shillong, June, 1999.

Fish Physiology and Biochemistry **21:** 303–311 1999

Stimulation of ureogenesis in the perfused liver of an Indian air-breathing catfish, *Clarias batrachus*, infused with different concentrations of ammonium chloride

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Abstract

The pattern of changes of activity of the urea cycle enzymes and the rate of urea-N excretion were studied in the perfused liver of an Indian air-breathing ureogenic walking catfish, *Clarias batrachus* The liver was perfused with different concentrations of NH_4Cl for a period of 60 min to determine the role of ammonia for stimulation of hepatic ureogenesis and the threshold level of ammonia loading needed to cause such stimulation Both the urea-N excretion and the ammonia uptake by the perfused liver were found to be a saturable process Ammonia accumulated significantly in the liver infused with 1 25 μmoles g liver $^{-1}$ min^{-1} of NH_4Cl, followed by a maximum accumulation of about 28 5 μmoles g wet wt^{-1} with the infusion of 5 08 μmoles g liver^{-1} min^{-1} The Vmax of the urea-N excretion (0 47 μmol g liver^{-1} min^{-1}) was obtained with the addition of 5 08 μmoles g liver^{-1} min^{-1} of NH_4Cl Both the tissue and the specific activity of the urea cycle enzymes, except ornithine transcarbamylase and arginase, were stimulated significantly with the infusion of either 1 25 or 5 08 μmoles g liver^{-1} min^{-1} of NH_4Cl Maximum stimulation of tissue activity of carbamoyl phosphate synthetase (about 120%) was seen with the infusion of 5 08 μmol g liver^{-1} min^{-1}, and for argininosuccinate synthetase (about 135%), and argininosuccinate lyase (about 50%) with the infusion of 10 81 μmol g liver^{-1} min^{-1} of NH_4Cl Higher accumulation of ammonia of about 10–15 μmol g wet wt^{-1} from the physiological level in the perfused liver while infusing with NH_4Cl was suggested to be one of the major causes of stimulation of ureogenesis The presence of such physiological adaptive strategy is probably necessary in this unique group of air-breathing walking catfish to survive under hyper-ammonia stress in their normal habitat or while living outside water or while burrowing inside mud

Introduction

Nitrogen metabolism is considered as one of the most sensitive physiological systems showing adaptive changes in response to environmental variations Accordingly, the nature of nitrogen excretory products in animals has changed with the evolution of vertebrates from water to the land habitat (Campell 1991, Wright 1995) Most teleostean fishes excrete ammonia as the major nitrogenous end product in response to their aquatic habitat (Randall and Wright 1987, Campbell 1991, Wood 1993, Saha and Ratha 1998) However, in special circumstances such as high ambient ammonia or aerial exposure, fish can hardly excrete ammonia, and toxic ammonia is concentrated in blood and body tissues Fishes are generally known to tolerate relatively higher accumulation of ammonia than mammals Plasma total ammonia ($NH_3 + NH_4^+$) normally remains between 0 05 to 2 mM in most teleost fishes (Campbell and Anderson 1991, Wood 1993) In contrast, blood ammonia levels greater than 0 05 mM can be toxic to the central nervous system of most mammals (Meijer et al 1990) It is, therefore, interesting to study the different mechanism(s) of management of accumulated ammonia especially in

those teleosts that are faced regularly with ammonia loading situations in normal part of their life cycle

Although the majority of teleost fishes are ammoniotelic, urea also constitutes about 10–30% of the total nitrogenous wastes in most species studied (Wood 1993, Saha and Ratha 1998) However, the sources of urea in teleosts and the involvement of the ornithine-urea cycle (hereafter referred to as urea cycle) is still not very clear A few species of teleost fish, such as the alkaline lake adapted tilapia, *Oreochromis alcalicus grahami* (Randall et al 1989), the marine toadfishes, *Opsanus tau* and *Opsanus beta* (Read 1971, Mommsen and Walsh 1989), and some Indian air-breathing teleosts (Saha and Ratha 1987,1989), have the enzymes required for a functional urea cycle Accordingly, interests in the study of the urea cycle, the expression of the urea cycle during early embryonic developmental stages, regulation of expression of the urea cycle enzymes, and nitrogen excretion patterns under different environmental constraints in different teleosts have recently increased

Several species of freshwater air-breathing teleosts exist in the Indian subcontinent They usually inhabit stagnant, slow flowing swampy water bodies or wet lands, that are usually unhabitable to purely aquatic fishes such as carps These swamps, which are often covered with macrovegetation such as water hyacinth, are characterized by a low dissolved oxygen, more free carbon dioxide and high ammonia levels (for review, see Saha and Ratha 1998) During summer, when the swamps dry up, they face more adverse ecological conditions and most of the air-breathing fishes burrow inside mud to avoid total dehydration Two of these air-breathing species, the singhi catfish (*Heteropneustes fossilis*) and the walking catfish (*Clarias batrachus*), have been reported to tolerate and survive well at a very high concentrations (up to 50–75 mM) of ammonium chloride (Saha and Ratha 1986, 1990, 1994, unpublished observations by Saha and Das), and this tolerance limit of external ammonia has been found to be much higher than any other teleost fish (Olson and Fromm 1971, Thurston et al 1981, Dabrowska and Wlasow 1986), including mudskippers (Iwata 1988) reported to date Although the presence of a functional urea cycle was reported in some of these amphibious fish species from the Indian subcontinent (Saha and Ratha 1987, 1989), the possibility of the regulation of ureogenesis under environmental constraints was not studied in any of these species except, the singhi catfish (*H fossilis*) under hyper-ammonia stress (Saha and Ratha 1990, 1994, Saha et al 1995, Ratha et al 1995) The present study was undertaken to find out the role of urea cycle for conversion of accumulated ammonia to urea under hyper-ammonia stress especially in the liver (most ureogenic tissue) of another unique amphibious fish from India, the walking catfish *(C batrachus)* by infusing different concentrations of NH_4Cl, and determining the level of ammonia accumulation needed to cause the stimulation of ureogenesis

Materials and methods

Animals

The fish, *Clarias batrachus*, weighing 50–80 g body wt (both sexes), were purchased from commercial sources and acclimatized in the laboratory for 4–6 weeks at about 30 °C with 12 h 12 h light and dark period before being used for experiments Minced pork liver and rice bran (5% of the body wt) was given as food, and the water, which was collected from a natural stream, was changed on alternate days

Liver perfusion technique

The fish were decapitated before operation for liver perfusion Livers were perfused via the portal vein in a non-circulating manner with haemoglobin-free media as used by French et al (1981) and with certain modifications made by Saha et al (1995) The isotonic media (265 mOsmol l^{-1}, determined by freezing point depression method) contained 119 mM NaCl, 5 mM KCl, 0 35 mM Na_2HPO_4, 0 44 mM KH_2PO_4, 0 81 mM $MgSO_4$ and 1 25 mM $CaCl_2$ as a basic solution The medium also contained 5 mM L-glucose and 2 mM L-ornithine Different concentrations of NH_4Cl (0 05–2 mM) were infused along with the perfusion media to study the effect of ammonia on the urea cycle enzymes activity, its accumulation rate and also its conversion to urea in the perfused liver The medium was gassed with O_2/CO_2 (99 1, v/v) before infusing into the liver at a flow rate of 4–5 ml g $liver^{-1}$ min^{-1} The pH of the medium was always maintained at 7 5 after gassing The temperature of the medium was 30 °C The effluent coming out of the perfused liver was collected through a cannula inserted at the superior vena cava at 2 min intervals for analysis of ammonia and urea-N

Livers were perfused for 20 min with the standard isotonic medium, followed by infusion of ammonium

chloride for 60 min Immediately after 60 min of infusion of NH_4Cl, the perfused liver was removed from the body, plunged into liquid nitrogen and stored at −60 °C until used for measurement of the urea cycle enzymes activity and the tissue ammonia level Another set of liver was perfused with isotonic solution containing only 5 mM glucose and 2 mM ornithine for 80 min and treated as control All the enzyme assays and tissue ammonia estimation were completed within 2–3 days of preserving the tissue

Estimations

Concentrations of ammonia and urea-N in the effluent were measured enzymatically (Kun and Kearney 1974), the details of which were mentioned earlier by Saha et al (1995) The effluent collected at different time intervals was deproteinized first by adding 20 μl of 2 M perchloric acid (PCA) to each 1 ml sample, centrifuged to precipitate out the protein and the supernatant was neutralized later by adding 20 μl of 2N NaOH to each tube before the measurement of ammonia and urea-N Ammonia level in the perfused liver was also measured by the same enzymatic method as mentioned above after processing the tissue (Saha and Ratha 1989)

Enzyme assay

A ten percent homogenate (w/v) of the liver tissue was prepared in 0 1% cetyltrimethyl ammonium bromide (CTB) for assaying the activities of the urea cycle enzymes (Brown and Cohen 1959) The homogenate was centrifuged at 600× g at 0 ± 2 °C for 15 min The supernatant was used for enzyme assays The five enzymes of the urea cycle such as carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) were assayed following the method mentioned earlier in detail (Saha et al 1995) This time, however, an extra 1 mM of uridine-5′-triphosphate (UTP) was added in the reaction mixture for the assay of urea synthesis-related CPS activity mainly to inhibit the pyrimidine synthesis-related CPS II activity which might interfere in the assay method It should be noted that the assay method used here for CPS activity does not distinguish between the two different forms of urea synthesis-related enzymes namely CPS I (ammonia- and N-acetyl-L-glutamate-dependent, mitochondrial) and CPS III (glutamine- and N-acetyl-L-glutamate-dependent, mitochondrial) The reaction for all the enzymes was stopped by adding 10% PCA in 1 0 5 ratio after a specific time of reaction, and centrifuged to precipitate out the protein Citrulline formed in the case of CPS and OTC, citrulline used in the case of ASS, and urea formed in the case of ASL and ARG were measured colorimetrically in the supernatant (Moore and Kauffman 1970) and expressed as enzyme activity All the enzyme assays were carried out at 30 °C One unit of enzyme activity was defined as that amount that catalyzed 1 μmol of product formed or substrate used per h at 30 °C The enzyme activity was expressed as both tissue (U g wet wt of tissue^{-1}) and specific (U mg protein^{-1}) activity Protein was estimated following the method of Lowry et al (1951) using crystalline bovine serum albumin as the standard

Data collected from three to four replicates were statistically analysed and presented as mean ± SEM Comparison of the paired mean values was made using Student's *t*-test (Croxton et al 1982) and $P > 0\ 05$ was taken as non-significant

Chemicals

All enzymes, co-enzymes, substrates, CTB and bovine serum albumin were obtained either from Sigma Chemical Co (St Louis, USA) or Boehringer Mannheim (Mannheim, Germany), and other chemicals used were of analytical grades obtained from indigenous sources Deionized double-glass-distilled water was used in all preparations

Results

Changes of ammonia level in the liver perfused with different concentrations of NH_4Cl

The changes in ammonia level in the liver perfused with different concentrations of NH_4Cl are shown in Table 1 No significant increase of ammonia level in the perfused liver was observed until increased the addition of 1 25 μmol g liver^{-1} min^{-1} of NH_4Cl The ammonia level in the liver raised from 7 64 to about 28 5 μmol g wet wt^{-1} of liver with the infusion of 5 08 μmol g liver^{-1} min^{-1} of NH_4Cl, followed by no further increase at a higher rate of infusion

Formation of urea-N in the perfused liver

The rate of urea-N released into the effluent by the perfused liver while infusing NH_4Cl at different rates

Table 1 Alteration of ammonia level (μmol g wet wt^{-1}) in the perfused liver of *C batrachus* infused with different concentrations of NH_4Cl Values are expressed as mean ± SEM (n = 3–4)

Ammonia infusion rate (μmol g liver^{-1} min^{-1})	Ammonia level in the perfused liver (μmol g wet wt^{-1})	p
0 (control)	7 64 ± 0 75	
0 4 ± 0 04	8 84 ± 0 66 (16)	N S
0 85 ± 0 09	10 45 ± 0 82 (37)	N S
1 25 ± 0 11	14 50 ± 1 02 (90)	<0 05
2 32 ± 0 26	20 05 ± 1 85 (162)	<0 001
5 08 ± 0 38	28 45 ± 2 24 (272)	<0 001
10 81 ± 0 81	28 65 ± 2 55 (275)	<0 001

Per cent increase of ammonia level with relation to control are given in parentheses NH_4Cl infusion at the rate of 5 μmol g liver^{-1} min^{-1} corresponds to 1 mM of NH_4Cl N S – not significant

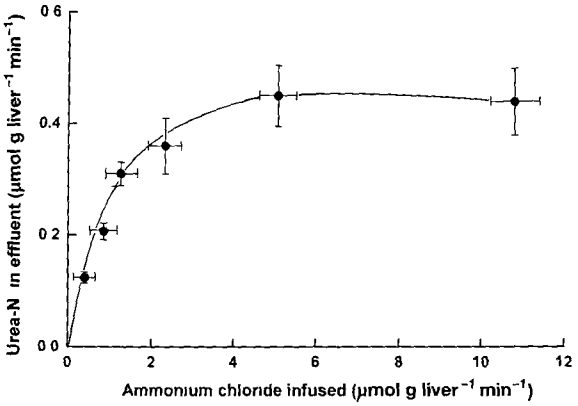

Figure 1. Urea-N excretion (μmol g liver^{-1} min^{-1}) in the effluent by the perfused liver of *C batrachus* infused with different concentrations of NH_4Cl Values were obtained between 50 and 60 min of infusion when the urea-N excretion already reached a steady state and are plotted as mean ± SEM (n = 3–4) NH_4Cl infusion of 5 μmol g liver^{-1} min^{-1} corresponds to 1 mM of NH_4Cl

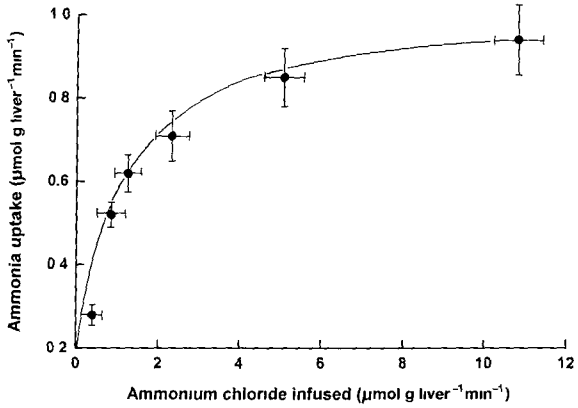

Figure 2 Ammonia uptake (μmol g liver^{-1} min^{-1}) by the perfused liver of *C batrachus* infused with different concentrations of NH_4Cl Values were obtained between 50 and 60 min of infusion when the ammonia uptake already reached a steady state and are plotted as mean ± SEM (n = 3–4) NH_4Cl infusion of 5 μmol g liver^{-1} min^{-1} corresponds to 1 mM of NH_4Cl

was found to be a saturable process (Figure 1). Initially at a lower rate of infusion of NH_4Cl, that is, up to 1.25 μmol g liver^{-1} min^{-1}, about 40–50% of the total ammonia taken up by the liver was converted to urea-N. Hence, the rate of urea-N excreted increased with the increase of infusion of ammonium chloride until it reached a Vmax of 0.47 μmol g liver^{-1} min^{-1} with the infusion rate of 5.08 μmol g liver^{-1} min^{-1}, followed by no further increase of urea-N excretion at a still higher infusion rate.

Uptake of ammonia by the perfused liver

The uptake of ammonia by the perfused liver, which was calculated by subtracting the amount of ammonia that was coming out into the effluent from the total ammonia infused in the liver, is shown in Figure 2. Initially up to the addition of 1.25 μmol g liver^{-1} min^{-1}, about 50–70% of the total ammonia infused were taken up by the liver. At higher rates of ammonia addition, the percentage uptake of ammonia gradually decreased. The rate of uptake of ammonia also appeared to be a saturable process reaching a Vmax of 1.34 μmol g liver^{-1} min^{-1} with the infusion of 10.81 μmol g liver^{-1} min^{-1} of NH_4Cl for 60 min (Figure 2). However, the Vmax of urea-N excretion was only 0.47 μmol g liver^{-1} min^{-1} indicating that the rate of uptake of ammonia was higher (about 3 fold) than the rate of urea-N excretion.

Table 2 Changes in the tissue (units g wet wt $^{-1}$) and the specific (units mg protein $^{-1}$) activity of the urea cycle enzymes in the perfused liver of *C batrachus* infused with different concentrations of NH_4Cl Values are expressed as mean ± SEM (n = 3–4)

Ammonia infusion rate	CPS		OTC		ASS		ASL		ARG	
(μmol g liver $^{-1}$ min $^{-1}$)	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
0 (control)	3 92 ± 0 31	0 048 ± 0 005	190 4 ± 11 1	2 33 ± 0 45	65 8 ± 6 2	0 8 ± 0 1	100 3 ± 7 2	1 23 ± 0 14	4035 ± 212	49 4 ± 5 4
0 85 ± 0 09	4 29 ± 0 44	0 05 ± 0 004	187 6 ± 15 2	2 16 ± 0 32	74 3 ± 4 5	0 8 ± 0 08	97 3 ± 6 5	1 12 ± 0 08	4132 ± 224	47 7 ± 4 2
1 25 ± 0 11	5 68 ± 0 56	0 067 ± 0 004	218 2 ± 12 2	2 57 ± 0 28	130 3 ± 6 1[a]	1 5 ± 0 7[a]	122 4 ± 11 4	1 44 ± 0 1	3945 ± 191	46 4 ± 5 7
2 32 ± 0 26	6 45 ± 0 55[a]	0 08 ± 0 006[a]	212 6 ± 14 5	2 54 ± 0 44	152 4 ± 7 2[b]	1 9 ± 0 1[b]	147 6 ± 8 2[a]	1 85 ± 0 1[a]	4345 ± 191	54 1 ± 6 1
5 08 ± 0 38	8 68 ± 0 71[b]	0 11 ± 0 02[b]	212 6 ± 14 5	2 78 ± 0 34	148 5 ± 8 5[b]	1 9 ± 0 1[c]	140 8 ± 6 2[a]	1 84 ± 0 1[a]	4402 ± 224	57 5 ± 4 8
10 81 ± 0 81	8 59 ± 0 6[b]	0 11 ± 0 6[b]	219 3 ± 11 2	2 70 ± 0 22	153 6 ± 6 4[c]	1 9 ± 0 1[c]	151 3 ± 6 4[a]	1 88 ± 0 1[a]	4378 ± 237	54 5 ± 4 2

1 unit of enzyme activity was defined as the amount that catalyzed 1 μmol of product formed or substrate used per h at 30 °C [a, b] and [c] p values significant at <0 05 <0 01 and <0 005 respectively CPS – carbamoyl phosphate synthetase OTC – ornithine transcarbamylase ASS – argininosuccinate synthetase ASL – argininosuccinate lyase and ARG – arginase

Pattern of changes of activity of urea cycle enzymes in the perfused liver

Pattern of changes of both the tissue and the specific activity of the urea cycle enzymes in the liver infused with different concentrations of NH_4Cl are shown in Table 2 At lower rates of ammonium chloride infusion, that is, up to 1 25 μmol g liver^{-1} min ^{1}, no significant increase of the tissue activity of any of the urea cycle enzymes were observed except for ASS, the activity of which increased significantly ($p < 0$ 05) even at 1 25 μmol g liver^{-1} min ^{1} of NH_4Cl in fusion Significant stimulation of tissue activities of other urea cycle enzymes were seen from 2 32 μmol g liver^{-1} min^{-1} of infusion The activity of CPS was stimulated maximally from 3 92 $\pm$ 0 31 to 8 68 $\pm$ 0 71 U g wet wt^{-1} with the addition of 5 08 μmol g liver^{-1} min ^{1} of NH_4Cl, and for ASS and ASL, from 65 8 $\pm$ 6 2 to 153 6 $\pm$ 6 4 and from 100 3 $\pm$ 7 2 to 151 3 $\pm$ 6 9 U g wet wt ^{1}, respectively, with the addition of 10 81 μmol g liver ^{1} min^{-1} of NH_4Cl The activities of CPS, ASS and ASL increased maximally by 121%, 133% and 51%, respectively However, the tissue activities of OTC and ARG did not show any significant change in all the concentrations of NH_4Cl infused

Significant stimulation of the specific activity was also observed for ASS from 1 25 μmol g liver^{-1} min^{-1} of NH_4Cl addition, and for CPS and ASL, it was from 2 32 μmol g liver ^{1} min^{-1} onwards (Table 2) The specific activities rose maximally from 0 048 $\pm$ 0 005 to 0 11 $\pm$ 0 02 for CPS, 0 81 $\pm$ 0 1 to 1 94 $\pm$ 0 12 for ASS and from 1 23 $\pm$ 0 14 to 1 88 $\pm$ 0 09 U mg protein^{-1} for ASL The specific activities of CPS, ASS and ASL increased maximally by 129%, 140% and 53%, respectively Like the tissue activity, the specific activities of OTC and ARG also did not change in any of the concentrations of NH_4Cl infused

Discussion

The liver of *C batrachus* has been reported to be the major ureogenic tissue for conversion of ammonia to urea (Saha and Ratha 1989, Saha et al 1999a) as in other ureotelic mammals and amphibia Thus, instead of exposing the whole fish in higher ambient ammonia, the liver of this walking catfish was directly loaded with different concentrations of NH_4Cl for 60 min by the perfusion technique to find out its role in the detoxification of ammonia The results obtained in the present study clearly indicated that the higher accumulation of ammonia could be one of the major factors for the stimulation of ureogenesis at least in the liver of this catfish No significant increase in the ammonia level was observed in the perfused liver at a lower rate of NH_4Cl infusion (up to 0 85 μmol g liver ^{1} min^{-1}) for 60 min (Table 1) The excretion of ammonia into the effluent while infusing NH_4Cl at a lower rate was also found to be less (about 30% out of the total in fused), indicating that most of the ammonia infused into the liver was either converted to urea N and/or to some non-essential amino acids (not determined in the present study) Further, out of the total ammonia taken up by the perfused liver at a lower rate (up to 1 25 μmol g liver^{-1} min^{-1}) only about 40 to 50% was converted to urea-N causing any significant change of activity of the urea cycle enzymes except for ASS (Table 2) The normal physiological level of activity of the urea cycle enzymes in the liver of this ureogenic teleost, possibly along with other detoxification pathways, might be sufficient to detoxify most of the ammonia that was taken up by the perfused liver causing any change of ammonia level *in vivo* Recently, the conversion of some part of accumulated ammonia to various non-essential amino acids under high ammonia load in the perfused liver has also been reported in this walking catfish (Saha et al 1999b) The maximum accumulation of ammonia of about 28 μmol g wet wt ^{1} of tissue was observed with 5 08 μmol g liver^{-1} min ^{1} of NH_4Cl infusion with no further increase in ammonia level in the liver at a higher rate of ammonia addition (Table 1) It is, therefore, evident from this perfusion experiment that the accumulation of ammonia in the liver is a saturable process

Both the urea-N excretion rate (Figure 1) and the rate of uptake of ammonia (Figure 2) by the perfused liver were also found to be a saturable process The Vmax of urea N excretion (0 47 μmol g liver^{-1} min ^{1}) was obtained with the addition of 5 08 μmol g liver ^{1} min ^{1} of NH_4Cl At a still higher rate of ammonia addition the urea-N excretion rate became independent of ammonia (substrate) load, while the Vmax of ammonia uptake (1 34 μmol g liver ^{1} min^{-1}) was obtained at a still higher rate of NH_4Cl addition (10 81 μmol g liver^{-1} min^{-1}) It may so happen that initially at a lower ammonia load, it is the induced urea cycle which is mainly responsible for the detoxification of ammonia, whereas, at a higher ammonia load other detoxification pathways are also actively involved in the detoxification process in addi-

tion to the induced urea cycle It has been shown that some extrahepatic tissues of this fish are also known to possess the activity of all or some of the urea cycle enzymes (Saha and Ratha 1989, Saha et al 1999a) It is, therefore, not possible to predict from this experiment what would be the Vmax of urea-N excretion by the whole animal under higher ammonia load except the Vmax of urea-N excretion by the perfused liver

Significant stimulation of activity (both tissue and specific) of the urea cycle enzymes except for OTC and ARG was seen with the addition of NH_4Cl at the rate of 2 32 μmol g liver^{-1} min^{-1}, which was accompanied by the rise of tissue ammonia level from 7 64 $\pm$ 0 75 to 20 05 $\pm$ 1 85 μmol g wet wt^{-1} within 60 min of infusion (Tables 1 and 2) Although the level of ammonia in the liver increased further at a still higher rate of NH_4Cl infusion, it did not cause any change of activity of the urea cycle enzymes except for CPS, whose activity increased till the addition of 5 08 μmol g liver^{-1} min^{-1} of NH_4Cl (Table 2) Therefore, these results indicated that an increase of 10–15 μmol g wet wt^{-1} of hepatic ammonia from its physiological level is possibly necessary to achieve any significant and/or maximum stimulation of activity of the urea cycle enzymes in this walking catfish Whereas, in the case of singhi catfish an increase of 3–5 μmol g wet wt^{-1} in the hepatic tissue was sufficient to cause any significant change of activity of the urea cycle enzymes (Saha et al 1995) In the case of ureotelic alkaline-lake Magadi tilapia, a 3 fold increase of urea excretion was reported on exposure to 0 5 mM NH_4Cl, without causing any significant induction of the urea cycle and some other ammonia detoxifying enzymes (Wood et al 1989, Walsh et al 1993) In this case, possibly the accumulation of ammonia *in vivo* might not have reached the threshold level to cause any significant induction of the urea cycle enzymes activity, as has been noticed in the liver of *C batrachus* infused with a lower rate of NH_4Cl The increase in the rate of urea-N by the perfused liver of the walking catfish at a lower rate of NH_4Cl infusion and by the tilapia (whole fish) exposed to NH_4Cl were mainly because of high physiological activity of the urea cycle enzymes In contrast, when a purely ammoniotelic teleost, the largemouth bass (*Micropterus salmoides*), where the levels of activity of various urea cycle enzymes are very low, was exposed to 0 25 and 1 mM of NH_4Cl, neither the change of urea-N excretion nor any change of urea cycle enzymes activity was observed (Kong et al 1998)

It is difficult to explain the mechanism of stimulation of ureogenesis in the walking catfish with the available data We may, however, hypothesize the possible mechanism(s) of stimulation of ureogenesis under hyper-ammonia stress, one of which could be the more synthesis of N-acetyl glutamate (NAG) (allosteric regulator) under hyper-ammonia stress, which can regulate at least the two urea cycle-related CPS, CPS I and III, as has been shown in rat liver (Lund and Wiggins 1984), and more recently in the toadfish (*O beta*) during confined (stress) condition (Julsrud et al 1998) Recently, the presence of both the urea cycle-related CPSs, CPS I-like and CPS III (most predominant in teleost species) have been reported in the walking catfish (Saha et al 1999a) But the assay method used here for CPS activity does not distinguish between these two different types of CPSs Therefore, it is not possible at present to identify whether both the CPSs or only one type was induced in the perfused liver under higher ammonia load Another possible mechanism could be the activation of pre-existing inactive enzymes serving ammonia as a regulatory molecule The third possibility could be the increase in the concentration of urea cycle enzymes under higher ammonia load However, it seems unlikely that the regulation at the transcriptional level would occur within 60 min of ammonia loading The physiological level of activity of OTC and ARG as such is high in this fish and even the highest ammonia load in the perfused liver possibly did not have any stimulatory effect on these two enzymes of the urea cycle for enhanced ureogenesis

Increase of pH in the external water was reported to stimulate the ureogenesis in Lahontan cutthroat, *Oncorhynchus clarki henshawi* (Wilkie et al 1993), in tilapia, *Oreochromis nilotica* (Wright 1993), and in rainbow trout, *Onchorhynchus mykiss* (Wilkie and Wood 1991) However, in the present study, while perfusing the liver of walking catfish, the pH of the media containing different concentrations of NH_4Cl were kept constant (pH 7 5) Therefore, the stimulation of ureogenesis under higher ammonia load noticed herein cannot be considered the effect of pH change of the media But, the intracellular pHi rising because of intracellular ammonia penetration was not taken into account in the present study and this might be another or an additional cause of induction of ureogenesis In ureogenic toadfish, *O beta* stimulation of ureogenesis was also reported while exposing to higher ambient ammonia and also to exposure to air for extended periods of time (Walsh et al 1994), and the reason of

this stimulation was concluded later to be due to stress related secretion of cortisol (Hopkins et al. 1995). In our walking catfish, however, it is evident from the perfusion experiment that higher ammonia load in the liver beyond a threshold level can cause stimulation of ureogenesis via the induced urea cycle. Such ammonia-induced ureogenesis has also been reported in ureotelic amphibians (Balinsky et al. 1961; Janssens and Cohen 1968; Janssens 1972). Presence of such physiological adaptive strategy is probably necessary in this unique group of air-breathing walking catfish to survive under hyper-ammonia stress in their normal habitat or while living outside water or while burrowing inside mud.

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Types of Carbamyl Phosphate Synthetases and Subcellular Localization of Urea Cycle and Related Enzymes in Air-Breathing Walking Catfish, *Clarias batrachus*

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ABSTRACT The types of carbamyl phosphate synthetase (CPS), the subcellular localization of urea cycle enzymes and glutamine synthetase (GS) in liver and kidney (potential ureogenic tissues) and also the possible involvement of some extra-hepatic tissues for urea synthesis via the urea cycle were studied in an Indian ureogenic amphibious air-breathing walking catfish, *Clarias batrachus*. Mitochondrial CPS III [glutamine- and N-acetyl-L-glutamate (NAG)-dependent] and cytosolic CPS II (glutamine-dependent) activities were found to be present in liver, analogous to that described for two other teleosts that have CPS III activity. The same activities and subcellular localization were found in kidney. Unexpectedly, a CPS I-like (ammonia- and NAG-dependent) activity was found to be present at levels higher than the CPS III activity in the mitochondrial fraction of both liver and kidney analogous to another Indian amphibious air-breathing teleost fish, *Heteropneustes fossilis*. The two other urea cycle enzymes, ornithine transcarbamylase (OTC) and arginase (ARG), and GS were found to be localized primarily in the mitochondria, and argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) in the cytosol analogous to *H. fossilis* and elasmobranchs. Significant levels of urea cycle enzymes along with GS activity could also be detected in muscle, intestine and brain tissues, except ASL in muscle and ASS in brain. Our results also indicated that probably all three different types of CPS activities are also expressed in all three extra-hepatic tissues. The urea cycle–related CPS III found in invertebrates and fish is considered to be the evolutionary precursor of the urea cycle–related CPS I in ureotelic mammalian and amphibian species. Whether or not the CPS I-like activity reported here (1) is due to the presence of a separate CPS I gene in addition to a CPS III gene or (2) represents an adapted CPS III activity in *C. batrachus*, these results suggest that the presence of both CPS I-like and CPS III activities along with the presence of urea cycle enzymes in extra-hepatic tissues may play an important physiological adaptive role for survival of these walking catfish in higher ambient ammonia or living in air or while burrowing inside mud during habitat drying. *J. Exp. Zool. 283:121–130, 1999.*

Most teleost fishes are ammoniotelic in terms of nitrogen excretion and, until recently, the presence of a functional urea cycle was not known to exist in teleosts (for reviews, see Campbell and Anderson, '91; Wood, '93; Anderson, '95a; Wright, '95; Saha and Ratha, '98). However, interest in the urea cycle and regulation of the expression of urea cycle enzymes in fish has increased in recent years due to (1) reports of a functional urea cycle in several different teleost fishes, namely some Indian air-breathing teleosts (including the walking catfish, *Clarias batrachus*) (Saha and Ratha, '87, '89), an alkaline lake-adapted tilapia (*Oreochromis alcalicus grahami*) (Randall et al., '89) and the marine toadfishes *Opsanus tau* and *O. beta* (Mommsen and Walsh, '89); (2) documentation of the expression of a specific type of urea cycle–related carbamyl phosphate synthetase (CPS, which catalyzes the first step of the urea cycle) in liver of largemouth bass (*Micropterus salmoides*) (Anderson, '76; Casey and Anderson, '83; Cao et al., '91), trout (*Oncorhynchus mykiss*) free embryo (Wright et al., '95), liver and kidney of the Indian air-breathing catfish (*Heteropneustes fossilis*) (Saha et al., '97), and midshipman (*Pori-*

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chthys notatus) (Mommsen and Walsh, '89; Anderson and Walsh, '95); and also (3) having certain alterations in subcellular localization of certain urea cycle–related enzymes (especially arginase and glutamine synthetase) (Mommsen and Walsh, '89; Anderson, '95a).

Three types of CPSs, which catalyze formation of carbamyl phosphate as the first step of both the urea cycle and pyrimidine nucleotide biosynthesis pathways, have been recognised (for a recent review, see Anderson, '95b). The urea cycle–related CPS I, found mainly in ureotelic mammalian and amphibian species, utilizes only ammonia as the nitrogen-donating substrate, requires N-acetyl-L-glutamate (NAG) as an allosteric activator for activity and is localized exclusively in the mitochondrial matrix in liver and small intestine. CPS II, which is responsible for pyrimidine 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.

Another urea cycle–related CPS, CPS III was first reported in invertebrates by Trammel and Campbell ('70, '71) and later in the liver of largemouth bass, a freshwater teleost, and, at much higher levels, in liver of marine ureosmotic elasmobranchs by Anderson ('76, '80). However, unlike CPS I, it utilizes glutamine as the nitrogen-donating substrate instead of ammonia, requires NAG for its activity and is localized exclusively in the mitochondria (Anderson, '81; Casey and Anderson, '83).

All the known ureogenic fishes, including one Indian amphibious singhi catfish (*H. fossilis*), in which the type of CPS has been characterized, use a CPS III-type enzyme, which has a preference for the substrate glutamine over ammonia as a nitrogen donor for catalyzing carbamyl phosphate formation (Campbell and Anderson, '91; Anderson, '95a,b; Saha et al., '97). The supply of glutamine to CPS III in these fishes is accomplished by glutamine synthetase (GS), which is believed to be co-localized in the mitochondria of hepatocytes (Chakravorty et al., '89; Mommsen and Walsh, '89). However, the mitochondrial localization of GS in liver and glutamine-dependent urea synthesis is not universal in fish, at least in teleost species, since GS is present in the cytosol of liver in some that appear to have a glutamine-dependent CPS III (Cao et al., '91; Anderson and Walsh, '95). Thus, in ureogenic fish, GS is considered the key first nitrogen-fixing step in the urea cycle in addition to its major role of trapping ammonia in brain.

Although the presence of a functional urea cycle has been reported in amphibious fish from the Indian subcontinent by Saha and Ratha ('87, '89) including the presence of CPS activity, the type of CPS present and also the subcellular localization of urea cycle and related enzymes have not yet been studied in any of these species except in one, the singhi catfish (*H. fossilis*) (Dkhar et al., '91; Saha et al., '97). Therefore, the main objectives of the present study were to determine the types of CPS present, the subcellular localization of urea cycle enzymes and GS both in liver and kidney tissues, and the involvement of some extra-hepatic tissues in urea synthesis via the urea cycle in another unique amphibious fish from India, the walking catfish, *Clarias batrachus*.

MATERIALS AND METHODS

Animal

The fish, *Clarias batrachus*, weighing 50–80 g body weight, were purchased from commercial sources and acclimatized in the laboratory for 4–6 weeks at about 30°C with 12 hr : 12 hr light and dark period before being used for experiments. No sex differentiation of fish was done while performing these studies. Minced pork liver and rice bran (5% of the body weight) was given as food, and the water, which was collected from a natural stream, was changed on alternate days.

Preparation of tissue extracts

Fish were sacrificed by decapitation and tissues such as liver, kidney, muscle, brain and intestine were excised. Liver and kidney tissue were used immediately for subcellular fractionation, and other tissues were frozen quickly by plunging immediately into liquid nitrogen and stored in –70°C until used for analysis of enzymes. For analysis of enzymes in muscle, intestine and brain, a 20% homogenate (w/v) was prepared in homogenizing buffer containing 100 mM Tris-HCl buffer (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiotheitol (DTT) and 0.25% Triton X-100. The homogenization was done with a motor-driven Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was subjected to sonication for proper breakage of mitochondria, centrifuged at 10,000*g* for 10 min, and the supernatant was used for assaying the enzymes. All steps were carried out at 4°C.

KCl, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4$ and 1.25 mM $CaCl_2$ as a basic solution for perfusion. The medium also contained 5 mM L-glucose and 2 mM L-ornithine. Ammonium chloride (2 mM), L-glutamine (5 mM) or a combination of these two was infused along with the perfusion media to study the rate of formation of urea by the perfused liver. The medium was gassed with O_2/CO_2 (99:1, v/v) before infusing into the liver at a flow rate of 4–5 ml/g liver/min. The pH of the medium was always maintained at 7.5 after gassing. The temperature of the medium was 30°C. The effluent coming out of the perfused liver was collected through a cannula inserted at the superior vena cava at 2-min intervals for analysis of ammonia and urea-N. The concentrations of ammonia and urea-N in the effluent collected at the 2-min intervals were measured enzymatically, based on the procedure of Kun and Kearney ('74). The rate of formation of ammonia from glutamine in the reaction mixture (by the action of glutaminase), while measuring the activity of CPS, was also determined following the same enzymatic method as mentioned previously. Protein was precipited out from the reaction mixture with 2 M PCA at different time of incubation, and the ammonia was measured in the supernatant after neutralizing with 2 N NaOH.

Chemicals

All enzymes, coenzymes, substrates, glycylglycine, Na-glycinate, DTT, UTP and MS 222 were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were of the highest quality obtained from indigenous sources. Deionised and double glass distilled water was used for all preparations.

RESULTS

CPS III activity in mitochondrial extracts

As noted by Anderson ('95a), both CPS III and CPS II can be identified by the fact that CPS III would be localized in the mitochondria (whereas CPS II would be localized in the cytosol), and although CPS III (like CPS II) utilizes glutamine as substrate, unlike CPS II, it would be activated by NAG and would not be inhibited by UTP. The same strategy was employed in the present study to identify these two enzymes both in liver and kidney. As shown in Tables 1 and 2, a significant level of glutamine-dependent CPS activity was present in the cytosolic fraction of both liver and kidney. This activity was significantly inhibited by UTP and was not affected by the presence of NAG. In addition, little activity was observed when glutamine was replaced by 5 mM ammonia, whether or not NAG was present. These kinetic and specificity properties are characteristic of CPS II. It should be noted that CPS II is also active with ammonia as substrate, but at pH 7.5 the ammonia concentration must be much higher (e.g., 100 mM) than the 5 mM used here to attain a maximum velocity comparable to that attained

TABLE 1. CPS activity (units/g wet wt) in different subcellular fractions of C. batrachus *liver in the presence of different nitrogen-donating substrates, NAG and/or UTP*[1]

Components reaction mixture	Homogenate	Mitochondrial fraction[2,3]	Cytosolic fraction[2,3]
Ammonia		1.25 ± 0.10	0.55 ± 0.12
Ammonia + UTP		0.75 ± 0.14	0.34 ± 0.09
Ammonia + NAG		5.04 ± 0.37	0.64 ± 0.11
Ammonia + NAG + UTP		4.75 ± 0.45	0.45 ± 0.05
Glutamine		1.45 ± 0.14	3.87 ± 0.29
Glutamine + UTP		1.15 ± 0.16	1.25 ± 0.07
Glutamine + NAG		4.85 ± 0.85	3.96 ± 0.65
Glutamine + NAG + UTP		4.17 ± 0.80	1.45 ± 0.11
Ammonia + Glutamine + NAG		6.55 ± 0.82	4.07 ± 0.81
Ammonia + Glutamine + NAG + UTP		6.05 ± 1.15	0.95 ± 0.14
GDH	616 ± 56	490 ± 36 (80)	141 ± 22 (23)
LDH	1245 ± 122	205 ± 45 (16)	1047 ± 102 (84)

[1]The reaction mixture for carbamyl phosphate synthetase (CPS) assay contained everything as mentioned in the Materials and Methods section, plus 5 mM ammonium chloride, 25 mM glutamine, 5 mM N-acetyl-L-glutamate (NAG) and/or 1 mM uridine-5′-triphosphate (UTP) wherever noted. One unit of enzyme activity was expressed as the amount that catalyzed 1 μmol of citrulline in the case of CPS, and 1 μmol of NADH utilized for glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH) per hr at 30°C.

[2]Values are expressed as mean ± SEM (n = 3).

[3]% of GDH and LDH activity out of the total are given in parentheses.

TABLE 2. CPS activity (units/g wet wt) in different subcellular fractions of C. batrachus *kidney in the presence of different nitrogen-donating substrates, NAG and/or UTP*[1]

Components reaction mixture	Homogenate	Mitochondrial fraction[2,3]	Cytosolic fraction[2,3]
Ammonia		1.25 ± 0.18	0.45 ± 0.07
Ammonia + UTP		0.65 ± 0.09	0.30 ± 0.08
Ammonia + NAG		4.42 ± 0.52	0.55 ± 0.10
Ammonia + NAG + UTP		4.05 ± 0.37	0.45 ± 0.06
Glutamine		1.25 ± 0.16	2.53 ± 0.22
Glutamine + UTP		0.85 ± 0.11	0.45 ± 0.05
Glutamine + NAG		3.85 ± 0.46	3.12 ± 0.35
Glutamine + NAG + UTP		3.45 ± 0.44	0.75 ± 0.12
Ammonia + Glutamine + NAG		5.63 ± 0.85	3.51 ± 0.47
Ammonia + Glutamine + NAG + UTP		5.24 ± 0.74	0.81 ± 0.18
GDH	335 ± 31	290 ± 30 (86)	74 ± 15 (22)
LDH	445 ± 45	56 ± 11 (13)	376 ± 50 (85)

[1]The concentrations of ammonium chloride, glutamine, NAG and/or UTP in the reaction mixture are the same as noted in Table 1. Abbreviations are as in Table 1.
[2]Values are expressed as mean ± SEM (n = 3).
[3]% of GDH and LDH activity out of the total are given in parentheses.

with glutamine (Anderson, '95a). Significant glutamine-dependent activity was also found in the mitochondrial fraction of both liver and kidney, but only when NAG was present; this glutamine- and NAG-dependent CPS activity was not significantly affected by the presence of UTP. These properties are characteristic of CPS III. Several observations made from the activity in liver mitochondrial extracts indicate that the glutamine-dependent activity is not an artifact in which activity is actually due to ammonia as a substrate, present as a contaminant of glutamine or arising from hydrolysis of glutamine by a glutaminase present in the extract. Product formation with either ammonia or glutamine as substrate was linear with time up to 45 min (data not shown). Some ammonia was formed with time in reaction mixtures with glutamine (e.g., 0.15, 0.34 and 0.51 mM after 15, 30 and 45 min, respectively), but the levels were too low to account for the rate observed with glutamine as substrate (note the Km values of 2.2 and 0.46 mM for ammonia and glutamine, respectively, below). Thus, these results appear to confirm the expectation that these fish have a urea cycle–related mitochondrial glutamine- and NAG-dependent CPS III and a pyrimidine-related cytosolic glutamine-dependent CPS II activity, analogous to the only three other teleosts in which the distribution of these CPSs has been documented, i.e., largemouth bass (Cao et al., '91), marine toadfish (Anderson and Walsh, '95) and singhi catfish (Saha et al., '97). The distribution of the activities of GDH and LDH, marker enzymes for mitochondrial and cytosolic fractions, respectively, indicates that although complete separation between the mitochondrial and cytosolic components was not attained, the subfractionation did provide significant enrichment of the two subcellular fractions, sufficient for the purposes described here.

CPS I-like activity in mitochondrial extracts

An unexpected finding was the presence, in the mitochondrial fractions of both liver and kidney, of a level of ammonia- and NAG-dependent CPS activity that was not affected by the presence of UTP, but almost at the level or a little bit higher than the glutamine- and NAG-dependent activity (Tables 1 and 2). Similar observations were also made recently in another amphibious singhi catfish, *H. fossilis* (Saha et al., '97). Further, inclusion of both ammonia and glutamine along with NAG in the reaction mixture resulted in an increase in the level of activity showing some additive effect (Tables 1 and 2). It should be noted here that this additive effect was not observed in the cytosolic fraction. (Tables 1 and 2). The observed additive effect might occur if either ammonia or glutamine can serve as nitrogen-donating substrate and the concentration of each is well below its Km value. This does not appear to be an explanation, however, because the measured Km values from ammonia and glutamine were found to be 2.2 and 0.46 mM, respectively.

Urea formation from ammonia and glutamine by perfused liver

Additional support for the probable occurrence of both ammonia- and glutamine-dependent urea cycle–related CPS activity was obtained from the liver perfusion studies. When ammonium chloride and glutamine were infused separately into liver of walking catfish, a significant rate of urea formation was observed with either substrate (Table 3). When ammonium chloride and glutamine were infused together, the rate of urea formation was increased further showing some additive effect.

Subcellular localization of urea cycle enzymes and glutamine synthetase

The subcellular localization of all the urea cycle enzymes (except CPS) and GS both in liver and kidney of walking catfish are presented in Tables 4 and 5. Most of the activity of OTC (86% and 87%) and ARG (76% and 77%) both in liver and kidney were found to be localized primarily in the mitochondrial fraction along with 83.5% and 92% of GDH activity in liver and kidney, respectively. ARG activity has been reported to be associated with the outer membrane of rat liver mitochondria due to non-specific binding, which could be solubilized by increasing the ionic (KCl) concentration in the homogenate (Rosenthal et al., '56; Soberon and Palacios, '76). The homogenizing medium used in the present study contained 0.1 M KCl, suggesting that the mitochondrial localization of ARG observed in liver and kidney of this fish is not an artifact due to non-specific binding to the outer membrane of the mitochondria. The activity of ASS (78% and 79%) and ASL (84% and 83%) were found to be localized in the cytosolic fraction along with 86% and 87% of LDH activity in liver and kidney, respectively. The GS activity was also found to be localized primarily in the mitochondrial fraction (83%) both in liver and kidney.

TABLE 3. Rate of urea synthesis from ammonia and glutamine separately, and from ammonia and glutamine together by the perfused liver of C. batrachus[1]

	Urea synthesis (μmol/g liver/hr)
Control	BLD[2]
Ammonia (2 mM)	11.2 ± 1.24
Glutamine (5 mM)	6.4 ± 1.11
Ammonia (2 mM) + Glutamine (5 mM)	13.6 ± 1.57

[1]Livers were perfused first with isotonic medium containing 5 mM glucose and 2 mM ornithine as mentioned in the Materials and Methods section for 20 min without ammonium chloride and glutamine, followed by the infusion of ammonium chloride (2 mM), glutamine (5 mM) or a combination of these two substrates. Values were obtained between 26 and 30 min of infusion and calculated as mean ± SEM (n = 4).
[2]BLD, below the level of detection.

Activity of urea cycle enzymes in extrahepatic tissues

All the urea cycle enzymes and GS were also measured in the present study in some extra-hepatic tissues other than kidney, such as muscle, intestine and brain of walking catfish without separating out the different subcellular fractions (Table 6). As noted above for testing the different types of CPS activity in the mitochondrial and cytosolic fractions of liver and kidney, the same strategy was also used for measurement of different types of CPSs in all the three non-ureogenic tissues. When ammonia alone was taken in the reaction mixture, no significant activity of CPS activity could be detected in any of the tissues. However, when NAG was taken along with ammonia, a significant level of activity could be detected in all three tissues, which was not affected by the presence of UTP. This may indicate that CPS I-like activity is present in all three tissues. Low levels of CPS activity could also be detected in all three tissues when glutamine alone was taken in the reaction mixture, which was activated by 113%, 185% and 187%, respectively, in presence of NAG, and partially inhibited by UTP in presence of glutamine and NAG by 36%, 47% and 23% in muscle, intestine and brain, respectively. This may indicate that both CPS III and II activity are also present in all three tissues. with a relatively higher level in brain. As noted above in the case of liver and kidney, in all three non-hepatic tissues also some additive effect of enzyme activity could be seen when both glutamine and ammonia were taken together in the reaction mixture along with NAG (Table 6). All other urea cycle enzymes could also be detected in the three different tissues except for ASS in brain and ASL in muscle, which could not be detected with the present assay method used. A very high level of GS activity could be detected in brain (as expected) with a relatively low level in muscle and intestine.

DISCUSSION

The urea cycle–related CPS activity, which has been reported to date in a limited number of fish species, have been suggested to be CPS III type (glutamine- and NAG-dependent) with the involvement of glutamine synthesis by GS as a first step of urea formation via the urea cycle. This has been directly confirmed in liver of *Squalus acanthias* (a

TABLE 4. Activity (units/g wet wt) of different urea cycle–related enzymes except CPS and marker enzymes in different subcellular fractions of Clarias batrachus *liver*[1]

Enzyme[2]	Homogenate	Nuclear fraction	Mitochondrial fraction	Cytosolic fraction	Recovered activity
OTC	203.5 ± 11.2	10.4 ± 1.5 (5.1)	175 ± 13.6 (86.1)	27.8 ± 4.2 (13.7)	213.6 (105)
ASS	53.3 ± 7.9	2.9 ± 0.7 (5.4)	14.2 ± 2.2 (26.6)	41.5 ± 8.1 (78)	58.6 (110)
ASL	42.4 ± 10.2	2.4 ± 0.4 (5.6)	7.8 ± 1.1 (18.4)	35.7 ± 3.9 (84.2)	45.9 (108)
ARG	4702 ± 178	410 ± 57 (8.7)	3615 ± 177 (77)	1084 ± 102 (23)	5109 (109)
GS	90.3 ± 14.4	8.1 ± 1.1 (9)	75.2 ± 10.6 (83.3)	24.2 ± 4.4 (26.8)	107.5 (119)
GDH	562.5 ± 52.4	87.5 ± 8.9 (15.5)	470.0 ± 48.6 (83.5)	140.0 ± 15.1 (24.9)	697.5 (124)
LDH	1006 ± 116	82.7 ± 11.2 (8.2)	163.2 ± 17.1 (16.2)	865.5 ± 56.3 (86)	1111 (110)

[1]1 unit of enzyme activity was defined as the amount of enzyme that catalyzed 1 μmol of product formed or substrate used per hr at 30°C for all the urea cycle related enzymes, and 1 μmol of NADH utilized per hr at 30°C for GDH and LDH; % of total enzyme activity are given in parentheses.
[2]OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase.

representative marine elasmobranch), where the GS is localized exclusively in the mitochondria and mitochondrial formation of citrulline from ammonia has been shown to involve obligatory intermediate formation of glutamine (Anderson and Casey, '84). This was accompanied by an absence of the pyrimidine-related CPS II in the liver, presumably because glutamine is utilized exclusively for urea formation (Hong et al., '95). The presence of CPS III activity has recently been documented in one Indian air-breathing singhi catfish (*H. fossilis*) both in liver and kidney (Saha et al., '97) along with reports of localization of GS (Chakravorty et al., '89) and arginase (Dkhar et al., '91) primarily in the mitochondria resembling that of elasmobranchs. In largemouth bass (Cao et al., '91), *O. tau* and *Porichthys notatus*, the liver GS is localized primarily in the cytosol; in *O. beta*, however, a significant level of the GS activity is present in the mitochondria as well as in the cytosol, and the level of mitochondrial GS may fluctuate in relation to the ureotelic nature of the fish's nitrogen metabolism (Walsh and Milligan, '95). Also, unlike elasmobranchs, cytosolic pyrimidine-related CPS II is present in the liver of those teleost species, where mitochondrial CPS III activity has

TABLE 5. Activity (units/g wet wt) of different urea cycle–related enzymes except CPS and marker enzymes in different subcellular fractions of Clarias batrachus *kidney*[1]

Enzyme[2]	Homogenate	Nuclear fraction	Mitochondrial fraction	Cytosolic fraction	Recovered activity
OTC	112.3 ± 12.1	8.8 ± 1.2 (7.8)	97.4 ± 12.8 (86.7)	18.4 ± 2.8 (16.4)	124.6 (111)
ASS	51.4 ± 6.8	5.5 ± 0.8 (10.7)	9.2 ± 1.6 (17.9)	40.7 ± 7.1 (79.2)	55.4 (108)
ASL	38.6 ± 6.62	3.8 ± 0.74 (9.8)	6.4 ± 1.1 (16.6)	31.9 ± 5.4 (82.6)	42.1 (109)
ARG	1150 ± 201	102 ± 21 (8.9)	876 ± 107 (76)	255 ± 32 (22)	1203 (105)
GS	48.3 ± 7.8	6.9 ± 0.7 (14.2)	40.1 ± 5.2 (83)	9.9 ± 1.2 (20.5)	56.9 (118)
GDH	312 ± 37.5	39 ± 5.9 (12.5)	287 ± 24 (92)	88 ± 10.7 (28)	414 (133)
LDH	420 ± 62	39 ± 8.21 (9.2)	48 ± 6.6 (11.4)	366 ± 58 (87)	451 (107)

[1]% of total enzyme activity are given in parentheses.
[2]Abbreviations are same as Table 4.

TABLE 6. Activity of urea enzymes and GS (units/g wet wt) in muscle, intestine and brain tissues of C. batrachus[1]

Enzyme[2]	Activity[3]		
	Muscle	Intestine	Brain
CPS			
Ammonia	0.35 ± 0.06	0.45 ± 0.08	0.85 ± 0.10
Ammonia + NAG	0.65 ± 0.08	1.07 ± 0.11	2.04 ± 0.25
Ammonia + NAG + UTP	0.59 ± 0.07	0.88 ± 0.10	1.96 ± 0.22
Glutamine	0.55 ± 0.07	0.65 ± 0.08	0.88 ± 0.10
Glutamine + NAG	1.17 ± 0.11	1.85 ± 0.14	2.53 ± 0.45
Glutamine + NAG + UTP	0.75 ± 0.07	0.98 ± 0.09	1.96 ± 0.12
Ammonia + Glutamine + NAG	1.55 ± 0.11	2.26 ± 0.16	4.02 ± 0.26
Ammonia + Glutamine + NAG + UTP	1.16 ± 0.12	1.26 ± 0.15	2.75 ± 0.36
OTC	5.61 ± 0.46	11.70 ± 0.51	5.49 ± 0.44
ASS	11.52 ± 0.65	10.65 ± 0.78	BLD
ASL	BLD	27.7 ± 1.82	3.0 ± 0.2
ARG	124.6 ± 14.1	110.8 ± 8.2	152.3 ± 12.4
GS	8.6 ± 0.84	29.7 ± 2.3	323.4 ± 25.5

[1]For CPS assay, the concentrations of ammonium chloride, glutamine. NAG and/or UTP in the reaction mixture are the same as noted in Table 1. One unit of enzyme activity was expressed as the amount which catalyzed 1 μmol of product formed, or 1 μmol of substrate utilized in case of ASS per hr at 30°C.
[2]Abbreviations are same as in Table 1.
[3]Values are expressed as mean ± SEM (n = 3); BLD, below the level of detection.

been reported and the presence of CPS II activity has been documented (Anderson, '80; Cao et al., '91; Anderson and Walsh, '95: Saha et al., '97). In the present study in walking catfish, high levels of GS activity were found both in liver and kidney, and were mainly localized in the mitochondria (Tables 4 and 5). In this respect, walking catfish resemble elasmobranchs and singhi catfish, and like other teleosts (including singhi catfish) cytosolic CPS II is present in liver (and kidney).

The presence of CPS III was not unusual in walking catfish with its known ureotelic capacity. However, the presence of significant ammonia- and NAG-dependent CPS (CPS I-like) activity in liver and kidney mitochondria of walking catfish, resembling that of singhi catfish (Saha et al., '97), is unique from that observed in other teleosts. The presence of a gene for both a CPS III and CPS I in walking catfish would not seem to be a likely explanation for these two activities within the context of the current understanding of the structural relationships between these two enzymes, their currently understood species distribution (CPS I is present only in ureotelic mammalian and amphibian species, CPS III is present only in invertebrates and fishes) and the prevailing view that CPS I evolved from CPS III (Mommsen and Walsh, '89; Campbell and Anderson, '91; Helbig and Atkinson, '94; Hong et al., '94; Anderson, '95a,b). Perhaps adaptation in walking and singhi catfish and closely related species was achieved as a separate event in which the CPS III gene underwent duplication and one gene subsequently lost the structural requirements for utilization of glutamine as substrate. Alternatively, perhaps the observed CPS I-like activity represents an adapted form of CPS III with separate ammonia and glutamine binding sites. It is perhaps difficult to say with the present study whether the glutamine-dependent CPS III described here is similar to the CPS III activities of dogfish and largemouth bass. Elucidation of the nature of the two types of activities will require purification and characterization of the enzyme(s) responsible for these activities and/or the mRNA(s) coding for the enzyme(s).

The subcellular localization of the urea cycle enzymes and GS in the liver and kidney of walking catfish was found to be analogous to that of singhi catfish (Chakravorty et al., '89; Dkhar et al., '91) and elasmobranchs (Casey and Anderson, '82), i.e., GS, CPS III, OTC and ARG are localized in the mitochondria, and ASS and ASL are localized in the cytosol (Tables 4 and 5). The co-localization of GS along with CPS III in mitochondria probably helps in the assimilation of ammonia more efficiently. Unlike that of elasmobranchs, CPS I-like activity has also been documented in the mitochondria of liver and kidney of this walking catfish. Pyrimidine synthesis via the CPS II probably needs glutamine to be transported out from the mitochondria to the cytosol. The presence of significant levels of both kinds of urea cycle–related CPS (CPS III and CPS I-like) activ-

ity, enabling this walking catfish to utilize ammonia either directly or indirectly as glutamine, as substrate for carbamyl phosphate formation in mitochondria, may be an adaptive physiological feature characteristic of these species that is related to their remarkable tolerance to high levels of ambient ammonium chloride and their ability to switch from ammoniotelism to ureotelism when exposed to high levels of ammonia and during exposure to air (unpublished observations by Saha and Das). The physiological significance of mitochondrial localization of ARG in this walking catfish and many other teleosts reported earlier is not very clear. One consequence of the localization of ARG in the mitochondria could be to supply ornithine directly for carbamyl phosphate synthesis inside the mitochondria and also could be due to replacement of transporter for ornithine by arginine in the mitochondrial membrane as suggested by Mommsen and Walsh ('89). Another consequence could be, as suggested in elasmobranchs (Anderson, '95a), that urea formed in the mitochondria serves as a feedback inhibitor for CPS III and also for GS.

In addition to two potential ureogenic tissues (liver and kidney), whether other tissues are involved in urea synthesis via the urea cycle, we surveyed the activities of GS and urea cycle enzymes in muscle, intestine and brain of this walking catfish. Clearly, all the enzymes appear to be present in all three tissues except ASS in brain and ASL in muscle (Table 6). Growing evidence suggests that extra-hepatic tissues could also participate in urea synthesis in fish as has been reported in several species of Indian air-breathing teleosts (Saha and Ratha, '87, '89), toadfish (Wood et al., '95), rainbow trout (Korte et al., '97), common carp and bowfin (Felskie et al., '98). There could also be the involvement of more than one tissue in the same species responsible for urea synthesis especially when one or two enzymes of urea cycle are lacking in specific tissues. For example, in our walking catfish the synthesis of citrulline or argininosuccinate may take place in brain and muscle, respectively, followed by the transportation of these intermediary products via the blood to either liver or kidney for ultimately converting to urea. Although the activity of different urea cycle enzymes in muscle were comparatively low, the muscle represents a high percentage of the body mass. Therefore, the total units of different enzymes could be physiologically significant in this fish. Very high levels of GS in brain in addition to the presence of various urea cycle enzymes probably help in avoiding accumulation of toxic ammonia by assimilating ammonia to glutamine again as a sort of physiological adaptation in this unique group of Indian air-breathing amphibious walking catfish.

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