STUDIES ON NITROGEN METABOLISM IN AN AIR-BREATHING CATFISH, *Clarias batrachus* DURING OSMOTIC STRESS

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DEDICATED

TO

MY BELOVED PARENTS

DECLARATION

I, Ms. Arundhati Bhattacharjee, hereby declare that the subject matter of this thesis is the record of the work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research 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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List of Abbreviations:

ADP	Adenosine 5'-diphosphate
ALT	Alanine aminotransaminase
AST	Aspartate aminotransaminase
AMP	*Adenosine 5'-monophosphate*
ARG	Arginase
E.C.	Enzyme Commission
EDTA	Ethylenediamine tetra-acetic acid
FAA	Free amino acid
g	gram
GDH	Glutamate dehydrogenase
GDP	Guanosine 5'-diphosphate
GSase	Glutamine synthetase
GTP	Guanosine 5'-triphosphate
HPLC	High Performance Liquid Chromatography
h	hour
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
µl	microlitre
µmole	micromole
mg	milligram
ml	milliliter
mM	millimolar
mOsmol/l	milliOsmol per liter
min	minute
M	Molar
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NADPH	**Nicotinamide adenine dinucleotide phosphate reduced form**
OUC	**Ornithine-urea cycle**
PCA	***Perchloric acid***
Tris	**Trihydroxy methyl aminomethane**
UV	**Ultra Violet**
v	**volume**

Abbreviations for amino acids:

Asp	Aspartic acid
Gly	Glycine
Ala	Alanine
Ser	Serine
Glu	Glutamic acid
Gln	Glutamine
Cit	Citrulline
Asn	Asparagine
Tyr	Tyrosine
Pro	Proline
GABA	Gamma amino butyric acid
Tau	Taurine
Thr	Threonine
Val	Valine
Met	Methionine
Ile	Isoleucine
Leu	Leucine
Trp	Tryptophan
His	Histidine
Lys	Lysine
Phe	Phenyl alanine
Orn	*Ornithine*
Arg	Arginine

CONTENTS

INTRODUCTION

Nitrogen metabolism is one of the most sensitive physiological systems in response to environmental changes (Gordon, 1970). The pathways leading to the biosynthesis of amino acids and nucleotides both share a requirement for nitrogen. But as soluble and biologically useful nitrogen compounds are scarce in natural environment. Therefore, ammonia, amino acids and nucleotides are used economically by most organisms. Animals are not capable of using inorganic nitrogen compounds to synthesize nitrogenous biomolecules. They receive organic nitrogenous compounds in the diet, which are taken in largely as amino acids, proteins and nucleic acids along with other biomolecules such as carbohydrates as the primary source for metabolic energy production releasing CO_2 and H_20 as the end products. Other biomolecules are used as energy source only when there is a depletion of carbohydrates in mammalian system (Phillips, 1969). However, in teleostean fishes the main source of energy is the catabolism of proteins and amino acids releasing ammnonia as one of the major end products (Goldstein and Forster, 1970; Watts and Watts, 1974; French et al., 1981; Walton and Cowey, 1982). Elevated levels of amino acids in plasma have been reported in fishes with high protein diets (Cowey et al., 1977). Excess amino acids cannot be stored (as stored proteins) as can carbohydrates (as glycogen) or lipids (as fat) in animals. In carnivorous fishes, the natural diet is rich in protein and low in carbohydrates. Cells utilize amino acids as energy sources during starvation either directly by the oxidation of carbon skeleton or indirectly by the conversion of carbon skeleton to glucose via the gluconeogenic pathway (Bever et al., 1981). Proteins and other nitrogen-containing biomolecules are regular and essential dietary components, and digestion and metabolism of these compounds results in excess production of nitrogenous wastes.

The nature of the nitrogenous excretory products in animals has changed with the evolution of vertebrates from water to the land habitat (Campbell, 1991; Wright, 1995; Saha and Ratha, 1998). Most teleost fishes excrete ammonia as the major nitrogenous end product in their aquatic habitat mostly through gills by simple diffusion (Walton and Cowey, 1982; Randall and Wright, 1987; Campbell, 1991; Wilkie, 1997; Saha and Ratha, 1998). 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 (Cooper and Plum, 1987; Campbell, 1991; Wood, 1993). Patterns of nitrogen excretion in animals broadly follow phylogenetic lines, but are also linked to the environment (Campbell, 1991). Ammonia, urea and uric acid differ in toxicity, solubility and production costs. Terrestrial animals, being water-limited, usually detoxify ammonia and excrete it in the form of urea or as purine such as uric acid (Campbell, 1991). Urea and uric acid are less toxic than ammonia and require 10 and 50 times less water, respectively, for storage and excretion at non-toxic concentrations (Wright, 1995). However, in circumstances such as high ambient ammonia or aerial exposure, fishes can hardly excrete ammonia, and toxic ammonia is concentrated in the blood and body tissues. In general, however, aquatic animals can tolerate more elevated levels of blood ammonia than terrestrial animals. Plasma total ammonia normally remains between 0.05 to 2 mmol/l in most teleosts (Campbell and Anderson, 1991; Wood, 1993; Wright et al., 1993) with the exception in air-breathing catfish, *Heteropneustes fossilis*, where it has been reported to raise to a level of 4 mmol/l in higher ambient ammonia concentration (Saha and Ratha, 1994). In contrast, blood ammonia levels

greater than 0.05 mmol/l can be toxic to the central nervous system of most mammals (Meijer et al., 1990). Changes in nitrogen metabolism and nitrogen excretory pattern is one of the important biochemical adaptations which had taken place when the animals migrated from aquatic to the terrestrial habitat, and this was again directly related to the availability of water in the surrounding environment (Gordon, 1970; Wright, 1995).

Hyper-ammonia stress

As mentioned above, ammonia is highly toxic to most of the living organisms. Ammonia toxicity to fish has been primarily attributed to the un-ionized form (NH_3) and the ionized form (NH_4^+) is relatively less toxic (EIFAC, 1970; Alabaster and Lloyd, 1982; Erickson, 1985; WHO, 1986; Hickey and Vickers, 1994). The proportion of unionized 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. 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).

More recently, the expression of high ornithine-urea cycle (OUC) enzymes with accompanying active urea cycle activity has been reported in several teleosts species as an adaptation to survive under unique environmental circumstances. Examples include the marine toadfishes, *Opsanus beta* and *Opsanus tau* (Read, 1971; Mommsen and Walsh, 1989), the alkaline lake-adapted tilapia, *Alcolapia grahami* (Randall et al., 1989), some Indian freshwater air-breathing fishes such as

Heteropneustes fossilis and *Clarias batrachus* (Saha and Ratha, 1987, 1989; Saha et al., 1999), and gobiid fish, *Mugilogobius abei* (Iwata et al., 2000). These fishes may excrete significant amount of urea in response to adverse environmental conditions such as confinement (stress), severly alkaline water, and ammonia loading during exposure to semidry conditions and higher ambient ammonia (Randall et al., 1989; Saha and Ratha, 1990, 1994, 1998; Walsh et al., 1990, 1994; Ratha et al., 1995; Walsh and Milligan, 1995; Saha and Das, 1999; Saha et al., 2001, 2002a, 2003).

Ammonia toxicity in various ammoniotelic teleosts has been studied extensively and the 96 h LC_{50} value for unionized ammonia was found to be well below 0.1 mmol/litre (Haywood, 1983; Thurston et al., 1983a,b; Dabrowska and Wlasow, 1986; Campbell 1991). The 48 h LC_{50} value of total ammonia (TA) for *Cyprinus carpio* was 0.28 mmol/l (Dabrowska and Wlasow, 1986), and the 24 h 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, *A. grahami* the 24 h 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). 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. Olson and Fromm (1971) found that goldfish, *Carassius auratus* subjected to increased ambient ammonia level showed increase in urea excretion rate. When purely ammoniotelic largemouth bass (*M. salmoides*), where the levels of activity of various OUC 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).

Furthermore, there could be other possible mechanism(s) present in this walking catfish to tackle the problem of ammonia toxicity such as the conversion of accumulated ammonia to various non-essential free amino acids (FAAs) under hyper ammonia stress as previously shown in the non-ureogenic ammoniotelic mudskippers, *Periopthalamus cantonensis* (Iwata et al 1981; Iwata, 1988), *Periopthalmus schlosseri* (Peng et al., 1998), and in the carp, *Cyprinus carpio* (Dabrowska and Wlasow, 1986). Ip et al. (2001) in a recent review, however, emphasized the importance of a decrease or suppression of proteolysis and/or amino acid catabolism by some fishes during aerial exposure and ammonia loading. However, the tolerance of such a high ambient ammonia by two air-breathing catfishes (*H. fossilis* and *C. batrachus*) suggests that possibly other ammonia detoxifying mechanism(s) such as conversion of ammonia to various non-essential free amino acids (FAAs) are also operating in this unique group of air-breathing catfish (Saha et al., 2002a)

Osmotic stress

Maintenance of cell volume is one of the pre-requisites for any living organism to sustain the osmolarity changes faced regularly by the organism either in the environment or within the body. It is a phenomenon, which not only occurs in vitro, but also in animal tissue cells in vivo, when the organism acts as an osmoconformer and is subjected to changes in the environment (for review, see Hoffmann, 1977). The cell volume is frequently being challenged either due to intestinal absorption of water, various amino acids and metabolites, or due to exposure to different osmotic environments specially in case of aquatic animals.

Therefore, maintenance of cell volume despite alterations in the osmolarity of the extracellular fluid has been demonstrated in vivo in different types of cells from

several invertebrate and vertebrate species. The mechanisms behind this property have been studied extensively in various cell systems in vitro (for review, see Chamberlin and Strange, 1989; Häussinger, 1994). However, the time taken to regain the original volume varies for different types of cells, even within the same organisms (Cheung et al., 1982). The recovery of the cell volume after swelling of the cell is achieved by a reduction in the content of certain cellular solutes, followed by an osmotically obligated efflux of water. The most important solutes of osmoeffectors are K^+, Cl^-, Na^+, methylamines, taurine, FAAs (such as glycine, alanine and GABA), disaccharides (such as sucrose and trehalose) and polyols (sorbitol and inositol), depending on the species and types of cells (Chamberlin and Strange, 1989). Like most mammalian cells, hepatocytes also possess mechanisms to regulate their cell volume during osmotic challenges (Cala, 1983; Häussinger et al., 1990, 1994; Häussinger and Lang, 1991).

Besides ions, the role of urea in osmoregulation has been known for a long time in fishes (Alexander et al., 1968; Goldstein and Forster, 1971; Pang et al., 1977), and an increase in urea production was observed when hypertonic medium was applied during the embryonic development of two teleost fishes, *Salmo gairdnerii* and *Poecilia reticulata* (Depeche et al., 1979). The presence of a functional OUC and high levels of tissues urea was found in some of the freshwater air-breathing teleosts such as *Amphipnous cuchia*, *Clarias batrachus*, *Anabas testudineus* and *Heteropneustes fossilis* are unique features of freshwater teleosts (Saha and Ratha, 1989; Saha et al., 1999). The observed activities of the OUC enzymes in these fishes were higher than those in exclusively freshwater teleosts (Brown and Cohen, 1960; Huggins et al., 1969; Wilson, 1973), freshwater stingrays (Goldstein and Forster, 1971) and in the

permanently aquatic lungfish, *Neoceratodus forsteri* (Goldstein et al., 1967) and were similar or closer to those of the aestivating lungfish, *Protopterus aethiopicus* (Goldstein et al., 1967; Huggins et al., 1969), the marine toadfishes, *O. beta* and *O. tau* (Read, 1971; Mommsen and Walsh, 1989) and the aquatic amphibian, *Xenopus laevis* (Huggins et al., 1969), where the synthesis of urea occurs via the OUC. Active ureogenesis through OUC for ammonia detoxification and waste nitrogen elimination have been confirmed in amphibians and terrestrial animals (Cohen, 1976) and in marine fishes (Campbell, 1973; Cohen, 1976; Pang et al., 1977; Hoar, 1983; Read, 1971). The accumulation of urea (an uncharged hydrophilic molecule) for osmotic equilibrium was suggested to have additional advantage as it had little detrimental effect on catalytic activity of enzymes in comparison to the increase in the concentration of inorganic ions (Yancey and Somero, 1979). The retention of urea in high concentrations in the tissues and body fluids has been peculiar to certain groups of fish, which Huggins et al. (1969) classified as ureosmotic. These fish synthesize and retain urea primarily for maintaining osmotic equilibrium in hypertonic environment such as in sea water. Accumulation of urea as a solute in intra- and extracellular fluids also seems to play a critical role in amphibians and appears to show an ability to adapt to a wide variety of osmotic environments.

In addition to urea, free amino acids (FAAs) are also known to play an important role as cellular osmolytes in many marine organisms in comparison to mammals due to osmolarity changes in their external environment at different seasons of the year. Apart from the role of supplying of metabolic energy, FAAs are also known as important solutes in the intercellular osmolyte pools of numerous marine invertebrates and marine fishes (Gilles, 1975; Goldstein and Perlman, 1995), because these raise

internal osmotic pressure without disturbing normal cellular functions (Yancey et al., 1982). Amino acids are also known to make a substantial contribution to the intracellular osmotic pressure in carp red blood corpuscles (RBCs), thereby playing an important role in cellular volume regulation in some euryhaline teleosts such as flounder, eel and trout (Fugelli, 1967; Fugelli and Zachariassen, 1976; Fincham et al., 1987; Garcia and Romeu et al., 1991). Accordingly, when confronted by stress of changing environmental salinity and extracellular fluid osmolarity, these cells minimize cell volume alterations largely by adjusting intracellular FAAs concentrations, while hypertonic stress causes their accumulation. The mechanisms regulating these changes in FAAs pools have been studied predominantly in invertebrates and also in mammalian tissues such as in cardiac cells (Thurston et al., 1981), Erlhich cells (Hoffmann and Lambert, 1983; Lambert, 1984; Lambert and Hoffmann, 1993), astrocytes (Pasantes-Morales et al., 1990, 1993; Sanchez-Olea et al., 1991) and renal MDCK cells (Sanchez-Olea et al., 1991). This regulatory mechanism is generally attributed to the changes in the cell permeability and transport of amino acids (Pierce and Greenberg, 1972) and intracellular amino acid catabolism and/or synthesis (Gilles, 1975; Schoffeniels, 1976; Bishop and Burton, 1993). Various investigations have also been made to address the mechanisms regulating intracellular amino acid concentrations in fish species. The hagfish, *Myxine glutinosa* (Chollete et al., 1970), the little skate, *Raja erinacea* (Forster and Goldstein, 1976; Goldstein and Mush, 1994), the rainbow trout (Michael et al., 1994), and several marine fish species (Thoroed and Fugelli, 1994) have been found to decrease intracellular amino acid pools significantly upon adaptation to hypotonic environments. Taurine was found to be the principal osmoeffector in the muscle of the mudskipper, *P. cantonensis* (Iwata et al., 1981) and

carp, *C. carpio*, and in the brain of a marine elasmobranch, *Raja erinacea* (Forster et al., 1976). In general, the principal FAAs involved in osmoregulation in fishes and invertebrates are taurine, glutamic acid, glycine, serine, proline, alanine and histidine (Jürrs and Bastrop, 1995). The amino acid oxidation has also been shown to play a vital role in the cell volume regulation in lower vertebrates (King et al., 1980; Moyes et al., 1986). Relatively large amount of glutamate plus glutamine was also found to be involved in osmoregulation in trout hepatocytes (Michael et al., 1994). The adjustment of intracellular FAA concentrations in response to osmotic stress has also been widely documented among crustaceans as well as in other marine invertebrates (for reviews, see Gilles, 1975; Schoffeniels, 1976; Pierce, 1981). Rapid accumulation of alanine and proline was observed during acclimatization to hypertonic stress in the intertidal copepod, *Tigriopus californicus* (Burton, 1986, Burton and Feldman, 1983). FAAs were also reported to be major organic osmolytes for intracellular osmoregulation in marine and brackish water mollusks (Lynch and Wood, 1966; Pierce, 1971; Bedford, 1971; Matsushima et al., 1984). Increase in the levels of various FAAs such as alanine, aspartate, glutamine, glutamate, glycine and tyrosine were reported under hypertonic media in mammalian tissues (Olson and Goldfinger, 1990; Beetsch and Olson, 1993; Hoffmann and Dunham, 1995; Beetsch and Olson, 1998; Lang et al., 1998).

Cell Volume Regulation

Cell volume depends on water fluxes across the cell membrane, which is generally highly permeable to water and cannot resist a hydrostatic pressure of magnitude, thus the cell and its environment always maintain osmotic equilibrium (Dick, 1970; Macknight and Leaf, 1977; Macknight, 1988). Hence, any osmotic imbalance across the cell membrane will lead to respective alterations in cell volume. The net

movements of water between cells and their surrounding fluids thus depend on the osmolalities of these compartments. Many factors challenge the constancy of cell volume. These include alterations of extracellular osmolarity, nutrient uptake, activation of ion channels and transport systems at the cell membrane, formation or cleavage of protein or glycogen from or to osmotically more active monomers, and the degradation of organic substances to CO_2 and water (for review, see Lang et al., 1998). Concentrations of intracellular electrolytes (in particular Na^+ and K^+) are highly conserved across all animal kingdoms and phyla. This high level of conservation reflects an evolutionary ancient optimization of the fundamental metabolic machinery of cells to function at a particular ionic milieu. Thus, cells restore their conserved ionic environment, chiefly by adjusting their levels of compatible osmolytes (Somero and Yancey, 1997). It is now generally recognized that cells of poikilosmotic animals have the capability of regulating their volume when submitted to osmotic shocks (Gilles, 1975, 1978, 1979). This phenomenon has considerable adaptive value since it enables the cells of these species to cope with very large osmolarity changes occurring at the blood level in different conditions of water availability in the environmental medium. The phenomenon of cell volume maintenance (at isotonic conditions) and regulation (in anisotonic conditions) were first studied on mammalian cells (Leaf, 1956; Kleinzeller, 1972).

Most of the present-day fishes (marine and freshwater) had a period of existence in their evolutionary history that required them to adapt to an environment that placed a severe osmotic stress on them and necessitated the development of osmoregulatory mechanisms that have enabled them to cope with this stress (Lockwood, 1966). These mechanisms had to deal with both the osmotic relationships

between the internal and external fluids. In fact there are fishes that leave the water habitat periodically (e.g., lungfishes, Smith, 1930) and are to cope with the desiccating effects of a terrestrial environment as well as the increase in extracellular fluid osmolality caused by accumulation of metabolic products that are concentrated in the extracellular fluids (e.g., sulfate). Thus, cells of these fishes must have mechanisms to prevent or decrease a reduction in cell volume caused by a hypertonic extracellular environment (Goldstein and Kleinzeller, 1987).

In invertebrates and lower vertebrates, changes in both inorganic electrolytes and organic solutes participate in the osmotic adjustments, which take place inside the cell during osmotic stress (Gilles, 1975). When living cells are suddenly exposed to hypotonic medium, they initially swell like a more or less perfect osmometer, because of water entrance, but within minutes retain almost their original volume. This behaviour has been considered a regulatory volume decrease (RVD). During the phase of volume regulation, under hypotonic stress cells lose ions like K^+, Na^+, Cl^- and possibly also some larger molecules like FAAs and/or other molecules, depending on cell types (Cholette et al., 1970; Schmidt-Nielson, 1975; Vislie and Fugelli, 1975; Hoffman and Hendil, 1976; Boyd et al., 1977). Conversely, upon sudden exposure to hypertonic medium, the cells shrink like more or less perfect osmometer, but display within minutes a volume regulatory increase (RVI) (Schmidt-Nielson, 1975, 1977), which brings back cell volume largely to the starting level. Although most of the cell types have RVD or RVI mechanisms to maintain the cell volume under anisotonic conditions created externally or internally, it is now well established that the cell remains partly swollen under hypotonic condition and partly shrunken under hypertonic conditions as long as the cells are exposed to anisotonic conditions (Fig.1)

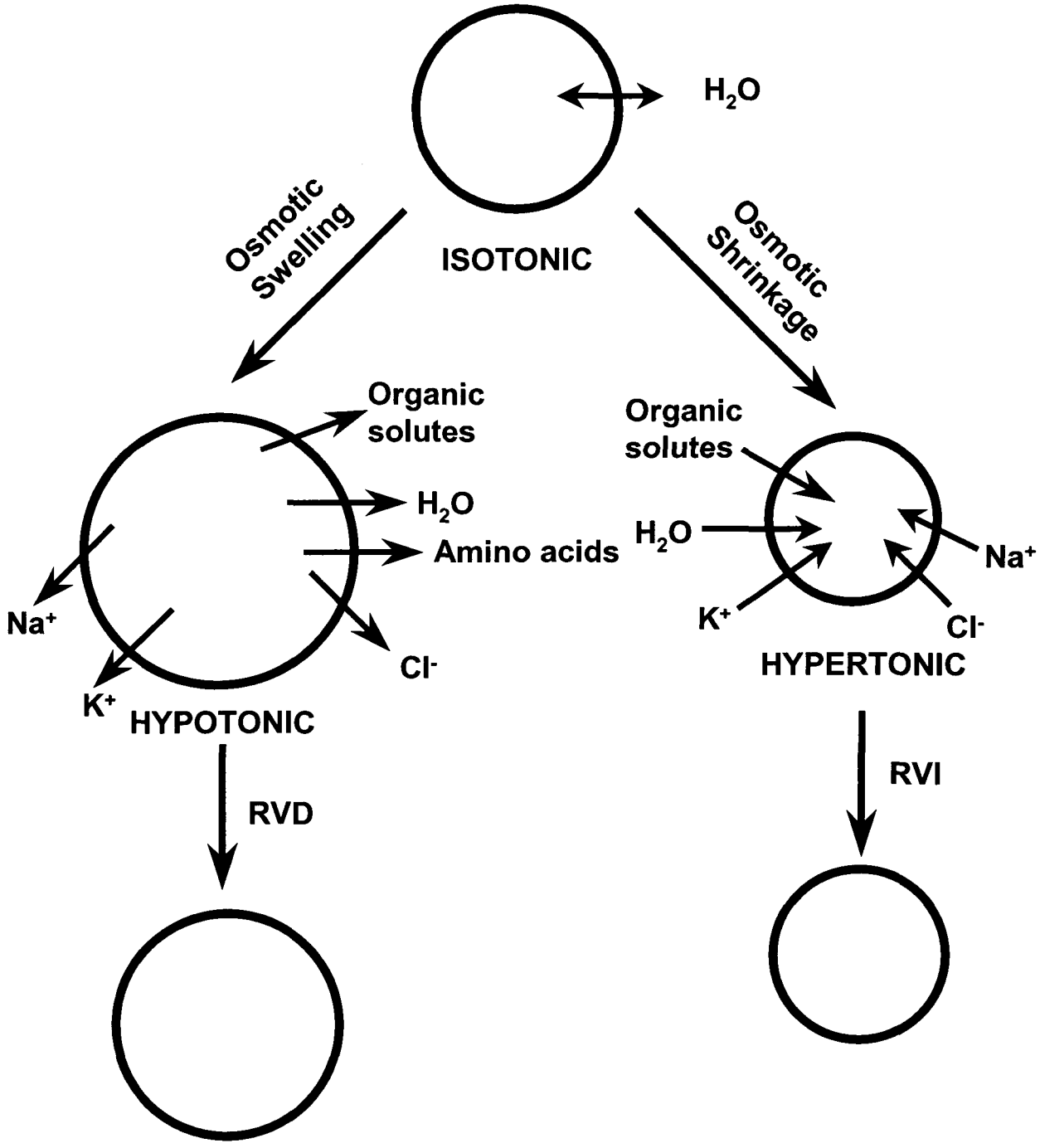


Fig: 1 Hepatic cell volume regulation under anisotonic conditions.

RVD - Regulatory Volume Decrease

RVI - Regulatory Volume Increase

(for review, see Häussinger and Lang, 1991a; Häussinger et al., 1994). These changes in cell volume and hydration status of the cell are reported to cause various metabolic changes in the mammalian system such as the changes in protein, carbohydrate, nitrogen and amino acid metabolism, lipogenesis, and urea synthesis from ammonia and other nitrogenous sources (for review, see Häussinger and Lang, 1991; Häussinger et al., 1994a). Further, cell volume changes also influence the reduced glutathione (GSH) and oxidized glutathione (GSSG) efflux from the rat liver (Saha et al., 1992, Häussinger et al., 1990a), taurocholate excretion in bile (Häussinger et al., 1992, 1993), taurine and betaine efflux (Wettstein et al., 1998, Warskulat et al., 1997), actin polymerization (Theodorpoulos et al., 1992), microtubule stability (Häussinger et al., 1994b), exocytosis (Häussinger et al., 1993), and intracellular pH (Schrieber et al., 1994; Lang et al., 1994) etc. Oxidative stress, resulted due to intracellular generation of hydrogen peroxide, is reported to cause cell shrinkage by opening of K^+ channel and more efflux of GSSG from the perfused rat liver (Saha et al., 1993). Further, it has been suggested that cellular shrinkage in skeletal muscle and liver in response to a variety of mediators of inflammation, including oxidative stress, might be one of the important triggers for the changes of protein catabolic state (Häussinger et al., 1993). Various studies have been carried out in fishes regarding the mechanisms of cell volume regulation (Bianchini et al., 1988; Jensen 1995; Perlman et al., 1996), but few informations are available on alterations of different metabolic processes in relation to cell volume changes. More recently, the effects of cell volume changes on glycogen metabolism (Goswami and Saha 1998), gluconeogenesis, hexose monophosphate pathway (HMP) (Goswami et al., 2004), protein synthesis, proteolysis and nitric oxide production (Saha and Biswas, unpublished results) have been reported in the perfused

liver of air-breathing catfish, *C. batrachus*. These effects of anisotonicity may add to other recently described effects of cell volume on hepatic carbohydrate metabolism, such as inhibition of glycogenolysis and gluconeogenesis and stimulation of glycogen synthesis during cell swelling, and vice versa during cell shrinkage.

Most cells in higher animals are isotonic to their external fluid. Intracellular osmotic effectors present in fish tissue include both inorganic and organic solutes; both are important for the volume regulation of the cell and together balance the osmolality of the extracellular fluid. Although the inorganic ions play a role in cell volume regulation in fish (Cala, 1977; Fugelli and Rohrs, 1980; Lauf, 1982), this role is limited by the need to keep the ionic composition of the cells relatively constant in order to provide a setting with metabolic processes and electrical properties of the cells. Despite the importance of osmoregulation, little is known about the distribution and relationship of ions and organic solutes between the major body compartments during processes of cell volume regulation in fish. In flounder RBC, volume regulation after swelling was characterized by net K^+ and water loss. When osmotically shrunken, these cells return to their original volume by influx of Na^+ and anions with water (Cala, 1977). In fact, mechanisms of volume-sensitive K^+ transport in RBC vary between different fish species. 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)-sensitive KCl transport and RVD in toadfish RBC was first demonstrated by Lauf (1982). The role of amino acids as intracellular osmolytes has also been demonstrated in a variety of fish species including hagfish (Cholette et al., 1970), the little skate and stingray, *Dasyatis sabina* (Forster and Goldstein, 1976; Boyd et al., 1977), and a variety of euryhaline teleosts (Lang and Fugelli, 1965; Huggins and Colley, 1971; Colley et al., 1974; Ashokas and Sorg, 1977).

The amino acid taurine (2-aminoethanesulfonic acid) is present in almost all animal tissues, and it is one of the most abundant free amino acids (Fig. 2). Taurine plays an important role in cell volume regulation in both vertebrates and invertebrates. Amongst the amino acids used for cell volume regulation in fish, taurine is reported to play most important function (Laserre and Gilles, 1971; Colley et al., 1974; Fugelli and Zachariassen, 1976; Boyd et al., 1977; Leader, 1981). In addition, taurine has long been recognized as an important intracellular osmolyte in invertebrates (Simpson et al., 1959), and more recently its role has been documented in the cell volume regulation of mammalian heart (Thurston et al., 1981). In metabolic terms, taurine is a relatively inert compound in fish tissues. Besides having protein stabilizing qualities, they do not interfere with metabolic processes such as protein biosynthesis. Taurine is not oxidized by fish tissues, thus it has to be eliminated from the extracellular fluid by excretion (King and Goldstein, 1986). Schrock et al. (1982) reported that taurine is excreted by kidney of marine fishes both by filtration and secretion. Secretion of taurine through renal sources, thus provides an important mechanism for the regulation of plasma taurine levels, thus playing a critical role in maintenance of cell volume in stress conditions by way of RVD or RVI. Many marine invertebrate species (Simpson et al., 1959), hagfish (Roberston, 1976) and elasmobranchs (for a review, see Goldstein and Perlman, 1995) accumulate amino acids in intracellular compartments to counterbalance the osmotic pressure of sea water. The most commonly occurring amino acids as osmolytes in these organisms are glycine, alanine, proline, β-alanine and taurine (for a review, see Yancey et al., 1982).

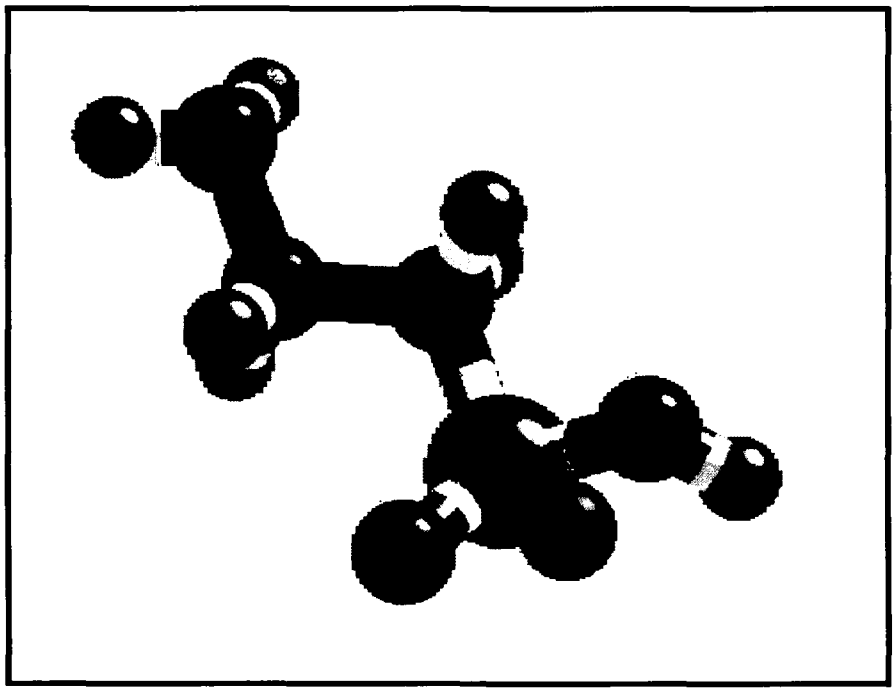


Fig: 2 Structure of 2-Aminoethanesulphonic Acid (Taurine)

$C_2H_7NO_3S$

There is a close relationship between cell volume homeostasis and amino acid transport. On one hand, cumulative, mostly Na^+- driven amino acid uptake into the cells creates an intra/extracellular osmotic gradient, which shifts free water into the cells. This leads to cell swelling, and volume regulatory mechanisms are required to defend the constancy of cell volume. On the other hand, some cells accumulate or release amino acids in response to cell volume challenges in order to balance the intracellular osmolarity. Thus, amino acid transport is involved in both, cell volume perturbations and regulation. It became increasingly clear that alterations of cell volume markedly influence a variety of metabolic pathways and modify cell growth and proliferation. Specifically in the liver, metabolic functions, such as protein and carbohydrate metabolism, which result in the formation or disappearance of osmotically active small molecules, are extremely sensitive to alterations of cell volume. Furthermore, indirectly or directly, cell volume changes modify a number of metabolic functions with no obvious impact on intracellular osmolarity. It appears that cell swelling and cell shrinkage lead to certain opposite patterns of cellular metabolic function. Apparently, amino acids and also hormones can trigger those patterns simply by altering cell volume. The freshwater amphibious air-breathing walking catfish (*Clarias batrachus*), that are found predominantly in the Indian subcontinent, spend a substantial part of their lives on mudflats in response to habitat drying and are observed to migrate terrestrially during wetter periods (Liem, 1987). This facultative air-breather usually inhabits stagnant, slow-flowing swampy water bodies or wet lands, which are often covered with macrovegetation such as water hyacinth, and these waters are also characterized by low dissolved oxygen, high bicarbonate and ammonia levels (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 the mud to avoid total dehydration. In addition to the presence of a functional OUC (Saha and Ratha, 1989, 1998; Saha et al., 1999), this air-breathing catfish has the potential to switch from ammoniotelism to ureotelism under hyper-ammonia stress (Saha and Das 1999, Saha et al., 2003), and also while exposed to air or living inside the mud (Saha and Das, unpublished data). Another unique characteristic feature reported in this catfish is the extreme tolerance to a very high concentration of ambient ammonia, surviving exposure upto 75 mM NH_4Cl for several weeks without any mortality (Saha et al., 2003, Das, 2000). One of the major reasons for tolerating such a high ambient ammonia by these catfishes was suggested to be due to the presence of a functional OUC both in hepatic and in some extra-hepatic tissues (Saha and Ratha, 1987; 1989; Saha et al., 1999; Das, 2000).

In addition of facing the problems of environmental ammonia toxicity as well as the endogenously produced ammonia toxicity, these air-breathing fishes also face the problem of osmotic stress in their natural habitat, such as in summer when the environmental water gets concentrated due to drying out of ponds and lakes, and in the monsoon season the environmental water gets diluted due to excessive rain. Therefore, it would be interesting to study the effect of osmotic stress on nitrogen metabolism in these air-breathing fishes.

OBJECTIVES:

Thus, from the foregoing studies and reports about the extreme habitat of different species of Indian air-breathing freshwater teleosts, and also from the reports mainly from our laboratory on various aspects of nitrogen metabolism, it would be

interesting to study the following in one of these air-breathing fishes (*Clarias batrachus*):

1. The effects of osmolarity stress on amino acid metabolism and ureogenesis in the perfused liver,
2. The effects of hypertonic stress on the whole animal on amino acid metabolism and ureogenesis,
3. The role of taurine in cell volume regulation in the isolated RBC in vitro condition.

PLAN OF WORK:

I To study the effects of anisotonicity on nitrogen metabolism and ureogenesis in the perfused liver of *C. batrachus*.

a. *C. batrachus* liver was perfused with isotonic (265 mOsmol/l) medium with/without 5 mM NH_4Cl, followed by infusion of hypotonic (-80 mOsmol/l) or hypertonic (+80 mOsmol/l) medium for 90 min, and the following parameters were measured:

- Changes in the concentration of different free amino acid (FAAs) in the perfused liver.
- Changes in the activity of different amino acid metabolism-related enzymes such as glutamine synthetase (GSase), glutamate dehydrogenase (GDH), aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) in the perfused liver.

b. *C. batrachus* liver was perfused with iso-, hypo- and hypertonic media along with infusion of 1 mM NH_4Cl, and the efflux of ammonia-N, urea-N,

glutamate and glutamine from the perfused liver in the effluent were measured.

c. *C. batrachus* liver was perfused with iso-, hypo- and hypertonic media along with the infusion of 0.25 mM glutamine, and the efflux of ammonia-N, urea-N, glutamate and glutamine from the perfused liver in the effluent were measured.

d. *C. batrachus* liver was perfused with iso-, hypo- and hypertonic media along with the infusion of amino acids, and the efflux of ammonia-N, urea-N, and total-N from the perfused liver in the effluent were measured.

II **To study the effect of hypertonicity on nitrogen metabolism in the whole fish.**

a. *C. batrachus* was exposed to hypertonic environment (250 mOsmol/l mannitol) for 7 days and the following parameters were measured:

- Changes in the concentrations of FAA, in different tissues such as liver, kidney, muscle, brain and in the plasma.
- Changes in the activity of different amino acid metabolism related enzymes as mentioned above in different tissues.
- Changes in the rate of excretion of ammonia and urea-N by the fish exposed to hypertonic medium.
- Changes in concentration of ammonia and urea-N in different tissues and in the plasma.
- Changes of activity of OUC enzymes i.e., carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase

(ASS), argininosuccinate lyase (ASL) and arginase (ARG) in liver and kidney tissues.

- Changes of osmolarity of blood plasma.

III To study the role of taurine (Tau) in cell volume regulation in the isolated RBC of *C. batrachus* during osmotic stress.

a. Erythrocytes were isolated from the *C. batrachus* blood by centrifugation and were exposed to iso- and anisotonic media, and the following parameters were measured:

- Tau concentration in the isolated RBC of *C. batrachus* under iso- and anisotonic conditions pre-incubated with/without 2 mM Tau.
- Tau uptake by the isolated RBC pre-incubated at different concentrations of Tau for 2 h under iso- and anisotonic conditions, while maintaining the osmolarity of the media with NaCl.
- Tau uptake by the isolated RBC pre-incubated at different concentrations of Tau for 2 h under iso- and anisotonic conditions, while maintaining the osmolarity of the media with mannitol.
- Maximum Tau uptake by the isolated RBC of *C. batrachus* under iso- and anisotonic conditions.
- Effect of time on Tau efflux by the isolated RBCs under hypotonic, hypotonic + DIDS, and hypertonic conditions while maintaining the osmolarity of the media with NaCl.

- Effect of time on Tau efflux by the isolated RBCs under hypotonic, hypotonic + DIDS, and hypertonic conditions while maintaining the osmolarity of the media with mannitol.
- Maximum Tau efflux by the isolated RBC of *C. batrachus* under hypotonic, hypotonic + DIDS, and hypertonic conditions.
- Effects of different osmolarities on Tau efflux.
- Effects of anisotonicity on water content in the isolated RBC preincubated with/without Tau.

MATERIALS AND METHODS

Experimental animal:

The fish, *Clarias batrachus*, weighing 85 ± 15 body weight, were purchased from commercial sources and acclimatized in the laboratory approximately for 1 month at a constant room temperature (28 ± 2 °C) with a 12 h : 12 h light and dark photoperiod before using for experiments. Minced pork liver and rice bran was given as food (5% of the body weight) everyday and the water was changed regularly after feeding. The fishes were used for experiments when death rate became zero and the food consumption was normal. Food was withheld 24 h prior to and during experiments. No sex differentiation of the fish was done while performing these studies.

Liver perfusion technique:

The fish was anaesthetized in neutralized 3-aminobenzoic acid ethyl ester (MS-222, 0.2 g/litre) for 2 min before operation for liver perfusion. Liver was perfused via the portal vein in a non-circulating manner with a haemoglobin-free medium as used by French et al. (1981) with certain modifications made by Saha et al. (1995). The blood osmolarity (determined by freezing point depression method) was found to be 265 mOsmol/litre. Therefore, the osmolarity of the standard medium was also maintained at same osmolarity, and was prepared by mixing 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 of $CaCl_2$ as a basic solution for perfusion. The medium also contained 1.2 mM lactate and 0.3 mM pyruvate. The medium was gassed with $0_2/C0_2$ (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 7.6 after gassing, since the blood pH of this walking catfish ranges between 7.5 to 7.7. The temperature of the medium was maintained at 30 °C. Ammonia, glutamine and amino

acids were infused along with the standard medium under different experimental conditions. Effluents were collected at 2 min intervals for various analysis. Anisotonicity of the medium was maintained either by withdrawing or by adding equivalent amount of NaCl or mannitol to the standard perfusion medium.

Perfusion experiment:

Livers were initially perfused for 30 min with isotonic medium, followed by infusion of hypo- or hypertonic medium in separate experiments for 90 min. After 90 min of perfusion with hypo- or hypertonic medium, the livers were removed, plunged into liquid nitrogen, and stored at – 60 °C for the analysis of free amino acids (FAAs) and also for assaying different amino acid metabolism-related enzymes. All the enzyme assays and analysis of FAAs were completed within two weeks of preserving the tissue. Another set of perfusion was also done under identical conditions, where, 5 mM NH_4Cl was also infused along with the hypo- or hypertonic medium for 90 min.

In another set of experiment, livers were perfused initially for 30 min with isotonic medium (265 mOsmol/l), containing either 1 mM NH_4Cl or 0.25 mM glutamine along with hypo- or hypertonic medium for 20 min in separate experiments. The effluents were collected at 2 min intervals for the measurement of efflux of ammonia, urea-N, glutamate and glutamine by the perfused liver.

In another set of experiment, livers were perfused initially for 20 min with isotonic medium (265 mOsmol/l), followed by infusion of different amino acids (such as glutamine, glutamatic acid, aspartic acid, alanine, glycine, threonine, serine) at a concentration of 5 mM along with either hypo- or hypertonic medium separately for 20 min, followed by infusion of again isotonic medium for 20 min. Effluents were collected

at 2 min intervals for the measurement of efflux of ammonia-N, urea-N and total-N from the perfused liver.

Mannitol exposure experiment:

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

A set of fish (preweighed) was exposed individually in plastic buckets containing 1 litre of 250 mOsmol/l mannitol in each bucket. Mannitol solution was prepared in bacteria free filtered water and the fishes were exposed to mannitol for 7 days. Another set of fish was kept individually in plastic buckets containing 1 liter of bacteria free filtered water, which served as controls. Both the mannitol solution and the water from each bucket were replaced with a fresh medium at 24 h interval. On day 7, all fishes treated either with mannitol or kept in water were removed, killed by decapitation after collection of blood from the caudal vein, and tissues such as the liver, kidney, muscle and brain were dissected out. Tissues were immediately plunged into liquid nitrogen and stored at – 60 °C for the analysis of free amino acids (FAAs), tissue ammonia and urea levels and also for assaying the activity of different enzymes related to amino acid metabolism and OUC. All the analysis were completed within two weeks of collecting the tissue.

Estimations:

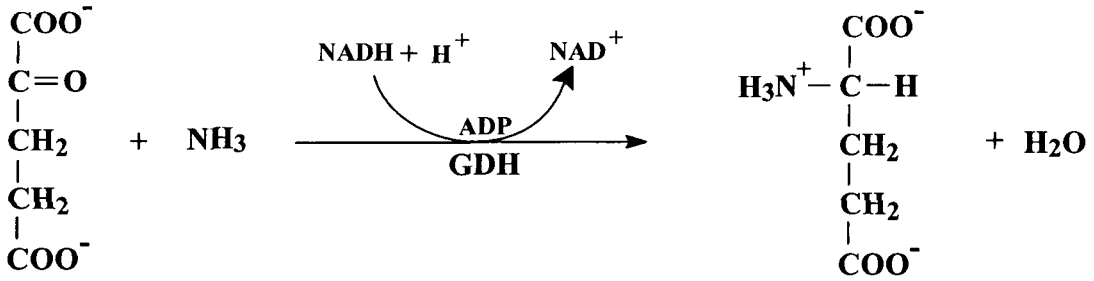
Estimation of ammonia and urea-N:

The rate of excretion of ammonia and urea-N by the fish as well as the concentrations in the effluents under different experimental conditions were measured enzymatically based on the procedure of Kun and Kearny (1974). To each 2 ml sample of effluent, collected during the perfusion or the samples collected during mannitol experiment, 20 µl of 2 M perchloric acid (PCA) was added to precipitate out the protein present in the sample. The precipitate was separated out by centrifugation and the supernatant was neutralized by adding 20 µl of 2 M NaOH before estimation 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 oxidised was equivalent to the amount of ammonia present in the sample.

The reaction takes place as follows:



α-Ketoglutarate L-Glutamate

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

Tris-HCl buffer (pH 8.0)	100 µmoles
α-ketoglutarate	2.5 µmoles
EDTA	0.2 µmole
ADP	1.0 µmole
NADH	0.4 µmole
GDH	2 units
Sample	0.2 ml

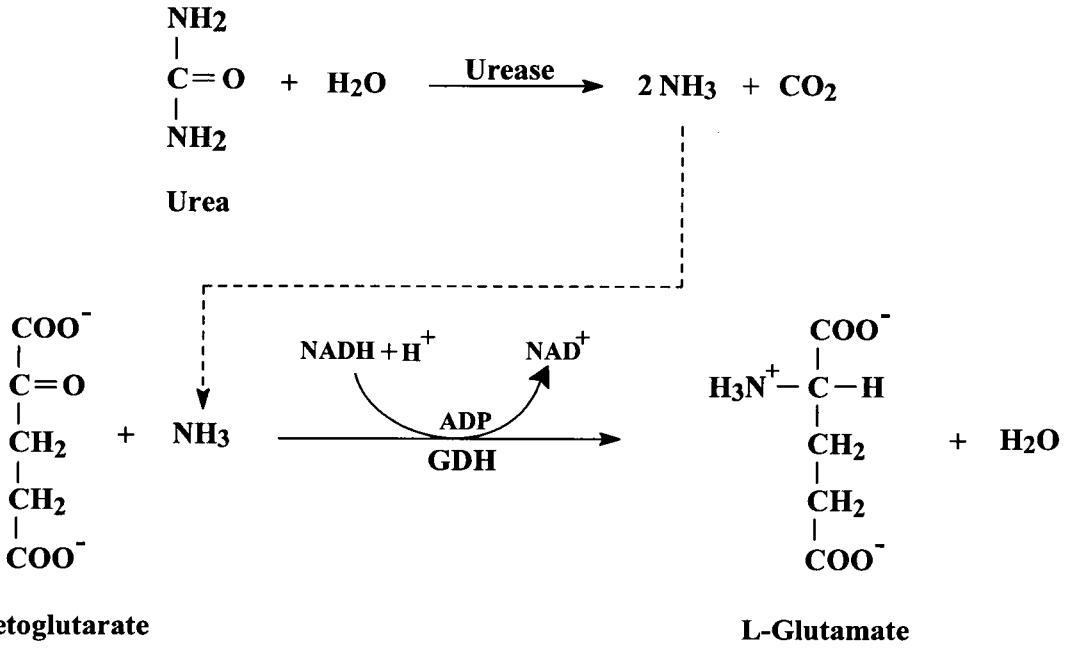
The reaction mixture was incubated for 30 min at 37 °C. A reagent blank was also prepared which contained everything in the reaction mixture as mentioned above except the sample, which was replaced by 0.2 ml of distilled water. The optical density (O.D.) was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer (Beckman, Model 640) both in the reagent blank and in the experimental samples. The differences in O.D. values obtained between these two

were used to calculate the concentration of ammonia present in the effluent taking 6.22 x 10^6 as molar extinction coefficient value for NADH.

Urea-N:

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

The enzymatic reaction takes place as follows:



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

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

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

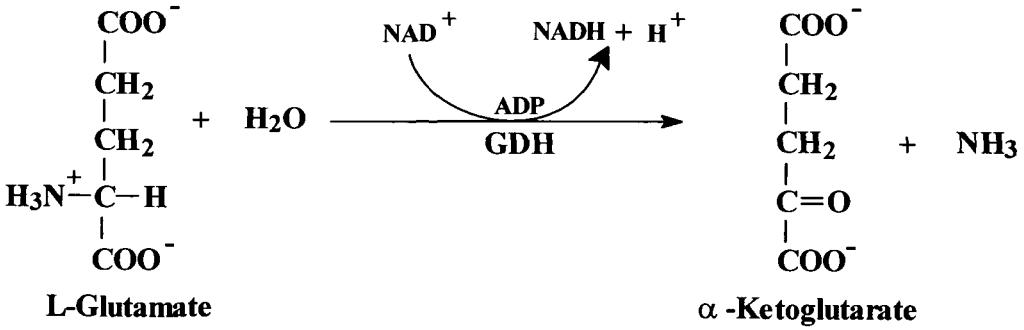
Glutamate and Glutamine:

Glutamate and glutamine concentrations were estimated enzymatically following the method of Bergmeyer (1974).

Glutamate:

For measurement of glutamate in the sample, all the glutamate was converted by the enzyme GDH to α-ketoglutarate and ammonia. The amount of NAD^+ reduced was equivalent to the amount of glutamate present in the sample.

The enzymatic reaction takes place as follows:



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

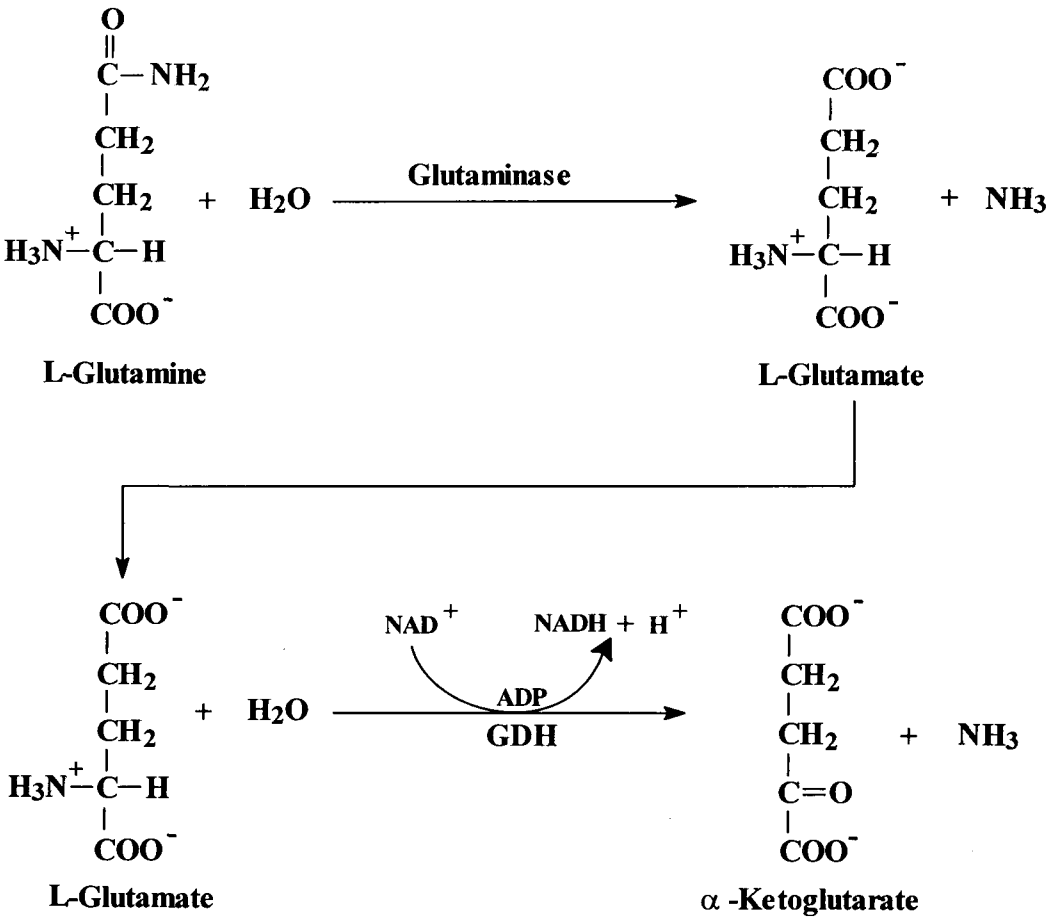
Na-acetate buffer (pH 5.O)	280 µmoles
Glycine	502 µmoles
ADP	0.96 µmole
NAD	0.63 µmole
Hydrazine hydrate	410 µmoles
GDH	10 units
Sample	0.1 ml

This reaction mixture was incubated for 30 min at 37 °C. A reagent blank was also prepared containing everything in the reaction mixture as mentioned above except the sample, which was replaced by 0.1 ml distilled water. Then the O.D. was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer both in the reagent blank and in the experimental samples. The differences in O.D. values obtained between these two were used to calculate the concentration of glutamate present in the sample taking 6.22×10^6 as molar extinction coefficient for NADH.

Glutamine:

For measurement of glutamine in the sample, all the L-glutamine was converted by the enzyme glutaminase to L-glutamate and ammonia. Then L-glutamate is converted by the enzyme GDH to α-ketoglutarate and ammonia. The amount of NAD reduced was equivalent to the amount of both glutamate and glutamine present in the sample. Further, the amount of glutamine was calculated by substrating the amount of glutamate present in each sample from that of total value of glutamate and glutamine.

The enzymatic reaction takes place as follows:



Initially, 0.2 ml of reaction mixture contained the following:

Na-acetate buffer (pH 5.0)	280 µmoles
Glutaminase	0.5 unit
Sample	0.1 ml

This reaction mixture was allowed to react for 1 h at 37 °C so that all the glutamine got converted to glutamate, followed by addition of 1 ml of same reaction mixture as used for glutamate estimation and incubated again at 37 °C for 30 min. A reagent blank was also prepared which contained everything in the reaction mixture as mentioned above except the sample, which was replaced by 0.1 ml distilled water. Then the O.D. was measured at 340 nm in a 1 ml quartz cuvette having 1 cm light path in a UV-visible spectrophotometer both in the reagent blank and in the experimental sample. The differences in O.D. values obtained between these two were used to calculate the concentration of glutamine present in the sample taking 6.22×10^6 as molar extinction coefficient for NADH.

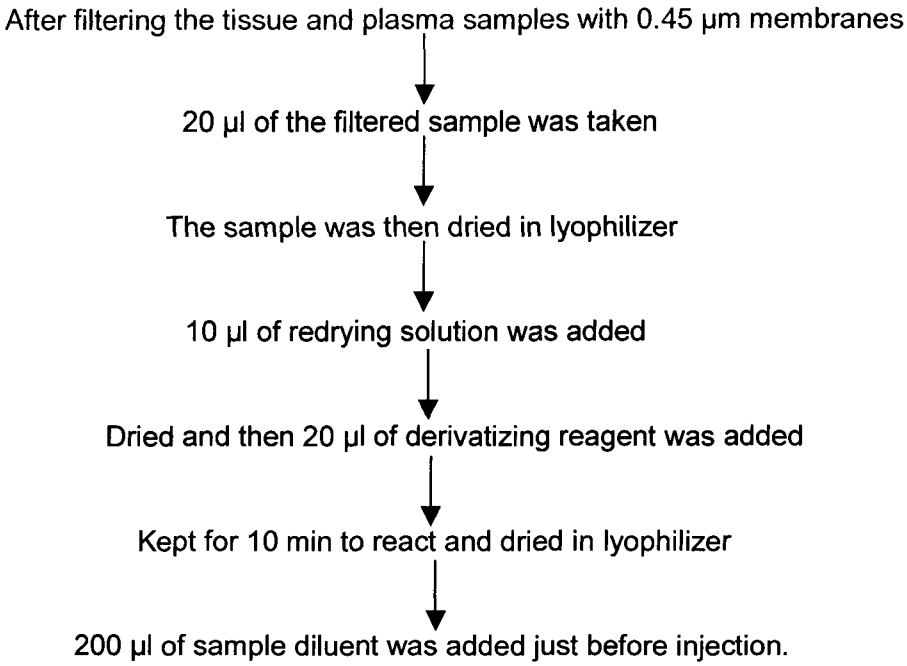
Protein Estimation:

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

Free Amino Acid Analysis:

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

Sample preparation for HPLC:



The derivatized sample was then analyzed with a Waters HPLC with a pre-column derivatization method. FAAs was derivatized with phenyl isothiocyanide (PITC) with the standard protocol provided by the company and was separated out with a Pico Tag column (Waters, USA). The eluted amino acids was detected in a UV-detector (Model 486) at 254 nm coupled with Millinium[32] data processor for quantification of the eluted peak areas. Two eluting mobile phases were used in a gradient for 72 min, to separate out all the FAAs. Eluent A (70 mM Na-acetate buffer, pH-6.5) and eluent B (60% acetonitrile). Before starting the run, the column oven temperature was set at 38 °C and the column was purged with 90% eluent A and 10% eluent B and equilibrated for stabilization of the baseline. The run was started with 100% mobile phase A and the flow rate was set at 0.5 ml/min through out the run. In the first 13.50 min, the linear gradient progressed to 3% mobile phase B, followed by increase to 6% mobile phase B

in 24 min, 9% increase of mobile phase B in 30 min, 34% linear increase of mobile phase B in 50 min and was held there till 62 min. The gradient was then increased to 100% mobile phase B from 62.01 min and continued till 72 min. After the gradient elution was complete, the column was washed thoroughly with HPLC grade water for 30 min and the column was again re-equilibrated with 100% eluent A before subsequent injections. The mixture of standard physiological FAAs (Sigma) containing 38 amino acids and some amino compounds was also eluted under identical conditions as mentioned above for identification and quantification of amino acids.

Tissue Processing for Assaying Amnio Acid Metabolism-Related Enzymes:

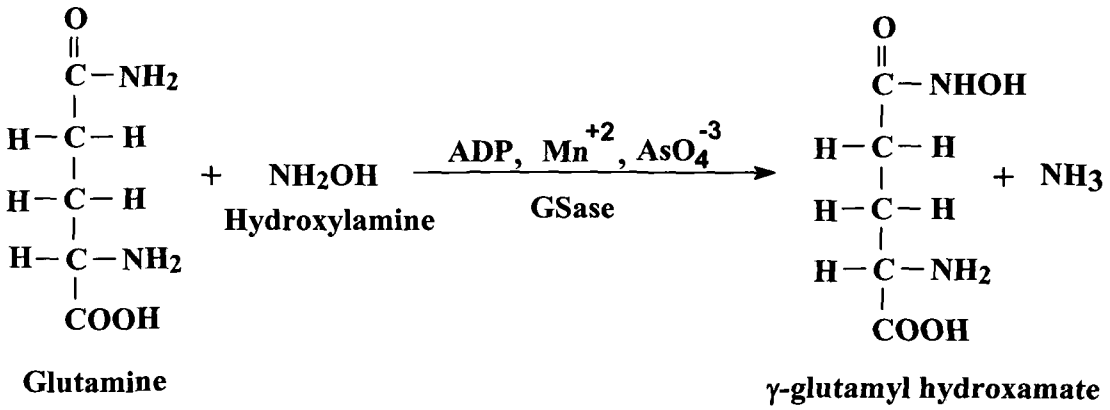
For assaying the activity of different amino acid metabolism-related enzymes, a portion of each frozen tissue was thawed on ice and a 10% homogenate (w/v) of each tissue was prepared with a motor driven Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle in a homogenizing buffer containing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

Enzyme Assay:

Glutamine synthetase (GSase; E.C. 6.3.1.2)

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

The enzymatic reaction takes place as follows:



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

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

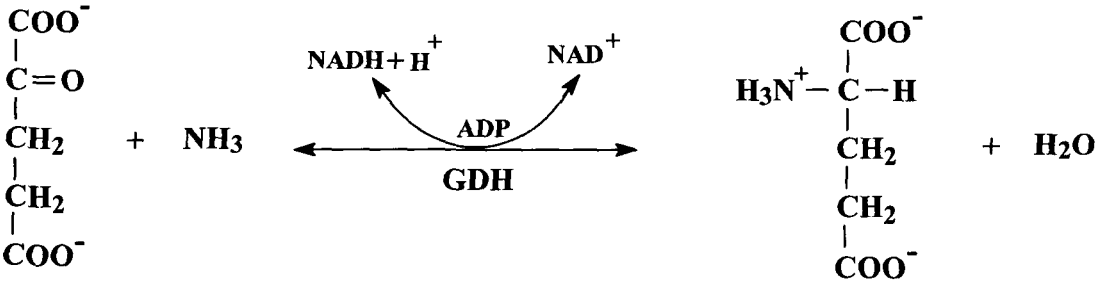
The assay mixture without the tissue extract was first preincubated for 5 min at 30 °C. The reaction was initiated by adding 0.2 ml of tissue extract and after 30 min of

incubation, the reaction was stopped by the addition of 1 ml of 20% PCA. A tissue blank was also prepared simultaneously by adding 1 ml of 20% PCA to the reaction mixture prior to the addition of the tissue extract. The precipitated protein was separated out by centrifugation at 10,000 rpm for 15 min and the amount of γ-glutamyl hydroxamate formed during the reaction was measured in the supernatant by adding 1.5 ml of $FeCl_3$ reagent (containing 0.37 M $FeCl_3$, 0.67 M HCl and 0.2 M TCA) and measuring the O.D. at 500 nm in a UV-visible spectrophotometer.

Glutamate dehydrogenase (GDH; E.C. 1.4.2.3)

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

The enzymatic reaction takes place as follows:



α-Ketoglutarate **L- Glutamate**

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

Tris-HCl buffer (pH: 7.5)	70 µmoles
α- Ketoglutarate	7 µmoles
NH_4Cl	225 µmoles
EDTA	0.25 µmole
ADP	3 µmoles
NADH	0.3 µmole
Tissue extract (suitably diluted)	25 µl

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

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

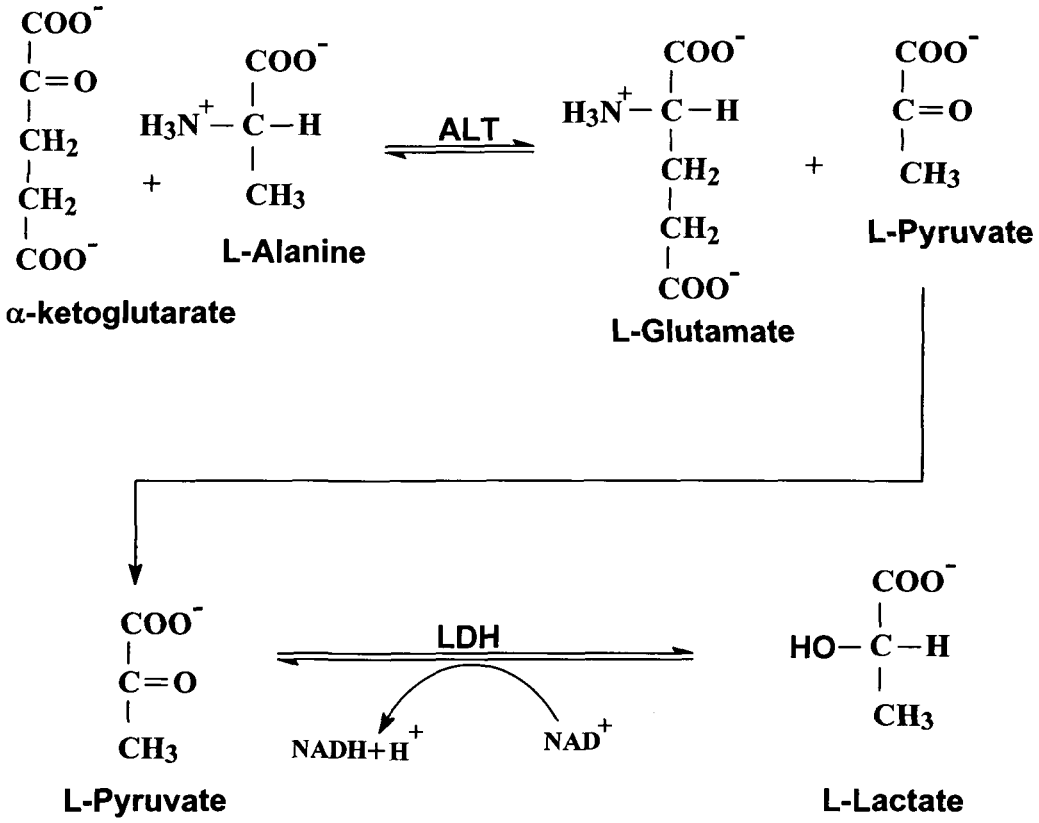
The assay mixture without the tissue extract (in both reductive amination and oxidative deamination) was pre-incubated at 30 °C for 5 min in a 1 ml quartz cuvette

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 was started by the addition of the tissue extract in the pre-incubated reaction mixture. The decrease in O.D. in case of reductive amination reaction and increase in O.D. in case of oxidative deamination reaction were recorded at 340 nm at 30 sec interval and the period of linear decrease / increase in O.D values were used for calculating the GDH activity. The amount of NADH oxidized in case of reductive amination reaction or the amount of NAD^+ reduced in case of oxidative deamination reaction was calculated taking 6.22 x 10^6 as the molar extinction co-efficient value for NADH for expressing the GDH activity. One unit of GDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 µmole of NADH to NAD^+ in case of GDH (reductive amination) or the reduction of 1 µmole of NAD^+ to NADH in case of GDH (oxidative deamination) per h at 30 °C.

Alanine aminotransaminase (ALT; E.C. 2.6.1.2)

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

The enzymatic reaction takes place as follows:



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

K-phosphate buffer (pH: 7.5)	125 μmoles
L-Alanine	125 μmoles
α-Ketoglutarate	15 μmoles
NADH	0.3 μmole

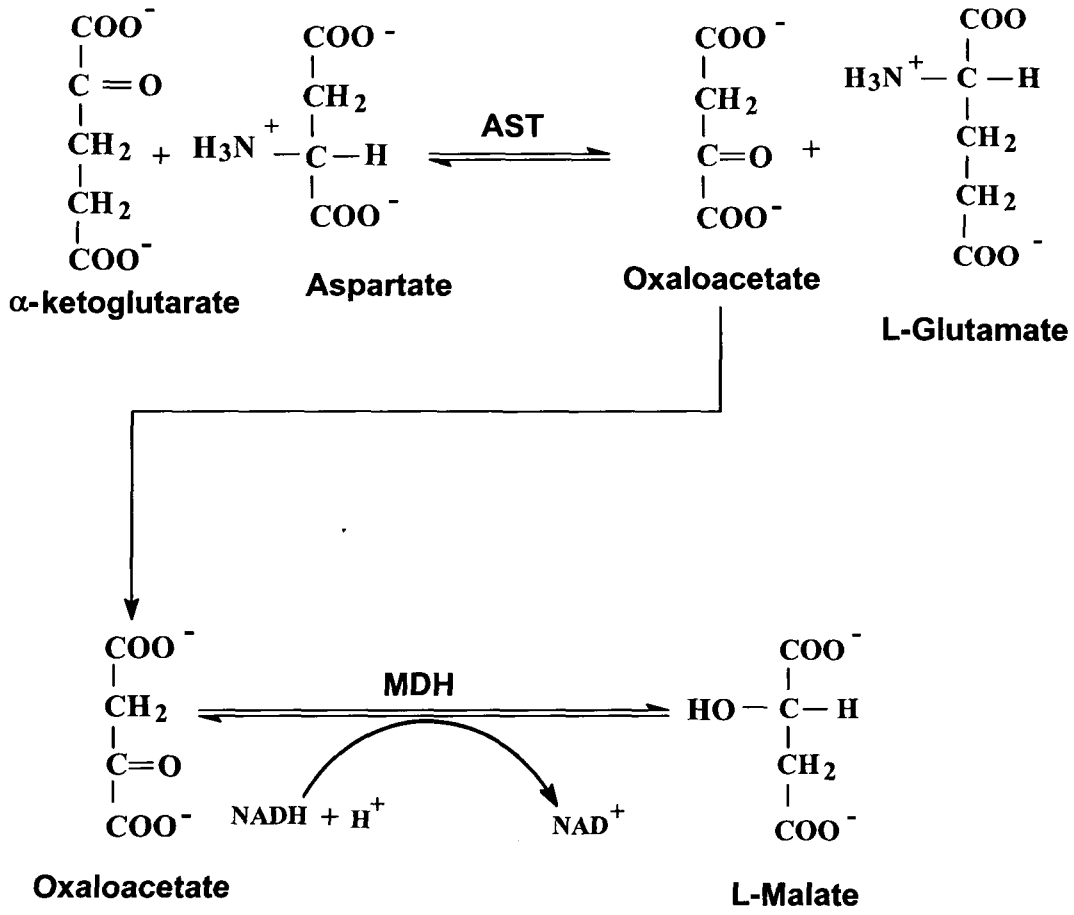
LDH (ammonia free)	10 units
Tissue extract (suitably diluted)	20 μl

The assay mixture was incubated at 30 °C in a 1 ml quartz cuvette having 1 cm light path directly in a UV-visible spectrophotometer (Beckman, Model DU 640) having a peltier temperature controlled system fitted to it. The reaction mixture without L-alanine was pre-incubated at 30 °C for 5 min. The reaction was started by addition of L-alanine into the reaction mixture. The decrease in O.D. was recorded at 340 nm at 30 sec interval. The period of linear decrease of O.D. was used for calculation of ALT activity. The amount of NADH oxidized per h was calculated taking 6.22×10^6 as molar extinction coefficient value for NADH. One unit of ALT activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to NAD^+ per h at 30 °C.

Aspartate aminotransaminase (AST; E.C. 2.6.1.1)

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

The enzymatic reaction takes place as follows:



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

K- phosphate buffer (pH: 7.5)	125 μmoles
L-Aspartate	125 μmoles
α-Ketoglutarate	15 μmoles
NADH	0.3 μmole
MDH (ammonia free)	10 units

Tissue extract (suitably diluted)	20 μl

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 DU 640) having a peltier temperature controlled system fitted to it. The reaction mixture without L-aspartate was pre-incubated at 30 °C for 5 min. The reaction was started by addition of L-aspartate into the reaction mixture. The decrease in O.D. was recorded at 340 nm at 30 sec interval. The period of linear decrease was used for calculation of AST activity. The rate of NADH oxidized per h was calculated taking 6.22×10^6 as molar extinction coefficient value for NADH. One unit of AST activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 μmole of NADH to NAD^+ per h at 30 °C.

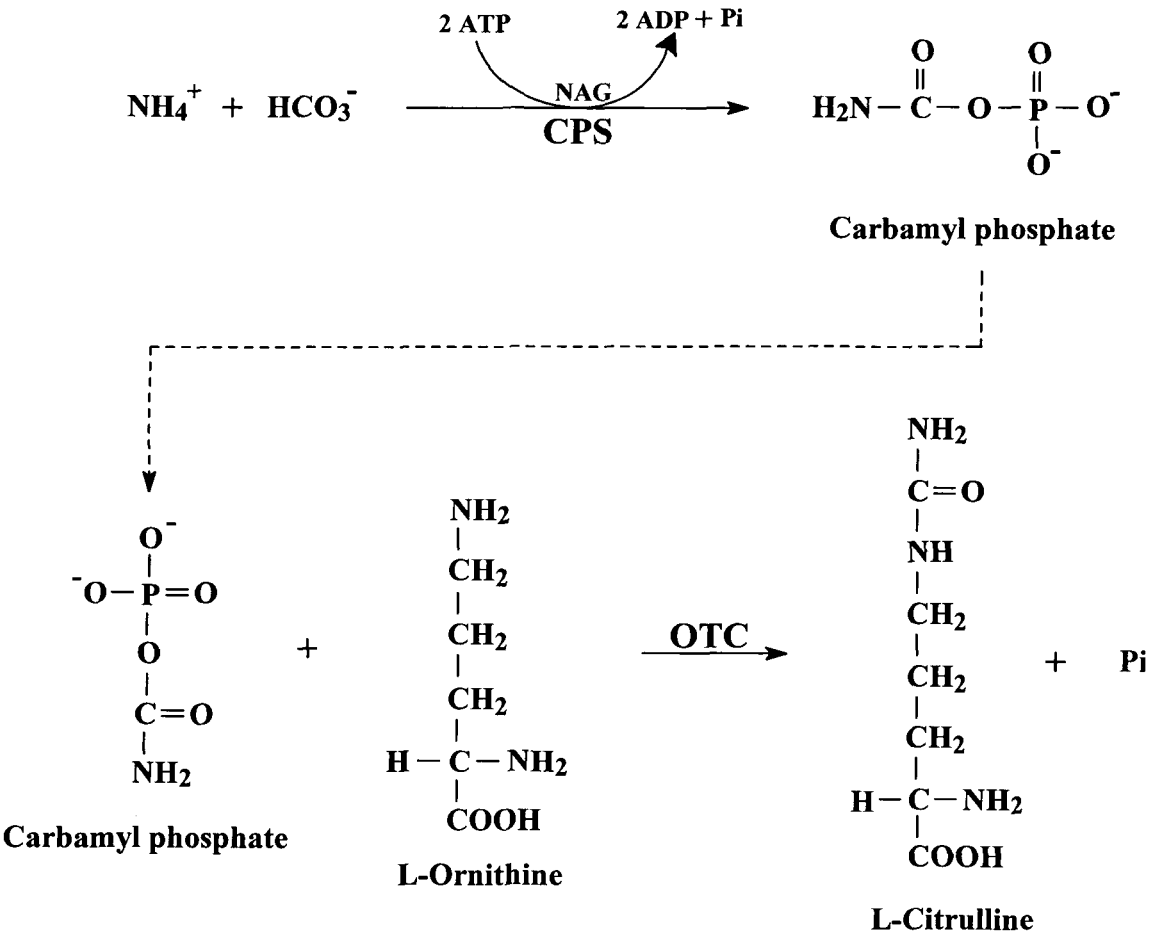
Tissue Processing for Assaying OUC enzymes:

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

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

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

The enzymatic reaction takes place as follows:



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

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

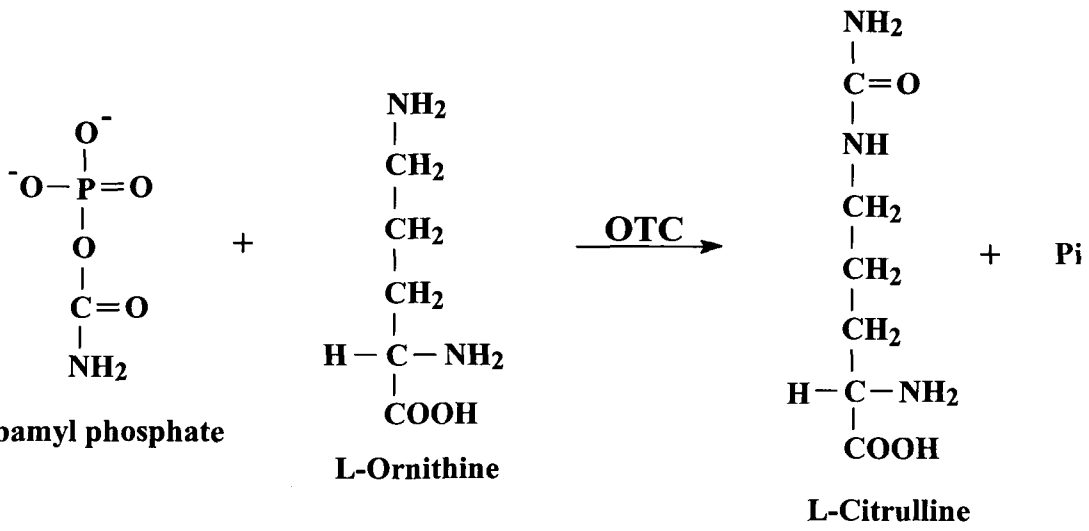
The assay mixture without the tissue extract was first preincubated for 5 min at 30 °C. The reaction was initiated by adding 0.3 ml of the tissue extract and after 30 min of incubation, the reaction was stopped by the addition of 0.5 ml of 10% PCA. A tissue blank was prepared simultaneously by adding 0.5 ml of 10% PCA to the reaction mixture prior to the addition of the tissue extract. The precipated protein was separated out by centrifugation and the supernatant was used for the estimation of citrulline following the method of Moore and Kauffman (1970). To 0.5 ml of the supernatant, 0.5 ml of distilled water, 2.5 ml of acid mixture (prepared by mixing 150 ml conc. H_3PO_4, 50 ml H_2SO_4, 0.118 g $MnSO_4$ and 0.015 g $FeCl_3$ in 500 ml of distilled water) and 0.25 ml of 3% (w/v) diacetyl monoxime were added. The mixture was kept for boiling in a hot water bath for 30 min, cooled and O.D. was measured at 490 nm in a UV-visible spectrophotometer (Beckman, DU 640) against the tissue blank. The amount of

citrulline formed by the enzymatic reaction was calculated from the standard graph, prepared by using different concentration of citrulline (0.01-0.1μmole), which was linear. One unit of CPS activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of citrulline per h at 30 °C.

Ornithine transcarbamylase (OTC; E.C. 2.1.3.3)

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

The enzymatic reaction takes place as follows:



Carbamyl phosphate **L-Ornithine** **L-Citrulline**

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

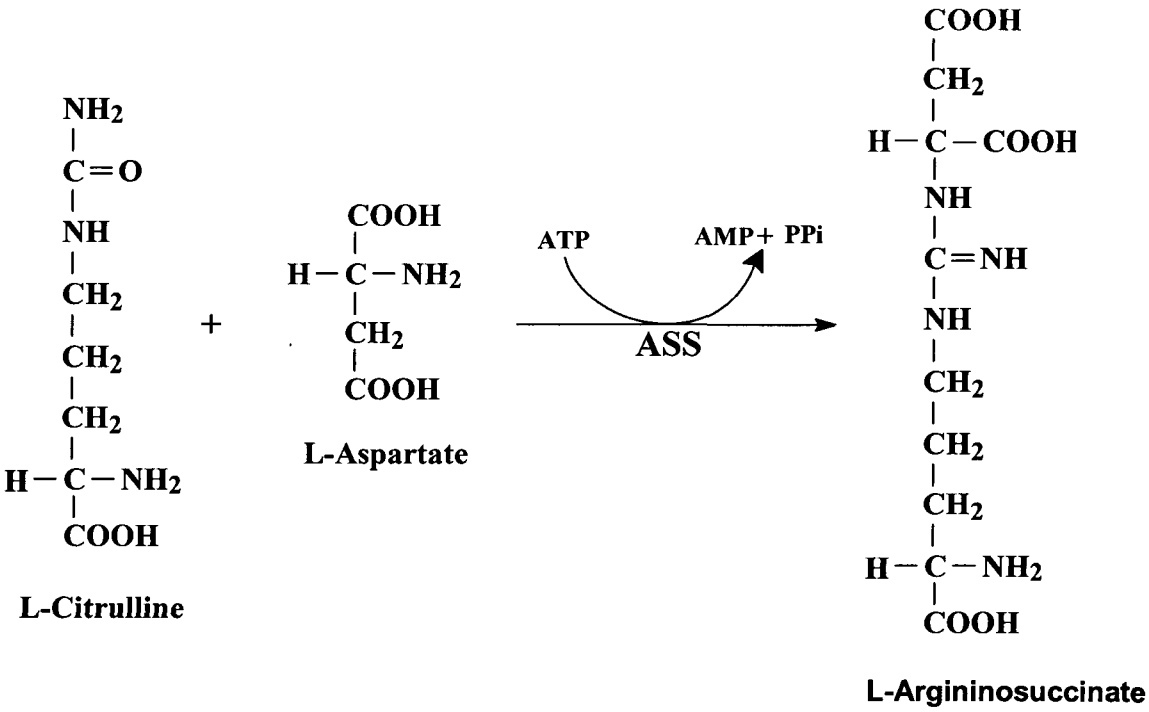
Glycyl-glycine buffer (pH 8.3)	50 μmoles
L-Ornithine	20 μmoles
Dilithium carbamyl phosphate	20 μmoles
Tissue extract	0.2 ml

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

Argininosuccinate synthetase (ASS; E.C. 6.3.4.5)

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

The enzymatic reaction takes place as follows:



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

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

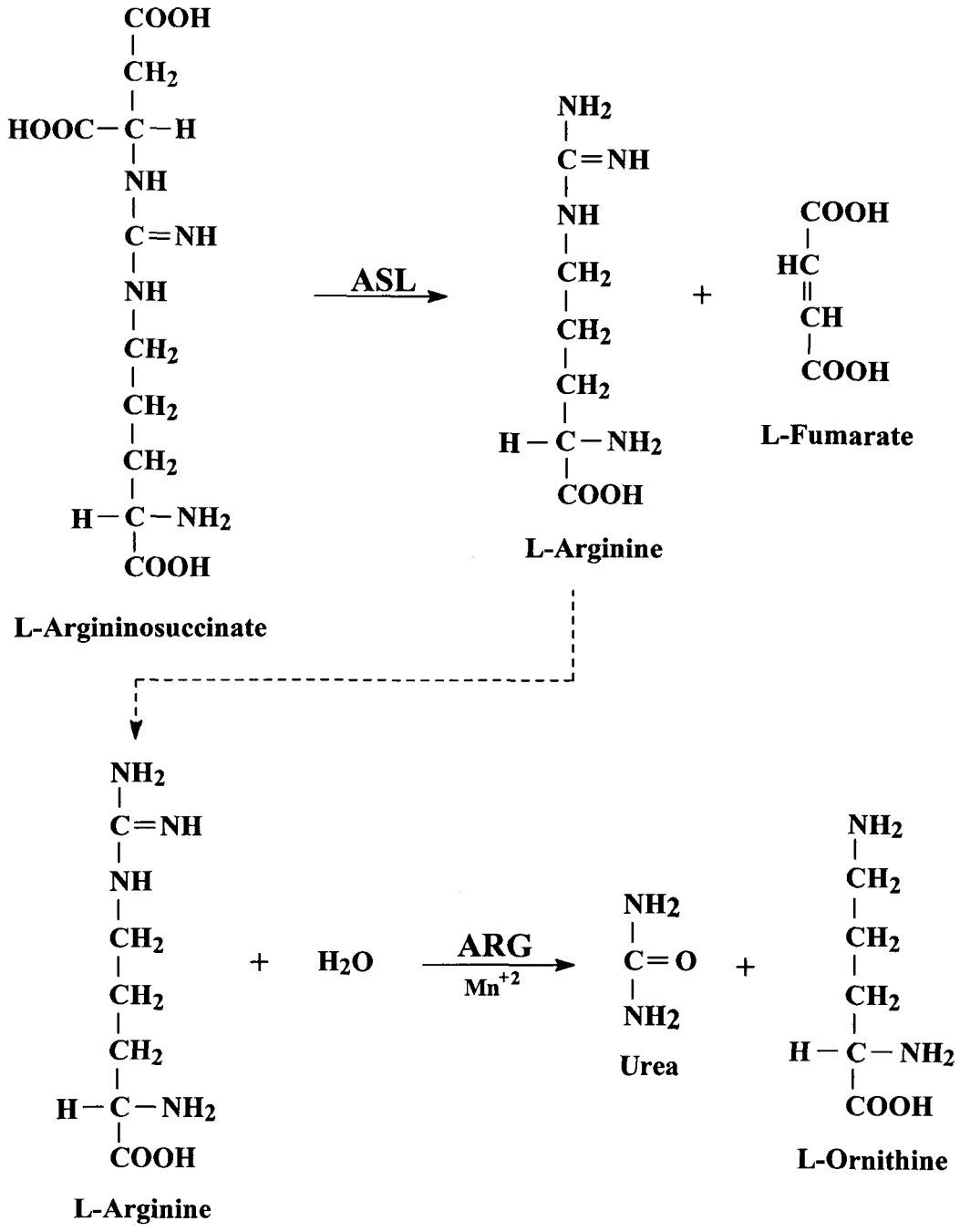
The reaction mixture without citrulline was first preincubated for 5 min at 30 °C. The reaction was initiated with the addition of citrulline and after 30 min of incubation,

the reaction was stopped by the addition of 0.5 ml of 10% PCA. In the tissue blank, PCA was added in the reaction mixture prior to the addition of citrulline. The precipitated protein was separated out by centrifugation and the amount of citrulline utilized during the incubation period was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above for CPS assay. One unit of ASS activity was expressed as that amount of enzyme which catalyzed the utilization of 1 μmole of citrulline per h at 30 °C.

Argininosuccinate lyase (ASL; E.C. 4.3.2.1)

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

The enzymatic reaction takes place as follows:



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

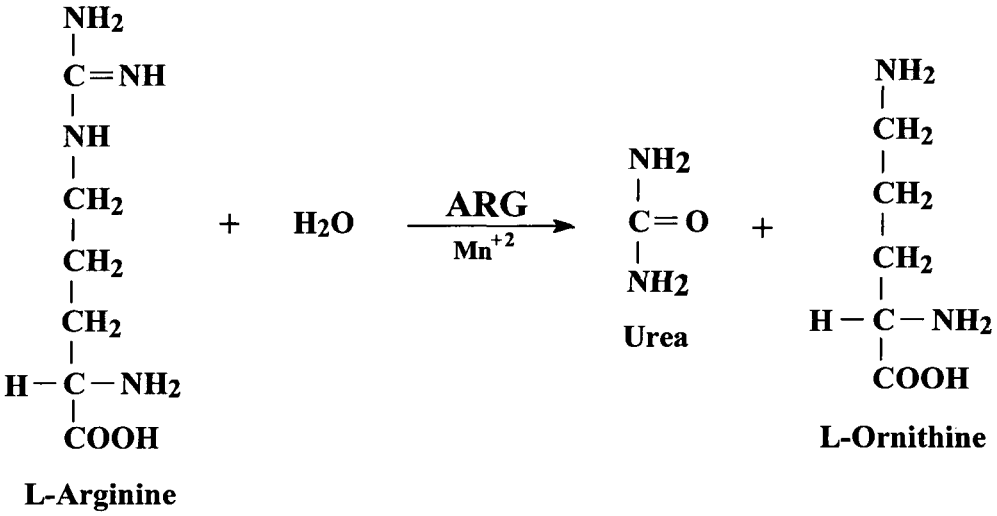
K-phosphate buffer (pH: 7.3)	60 μmoles
L-Argininosuccinate	4 μmoles
Arginase	10 units
Tissue extract	0.2 ml

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

Arginase (ARG; E.C. 3.5.3.1)

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

The enzymatic reaction takes place as follows:



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

Na- glycinate buffer (9.5)	100 μmoles
L-Arginine	50 μmoles
$MnCl_2$	0.5 μmole
Tissue extract	0.1 μmole

The reaction mixture without L-arginine was first preincubated for 5 min at 30 °C. The reaction was initiated by the addition of L-arginine, and after 15 min of incubation, the reaction was stopped by adding 1.0 ml of 10% PCA. A tissue blank was also prepared simultaneously by adding 1.0 ml of 10% PCA to the reaction mixture prior to the

addition of L-arginine. The precipitated protein was separated out by centrifugation and the supernatant was used for urea estimation. The amount of urea formed during the incubation period was estimated following the method of Moore and Kauffman (1970) as described above for ASL assay. One unit of arginase activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmole of urea per h at 30 °C.

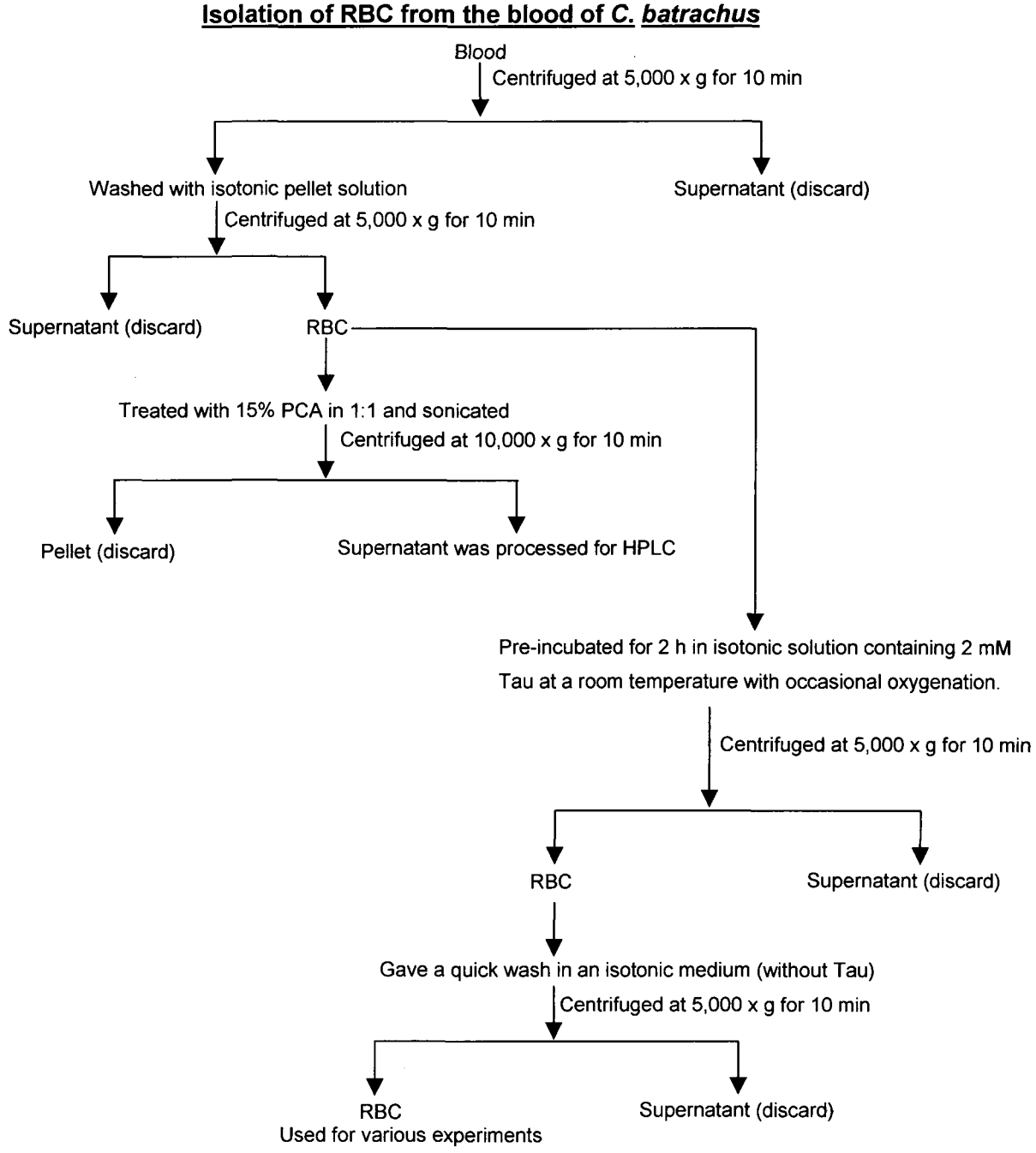
Measurement of osmolarity:

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

Isolation of RBC from the blood of *C. batrachus*:

Blood was collected from the caudal vein with a heparinized syringe, centrifuged immediately at 5,000 x g for 10 min to settle the RBC, supernatant was discarded. The RBC were then washed in isotonic solution containing 119 mM NaCl/mannitol, 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 of $CaCl_2$ as a basic solution. The medium also contained 1.2 mM L-lactate and 0.3 mM L-pyruvate. It was then centrifuged at 5,000 x g for 10 min, supernatant was discarded. RBC were then isolated in two sets. One set of RBC after washing in isotonic solution was centrifuged at 5,000 x g for 10 min, supernatant was discarded and the packed RBC was processed for Tau analysis by HPLC. The other set of RBC were incubated for 2 h in isotonic solution containing 2 mM Tau at room temperature with occasssional oxygenation. Erythrocytes were then centrifuged at 5,000 g for 10 min. Supernatant was discarded and RBC was used for

various experiments. The schematic representation for the isolation of RBC from the blood of *C. batrachus* is given in the following chart:



Measurement of water content or hydration status:

The pre-weighed isolated RBC was kept in an oven at 70 °C for 24 h, sufficient enough to evaporate out all the water content in the RBC, followed by measuring the weight of dried RBC. The differences in the wet weight and dry weight of RBC were taken as water content of RBC.

Chemicals:

All the enzymes, co-enzymes, substrates, mixtures of physiological amino acids and bovine serum albumin were obtained either from Sigma Chemical Co., St. Louis, U.S.A. or from Roche, Germany. All the 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 five observations at each point and presented as mean ± standard error of the mean (SEM). One-way ANOVA test was performed when the control value was compared with more than one experimental data. Linear regression analysis was also done in one experiment to find a correlation between the efflux of Tau and osmolarity changes by the isolated RBC of *C. batrachus* following the method of Croxton et al. (1982). Besides the presentation of data in a tabulated forms, histograms, and line diagrams were prepared to highlight the results.

RESULTS

Effects of anisotonicity on amino acid metabolism in the perfused liver of *C. batrachus*

Effects of anisotonicity on the levels of different FAAs in the perfused liver of *C. batrachus*:

As shown in table 1 and Fig. 3, when the liver of *C. batrachus* was perfused with hypotonic medium (-80 mOsmol/l) for 90 min after initial perfusion with isotonic medium (265 mOsmol/l) for 30 min, there was a decrease in the levels of almost all the non-essential and essential free amino acids (FAAs). Out of all the non-essential FAAs, maximum decrease was seen in the case of Ala, Cit, Ser, Asp, Asn and Tau, and out of all the essential FAAs, maximum decrease was seen in the case of Ile, Lys, Val and Met. In contrast, when the liver was perfused with hypertonic medium (+80 mOsmol/l), there was a significant increase in the levels of many of the non-essential and essential FAAs. Out of all the non-essential FAAs, maximum increase was seen in the case of Asp, Ala, Glu, Gln, Cit and Tau, and out of all the essential FAAs, maximum increase was seen in the case of Thr, Orn and Arg. Under hypotonic condition, the total non-essential FAAs decreased significantly by 41% (P <0.05) and total essential FAAs by 36% (P <0.05). Whereas, under the hypertonic condition, percentage increase of total non-essential FAAs was 71% (P <0.01) and total essential FAAs by 51% (P <0.01) (Fig. 4).

Effects of anisotonicity on the levels of different FAAs in the perfused liver of *C. batrachus* infused with 5 mM NH_4Cl:

In another set of fish, livers were perfused with anisotonic media while infusing continuously with 5 mM NH_4Cl (Table 2; Fig. 5). When the liver of *C. batrachus* was

perfused with hypotonic medium (-80 mOsmol/l) for 90 min after initial perfusion with isotonic medium (265 mOsmol/l) for 30 min, a significant decrease in the levels of many of the non-essential and essential (FAAs) was seen. Out of all the non-essential FAAs, maximum decrease was seen in the case of Ala, Pro, Glu, Gln and Tau and out of all the essential FAAs, maximum decrease was seen in the case of His, Ile, Met and Val. In contast, when the liver was perfused with hypertonic medium (+80 mOsmol/l), there was a significant increase in the levels of both non-essential and essential FAAs. Out of all the non-essential FAAs, maximum increase was seen in the case of Asp, Ala, Glu, Gln, Cit and Tau, and out of the essential FAAs, maximum increase was seen in the case of Thr, Orn and Arg. Under hypotonic condition the total non-essential FAAs decreased significantly by 43% (P <0.05) and the total essential FAAs decreased significantly by 32% (P <0.05). Whereas under hypertonic condition, percentage increase of total non-essential FAAs was 74% (P <0.01) and total essential FAAs percentage by 55% (P <0.01) (Fig. 6).

Effects of anisotonicity on the tissue activity of some amino acid metabolism related enzymes in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl:

The changes of tissue activity of GSase, GDH (both O.D and R.A.) in the perfused liver of *C. batrachus* while perfusing under iso- and anisotonic conditions for 90 min both in the absence and presence of 5 mM NH_4Cl are presented in table 3 and Figs. 7 & 8. No significant changes of GSase activity was observed in the perfused liver while perfusing with hypotonic perfusion medium both in the absence or presence of NH_4Cl. Whereas under hypertonic perfusion condition, the GSase activity significantly increased from 76.11 ± 3.56 to 108.69 ± 4.87 units/g wet wt (43%, P <0.05) and from

113.62 ± 5.21 to 149.35 ±6.12 units/g wet wt (31%, P <0.05) in the absence and presence of NH_4Cl, respectively. Whereas for GDH (both in O.D. and R.A. direction) the enzyme activity did not show any significant changes under hypotonic condition both in the presence and absence of NH_4Cl. But under hypertonic perfusion condition, GDH activity in oxidative deamination direction increased significantly from 369 ± 13.74 to 474 ± 15.87 units/g wet wt (28%, P <0.05) and from 489 ± 17.02 to 672 ± 21.63 units/g wet wt (37%, P <0.05) in the absence and presence of NH_4Cl, respectively. The GDH activity in the reductive amination direction increased significantly from 393 ± 18.50 to 528 ± 12.0 units/g wet wt (34%, P <0.05) and from 528 ± 12.87 to 864 ± 54.99 units/g wet wt (64%, P <0.01) in the absence and presence of NH_4Cl, respectively.

The changes of tissue activity of AST and ALT in the perfused liver of *C. batrachus* both under iso- and anisotonic conditions are presented in table 4 and Figs. 7 & 8. The AST activity under hypotonic condition decreased significantly from 380 ± 14.44 to 324 ± 10.39 units/g wet wt (15%, P <0.05), and from 492 ± 17.8 to 354 ± 6.0 units/g wet wt (28%, P <0.05), respectively, in the absence or presence of NH_4Cl. Whereas the hypotonicity did not cause any significant changes of ALT activity in the perfused liver either in the absence or presence of NH_4Cl. But, hypertonicity caused a significant increase of ALT activity in the perfused liver from 411 ± 16.37 to 512 ± 7.04 units/g wet wt (25 %, P <0.05), and from 384 ± 10.04 to 622 ± 5.78 units/g wet wt (62%, P <0.01), respectively, in the absence and presence of NH_4Cl.

It is to be noted here that the activities of all the enzymes except for ALT increased significantly in the perfused liver infused with 5 mM NH_4Cl compared to the activities in the perfused liver without infusing with NH_4Cl under isotonic conditions (Tables 3 and 4).

Effects of anisotonicity on the specific activity of some amino acid metabolism related enzymes in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl:

The pattern of changes of specific activity of all the above mentioned enzymes in the perfused liver of *C. batrachus* under anisotonic perfusion conditions was almost similar to that of tissue activity (Tables 3 & 4; Figs. 9 & 10). No significant changes of GSase activity was observed in the perfused liver while perfusing with hypotonic perfusion medium both in the absence or presence of NH_4Cl. Whereas under hypertonic perfusion condition, the GSase activity significantly increased from 1.08 ± 0.14 to 1.56 ± 0.10 units/mg protein (44%, P <0.05) and from 1.55 ± 0.13 to 2.13 ± 0.11 units/mg protein (37%, P <0.05) in the absence and presence of NH_4Cl, respectively. Likewise for GDH (both in O.D. and R.A. direction), the enzyme activity did not show any significant changes under hypotonic condition both in the presence and absence of NH_4Cl. But under hypertonic perfusion condition, GDH activity in oxidative deamination direction increased significantly from 6.79 ± 0.26 to 8.56 ± 0.24 units/mg protein (26%, P <0.05) and from 9.00 ± 0.32 to 12.13 ± 0.16 units/mg protein (35%, P <0.05) in the absence and presence of NH_4Cl, respectively. The GDH activity in the reductive amination direction increased significantly from 7.24 ± 0.28 to 9.55 ± 0.37 units/mg protein (32%, P <0.05) and from 9.72 ± 0.21 to 15.64 ± 1.17 units/mg protein (61%, P <0.01) in the absence and presence of NH_4Cl, respectively.

The changes of specific activity of AST and ALT in the perfused liver of *C. batrachus* both under iso- and anisotonic conditions are presented in table 4, and Figs. 9 & 10. The AST activity under hypotonic condition decreased significantly from 7.01 ± 0.24 to 5.85 ± 0.19 units/mg protein (17%, P <0.05), and from 9.05 ± 0.30 to 6.39 ± 0.03

units/mg protein (29%, P <0.05), respectively, in the absence or presence of NH_4Cl. The effect of hypotonicity on ALT activity in the perfused liver was not seen much either in the absence or presence of NH_4Cl. Likewise, hypertonicity also did not cause any significant increase of ALT in absence of NH_4Cl, but in the presence of NH_4Cl, there was a significant increase of ALT from 7.10 ± 0.25 to 8.39 ± 0.44 (18%, P <0.05).

It is to be noted further that the specific activities of all the enzymes increased significantly in the perfused liver infused with 5 mM NH_4Cl compared to the activities in the perfused liver without NH_4Cl under isotonic conditions (Tables 3 and 4).

Effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine in the perfused liver of *C. batrachus* infused with 1 mM NH_4Cl:

Table 5 and Fig. 11 represent the effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine from the perfused liver of *C. batrachus* while infusing with 1 mM NH_4Cl. The ammonia-N efflux, which was recorded to be 3.06 ± 0.17 µmoles/g liver/min under isotonic condition, significantly increased to 3.55 ± 0.10 µmoles/g liver/min (16%, P <0.05) under hypotonic (-80 mOsmol/l) condition, and significantly decreased from 2.82 ± 0.15 to 2.31 ± 0.12 µmoles/g liver/min (18%, P <0.05) under hypertonic condition. Whereas the efflux of urea-N significantly decreased from 0.44 ± 0.07 to 0.19 ± 0.05 µmoles/g liver/min (57%, P <0.01) under hypotonic condition, and significantly increased from 0.51 ± 0.1 to 0.85 ± 0.06 µmoles/g liver/min (67%, P <0.01) under hypertonic condition. Likewise, glutamate efflux significantly decreased from 0.08 ± 0.02 to 0.04 ± 0.02 µmoles/g liver/min (50%, P <0.01) under hypotonic condition, and significantly increased from 0.13 ± 0.03 to 0.21 ± 0.06 µmoles/g liver/min (62%, P <0.01) under hypertonic condition. In case of glutamine, the efflux decreased significantly under both hypotonic and hypertonic conditions from 0.13

± 0.03 to 0.02 ± 0.04 µmoles/g liver/min (85%, P <0.01) and from 0.09 ± 0.03 to 0.06 ± 0.01 µmoles/g liver/min (33%, P <0.05), respectively.

Effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine in the perfused liver of *C. batrachus* infused with 0.25 mM glutamine:

Table 6 and Fig. 12 represent the effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine from the perfused liver of *C. batrachus* while infusing with 0.25 mM glutamine. The ammonia-N efflux, which was recorded to be 0.16 ± 0.01 µmoles/g liver/min under isotonic condition, significantly increased to 0.34 ± 0.03 µmoles/g liver/min (113%, P <0.001) under hypotonic (-80 mOsmol/l) condition, and significantly decreased from 0.71 ± 0.06 to 0.28 ± 0.04 µmoles/g liver/min (61%, P <0.01) under hypertonic condition. Whereas the efflux of urea-N significantly increased from 0.12 ± 0.04 to 0.25 ± 0.07 µmoles/g liver/min (108%, P <0.001), and from 0.14 ± 0.03 to 0.34 ± 0.04 µmoles/g liver/min (143%, P <0.001) both under hypotonic and hypertonic conditions, respectively. Likewise, glutamate efflux significantly increased from 0.07 ± 0.01 to 0.11 ± 0.01 µmoles/g liver/min (57%, P <0.01) under hypotonic condition, and significantly decreased from 0.33 ± 0.02 to 0.12 ± 0.02 µmoles/g liver/min (64%, P <0.01) under hypertonic condition. In case of glutamine, the efflux decreased significantly under hypotonic condition from 0.49 ± 0.05 to 0.33 ± 0.07 µmoles/g liver/min (33%, P <0.05) and increased from 0.54 ± 0.05 to 0.60 ± 0.06 µmoles/g liver/min (11%, P <0.05) under hypertonic condition.

Effects of anisotonicity on the efflux of ammonia-N, urea-N, and total-N from the perfused liver of *C. batrachus* infused with different amino acids:

Table 7 and Fig. 13 represent the efflux of ammonia-N, urea-N and total-N (as ammonia-N + urea-N) from the perfused liver of *C. batrachus* under iso- and anisotonic conditions while infusing with different amino acids (5 mM). The efflux of ammonia-N from the perfused liver under isotonic condition while infusing with different amino acids was in the order of Gln>Glu>Asp>Ala>Gly>Thr>Ser, whereas the urea-N and total-N efflux under isotonic condition was in the order of Gln>Glu>Asp>Ala>Gly>Thr>Ser.

Under hypotonic perfusion condition the efflux of ammonia-N by the perfused liver while infusing with different amino acids increased significantly, whereas under hypertonic condition there was a significant decrease of ammonia-N efflux from all the amino acids infused. The percentage increase of ammonia-N under hypotonic condition was 69, 60, 48, 73, 58, 58 and 95%, respectively, for Gln, Glu, Asp, Ala, Gly, Thr and Ser. The percentage decrease of ammonia-N under hypertonic condition was 39, 48, 35, 51, 38, 38 and 43, respectively, for Gln, Glu, Asp, Ala Gly, Thr and Ser. Similarly, the efflux of urea-N from the perfused liver while infusing with different amino acids significantly increased under hypotonic condition and decreased under hypertonic condition. The percentage increase of urea-N under hypotonic condition was 62, 165, 157, 144, 93, 80 and 200%, respectively, for Gln, Glu, Asp, Ala, Gly, Thr and Ser. The percentage decrease of urea-N under hypertonic condition was 53, 54, 48, 63, 47, 50 and 50%, respectively, for Gln, Glu, Asp, Ala, Gly, Thr and Ser.

Likewise, the total-N efflux from different amino acids by the perfused liver followed the similar trend, i.e., increase under hypotonic condition and decrease under hypertonic condition. The percentage increase of total-N efflux under hypotonic

condition was 66, 109, 157, 93, 72, 53, and 124%, respectively, for Gln, Glu, Asp, Ala, Gly, Thr and Ser. The percentage decrease of total-N efflux under hypertonic condition was 44, 51, 48, 54, 41, 47, and 45, respectively, for Gln, Glu, Asp, Ala, Gly, Thr and Ser.

Effect of hypertonicity on amino acid metabolism and ureogenesis in *C. batrachus*

Changes in the concentration of different FAAs in different tissues and in the plasma of *C. batrachus* following exposure to 250 mOsmol/l mannitol:

Liver:

The changes in the concentration of FAAs in the liver of *C. batrachus* following exposure to 250 mOsmol/l mannitol for a period 7 days with relation to controls are presented in table 8 and Figs. 14 & 15. Most of the non-essential FAAs increased significantly in the liver exposed to 250 mOsmol/l mannitol. There was significant increase of both non-essential and essential FAAs in the liver of *C. batrachus* following exposure to mannitol for 3 days, followed by further increase after 7 days. The non-essential FAAs increased significantly by 67% and 93%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Asp (86%), Gly (74%), Ala (102%), Asn (217%), Glu (87%), Gln (133%) and Tau (112%) concentrations. The essential FAAs also increased significantly by 10% and 13%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Met (29%), Leu (23%), Trp (34%) and Phe (33%).

Kidney:

The changes in the concentration of FAAs in the kidney of *C. batrachus* following exposure to 250 mOsmol/l mannitol for a period 7 days with relation to controls are presented in table 9 and Figs. 16 & 17. Most of the non-essential FAAs increased significantly in the kidney exposed to 250 mOsmol/l mannitol. There was significant increase of both non-essential and essential FAAs in the kidney of *C. batrachus* following exposure to mannitol for 3 days, followed by further increase after 7 days. The non-essential FAAs increased significantly by 37% and 64%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Asp (66%), Gly (64%), Ala (76%), Asn (78%), Gln (154%) and Tau (73%) concentrations. The essential FAAs also increased significantly by 32% and 33%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Met (116%), Ile (52%), Trp (51%) and Phe (48%).

Muscle:

The changes in the concentration of FAAs in the muscle of *C. batrachus* following exposure to 250 mOsmol/l mannitol for a period 7 days with relation to controls are presented in table 10 and Figs. 18 & 19. Most of the non-essential FAAs increased significantly in the muscle exposed to 250 mOsmol/l mannitol. There was significant increase of both non-essential and essential FAAs in the muscle of *C. batrachus* following exposure to mannitol for 3 days, followed by further increase after 7 days. The non-essential FAAs increased significantly by 36% and 40%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Asp (82%), Gly (23%), Ala (67%), Asn (143%), Glu (341%), Gln (425%) and Tau (21%) concentrations. The essential FAAs also increased significantly by 8% and 48%,

respectively, after the 3rd and 7th day of exposure. This was mainly attributable to the increase of Thr (64%), Val (71), Met (42%), Leu (60%) and Phe (44%).

Brain:

The changes in the concentration of FAAs in the brain of *C. batrachus* following exposure to 250 mOsmol/l mannitol for a period 7 days with relation to controls are presented in table 11 and Figs. 20 & 21. Most of the non-essential FAAs increased significantly in the brain exposed to 250 mOsmol/l mannitol. There was significant increase of both non-essential and essential FAAs in the brain of *C. batrachus* following exposure to mannitol for 3 days, followed by further increase after 7 days. The non-essential FAAs increased significantly by 32% and 52%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Asp (148%), Asn (88%), Glu (56%), Gln (50%), Tyr (165) and Pro (240%) concentrations. The essential FAAs also increased significantly by 73% and 59%, respectively, after the 3rd and 7th day of exposure, which was mainly attributable to the increase of Val (36%), Phe (250%), His (112%) and Orn (164%).

Plasma:

The changes in the concentration of FAAs in the plasma of *C. batrachus* following exposure to 250 mOsmol/l mannitol for a period 7 days with relation to controls are presented in table 12 and Figs. 22 & 23. Most of the non-essential FAAs increased significantly in the plasma exposed to 250 mOsmol/l mannitol. There was significant increase of both non-essential and essential FAAs in the plasma of *C. batrachus* following exposure to mannitol for 3 days, followed by further increase after 7 days. The non-essential FAAs increased significantly by 136% and 232%, respectively, after the 3rd and 7th day of exposure. This was mainly attributable to the increase of Asp

(221%), Gly (217%), Ala (189%), Glu (253%), Gln (157%), Tyr (188%) and Tau (293%) concentrations. The essential FAAs also increased significantly by 142% and 139%, respectively, after the 3rd and 7th day of exposure. This was mainly attributable to the increase of Thr (313%), Val (125%), Met (240%), Leu (367%), Phe (275%) and Orn (200%).

Changes in the tissue and specific activities of different amino acid metabolism related enzymes in different tissues of *C. batrachus* exposed to 250 mOsmol/l mannitol:

Liver:

The changes of tissue activity related to amino acid metabolism in the liver of *C. batrachus* exposed to 250 mOsmol/l for 7 days are presented in the table 13 and Figs. 24 & 32. The tissue activity of GSase, GDH (R.A), GDH (O.D) and ALT increased significantly in the fish liver exposed to 250 mOsmol/l mannitol, whereas the AST activity did not show any significant change. The GSase activity increased maximally from 136 to 185 units/g wet wt (37%) after the 7th day, GDH (R.A) from 672 to 1328 units/g wet wt (98%) after the 3rd day, GDH (O.D) from 218 to 363 units/g wet wt (66%) after the 3rd day and ALT from 310 to 606 units/g wet wt (95%) after the 7th day of exposure.

The specific activity of all the above mentioned enzymes except for AST increased significantly in the fish liver during exposure to 250 mOsmol/l mannitol (Table 13; Figs. 25, 33). The specific activity of GSase increased maximally from 3.13 to 4.30 units/mg protein (37%) after the 7th day, GDH (R.A) from 7.44 to 18.06 units/g wet wt (143%) after the 3rd day, GDH (O.D) from 2.52 to 4.86 units/g wet wt (93%) after the 3rd day, and ALT from 11.76 to 22.31 units/mg protein (90%) after the 7th day of exposure.

Kidney:

The changes of tissue activity related to amino acid metabolism in the kidney of *C. batrachus* exposed to 250 mOsmol/l are presented in table 14 and Figs. 26 & 32. The tissue activity of all the enzymes studied such as GSase, GDH (R.A), GDH (O.D), AST and ALT increased significantly in the fish kidney during exposure to 250 mOsmol/l mannitol after 3 days, followed by further increase after 7 days for some of the enzymes. The tissue activity of GSase increased maximally from 103.9 to 136.5 units/g wet wt (31%) after the 7th day, GDH (R.A) from 592 to 1072 units/g wet wt (81%) after the 3rd day, GDH (O.D) from 192 to 336 units/g wet wt (75%) after the 3rd day, AST from 254 to 414 units/g wet wt (63%) after the 7th day and ALT from 290 to 432 units/g wet wt (49%) after the 7th day of exposure.

The specific activity of all the above mentioned enzymes also increased significantly in the similar pattern in the fish kidney exposed to 250 mOsmol/l mannitol (Table 14; Figs. 27, 33). The specific activity of GSase increased maximally from 2.43 to 2.92 units/mg protein (20%) after the 7th day, GDH (R.A) from 12.74 to 19.37 units/g wet wt (52%) after the 7th day, GDH (O.D) from 6.25 to 9.13 units/g wet wt (46%) after the 7th day, AST from 4.66 to 7.33 units/g wet wt (57%) after the 3rd day and ALT from 7.61 to 17.03 units/mg protein (124 %) after the 7th day of exposure.

Muscle:

The changes of tissue activity related to amino acid metabolism in the muscle of *C. batrachus* following exposure to 250 mOsmol/l are presented in table 15 and Figs. 28 & 32. The tissue activity of all the enzymes studied such as GSase, GDH (R.A), GDH (O.D), AST and ALT increased significantly in the fish muscle exposed to 250

mOsmol/l mannitol. The tissue activity of GSase increased maximally from 21.1 to 27.3 units/g wet wt (29%) after the 7th day, GDH (R.A) from 213.3 to 362.7 units/g wet wt (70%) after the 3rd day, GDH (O.D) from 205 to 296.7 units/g wet wt (44%) after the 7th day, AST from 256 to 396 units/g wet wt (55%) after the 7th day and ALT from 260 to 492 units/g wet wt (89%) after the 7th day of exposure.

The specific activity of all the above mentioned enzymes also increased significantly in the fish muscle exposed to 250 mOsmol/l mannitol (Table 15; Figs. 29, 33). The specific activity of GSase increased maximally from 0.78 to 1.06 units/mg protein (36%) after the 7th day, GDH (R.A) from 6.96 to 17.49 units/g wet wt (151%) after the 7th day, GDH (O.D) from 6.28 to 8.32 units/g wet wt (32%) after the 7th day, AST from 6.58 to 9.73 units/g wet wt (48%) after the 3rd day and ALT from 6.72 to 9.07 units/mg protein (35 %) after the 3rd day of exposure.

Brain:

The changes of tissue activity related to amino acid metabolism in the brain of *C. batrachus* exposed to 250 mOsmol/l are presented in table 16 and Figs. 30 & 32. The tissue activity of all the enzymes studied such as GSase, GDH (R.A), GDH (O.D), AST and ALT increased in the fish brain exposed to 250 mOsmol/l mannitol. The tissue activity of GSase increased maximally from 329 to 398 units/g wet wt (21%) after the 7th day, GDH (R.A) from 2303 to 2485 (8%) after the 7th day, GDH (O.D) from 266 to 282 (6%) after the 7th day, AST from 64 to 73 (14%) after the 3rd day and ALT from 66 to 117 (77%) after the 7th day of exposure.

The specific activity of all the above mentioned enzymes also increased in the fish brain exposed to 250 mOsmol/l mannitol (Table 16; Figs. 31 & 33). The specific activity of GSase increased from 10 to 14.4 units/mg protein (44%) after the 7th day,

GDH (R.A) from 41.13 to 45.23 (10%) after the 3rd day, GDH (O.D) from 4.29 to 5.35 (25%) after the 3rd day, AST from 2.97 to 3.22 (8%) after the 3rd day and ALT from 4.28 to 6.48 units/ mg protein (51%) after the 7th day of exposure.

Pattern of excretion of ammonia and urea-N by *C. batrachus* during exposure to 250 mOsmol/l mannitol for 7 days:

The rate of excretion of both ammonia and urea-N by *C. batrachus* during exposure to 250 mOsmol/l mannitol for 7 days are presented in table 17 and Fig. 34.

Ammonia:

The rate of excretion of ammonia by the control fish averaged to be about 320 µmoles/kg body wt/ h throughout the experimental period of 7 days. However, ammonia excretion by the fish exposed to 250 mOsmol/l mannitol decreased significantly by 54% after the 1st day, followed by further decrease to 68% after the 4th day and this level was maintained until the 7th day.

Urea-N:

The rate of excretion of urea-N by the control fish averaged to be about 85 µmoles/kg body wt/ h throughout the experimental period of 7 days. However, the rate of urea-N excretion by the treated fish increased significantly by (47%) after the 1st day of exposure, followed by further increase to a maximum of 67% after the 3rd day and this level was almost maintained at later stages of mannitol exposure.

Changes in the concentrations of ammonia and urea-N in different tissues and in the plasma of *C. batrachus* following exposure to 250 mOsmol/l mannitol:

Ammonia:

The changes in the concentrations of ammonia in different tissues and in plasma of *C. batrachus* during exposure to 250 mOsmol/l mannitol are presented in table 18 and Figs. 35 & 36. Ammonia accumulated significantly in the liver from 10.7 to 12.9 (21%) on the 3rd day and maintained this level until the 7th day. In the kidney also ammonia accumulated significantly on the 3rd day from 9.7 to 12.2 (26%), followed by no further increase at later stages. Whereas in the muscle, brain and plasma there was no significant increase of ammonia concentration in the mannitol treated fish throughout the period of experiment.

Urea-N:

As seen in table 19 and Figs. 37 & 38, urea-N concentration increased in all the tissues and plasma of the fish exposed to 250 mOsmol/l mannitol for 7 days. In the liver, urea-N concentration increased from 8.2 to 12.8 µmoles/g wet wt (56%) on the 3rd day, followed by further increase on the 7th day from 8.4 to 13.4 µmoles/g wet wt (61%). In the kidney, urea-N concentration increased from 7.2 to 9.5 µmoles/g wet wt (32%) on the 3rd day, followed by further increase on the 7th day from 7.6 to 11.4 µmoles/g wet wt (50%). In the muscle, urea-N concentration increased from 3.2 to 5.6 µmoles/g wet wt (75%) on the 3rd day, which did not increase further at later stages. In the brain, urea-N concentration increased from 2.5 to 3.1 µmoles/g wet wt (24%) on the 3rd day, followed by further increase on the 7th day from 2.6 to 3.4 µmoles/g wet wt (31%). In the plasma,

urea-N concentration increased from 0.81 to 1.68 µmoles/ml (107%) on the 3rd day, followed by further increase on the 7th day from 0.82 to 1.94 µmoles/ml (137%).

Changes in tissue and specific activities of ornithine-urea cycle (OUC) enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol:

There was a significant increase of tissue activity of all the enzymes of OUC except the OTC and ARG in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol (Table 20; Figs. 39 & 40). Tissue activity of CPS increased from 3.15 to 4.05 units/g wet wt (29%) on the 3rd day, followed by further increase on the 7th day from 3.21 to 4.38 units/g wet wt (36%). Tissue activity of ASS increased significantly from 44.2 to 57.4 units/g wet wt (30%) on the 3rd day, followed by further increase on the 7th day from 47.3 to 63.4 units/g wet wt (34%). Tissue activity of ASL increased significantly from 51.4 to 68.3 units/g wet wt (33%) on the 3rd day, followed by further increase on the 7th day from 49.7 to 71.2 units/g wet wt (43%).

There was a significant increase of specific activity of at least three enzyme of OUC in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol (Table 21; Figs. 41 & 42). Specific activity of CPS increased from 0.06 to 0.08 units/mg protein (33%) on the 3rd day, followed by further increase on the 7th day from 0.06 to 0.09 (50%). Specific activity of ASS increased from 0.82 to 1.10 units/mg protein (34%) on the 3rd day, followed by further increase on the 7th day from 0.89 to 1.22 units/mg protein (37%). Specific activity of ASL increased from 0.95 to 1.31 units/mg protein (38%) on the 3rd day, followed by further increase on the 7th day from 0.94 to 1.37 units/mg protein. For OTC and ARG, no significant changes of activity was seen in the liver of treated fish.

Changes in tissue and specific activities of OUC enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol:

There was a significant increase of tissue activity of all the OUC enzymes except for the OTC and ARG in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol (Table 22; Figs. 43 & 44). Tissue activity of CPS increased significantly from 2.45 to 3.05 units/g wet wt (24%) on the 3rd day, followed by further increase on the 7th day from 2.51 to 3.55 units/g wet wt (41%). Tissue activity of ASS increased significantly from 41.5 to 52.4 units/g wet wt (26%) on the 3rd day, followed by further increase on the 7th day from 40.7 to 56.9 units/g wet wt (40%). Tissue activity of ASL enzyme increased significantly from 38.9 to 53.1 units/g wet wt (37%) on the 3rd day, followed by further increase on the 7th day from 39.4 to 55.7 units/g wet wt (41%).

The pattern of changes of the specific activity of OUC enzymes in the kidney of mannitol-treated fish was almost similar with that of tissue activity (Table 23; Figs. 45 & 46). Specific activity of CPS increased significantly from 0.05 to 0.06 units/mg protein (20%) on the 3rd day, followed by further increase on the 7th day from 0.05 to 0.08 units/mg protein (60%). Specific activity of ASS increased significantly from 0.82 to 1.11 units/mg protein (29%) on the 3rd day, followed by further increase on the 7th day from 0.87 to 1.28 units/mg protein (47%). Specific activity of ASL increased significantly from 0.81 to 1.31 units/mg protein (40%) on the 3rd day, which did not increase further on the 7th day. The specific activity of OTC and ARG did not show any changes in the kidney of mannitol-treated fish.

Changes in the osmolarity of blood plasma (mOsmol/l) of *C. batrachus* exposed to 250 mOsmol/l mannitol:

The plasma osmolarity of control *C. batrachus* was recorded to be 265 ± 1.45 mOsmol/l, but increased significantly to 317 ± 9.21 mOsmol/l (20%) after the 3rd day, and to 347 ± 2.52 mOsmol/l (30%) after the 7th day of exposure to 250 mOsmol/l (Table 24; Fig. 47).

Effects of anisotonicity on the taurine (Tau) efflux/uptake by the isolated RBC of *C. batrachus*

Tau concentration in the isolated RBC of *C. batrachus* under iso- and anisotonic conditions pre-incubated with/without 2 mM taurine:

Tau concentration in the isolated RBC of *C. batrachus* under isotonic condition was recorded to be 0.787 ± 0.09 µmoles/g dry wt, which decreased significantly under hypotonic condition to 0.254 ± 0.05 µmoles/g dry wt (68%), and under hypertonic condition there was no significant changes of Tau concentration in the isolated RBC (Table 25).

When the isolated RBC was pre-incubated in an isotonic medium containing 2 mM Tau for 2 h, the Tau concentration in the RBC raised to 4.63 ± 0.28 µmoles/g dry wt. But the Tau concentration in the preincubated RBC decreased significantly to 1.25 ± 0.12 µmoles/g dry wt (73%) under the hypotonic condition, whereas the hypertonic exposure did not cause any significant changes of Tau concentration in the isolated RBC (Table 25).

Tau uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentrations of Tau for 2 h both under iso- and anisotonic conditions while maintaining the osmolarity of the media with NaCl:

As shown in table 26 and Fig 48, Tau uptake by the isolated RBC under isotonic condition increased gradually with the increase of Tau concentration in the incubation medium reaching to a maximum level of 4.41 ± 0.29 µmoles/g dry wt/2 h at 1 mM Tau concentration with no further changes at higher concentration. Under hypotonic condition, no significant uptake of Tau by the isolated RBC was seen in any of the concentrations of Tau in the external medium. Whereas under hypertonic condition, the Tau uptake was maximum reaching to a level of 9.41 ± 0.59 µmoles/g dry wt/2 h at 1 mM taurine in the external medium with slight increase of Tau level at a still higher Tau concentration in the external medium.

Tau uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentrations of Tau for 2 h both under iso- and anisotonic conditions while maintaining the osmolarity of the media with mannitol:

As shown in table 27 and Fig 49, Tau uptake by the isolated RBC under isotonic condition increased gradually with the increase of Tau concentration in the medium reaching to a maximum level of 1.05 ± 0.06 µmoles/g dry wt/2 h at 1 mM Tau concentration in the external medium with no further changes at higher concentrations. Under hypotonic condition, no significant uptake of Tau by the isolated RBC was seen in any of the concentrations of Tau in the external medium. Whereas under hypertonic condition, the Tau uptake was maximum reaching to a level of 2.51 ± 0.21 µmoles/g dry wt/2 h at 1 mM Tau concentration with slight increase at a still higher Tau concentration

in the external medium. However it is to be noted that the uptake of Tau by the isolated RBC both under iso- and hypertonic conditions was much lower when the NaCl was replaced by mannitol to maintain the osmolarity of the medium.

Effect of time on Tau efflux by the isolated RBC of *C. batrachus* under hypo- and hypertonic conditions while maintaining the osmolarity of the media with NaCl:

Table 29 and Fig. 50 represent the Tau efflux by the isolated RBC of *C. batrachus* under iso- and anisotonic conditions when the osmolarity of the medium was maintained with NaCl. Maximum efflux of Tau upto the level of 81 ± 8% of the total under hypotonic condition was observed with no further increase of Tau efflux at later stages. Whereas, when DIDS was added along with the hypotonic medium, Tau efflux was greatly reduced to 26 ± 4% of the total within 10 min of incubation. Under hypertonic condition the Tau efflux by the isolated RBC was found to be negligible.

Effect of time on Tau efflux by the isolated RBC of *C. batrachus* under hypo- and hypertonic conditions while maintaining the osmolarity of the media with mannitol:

Table 30 and Fig. 51 represent the Tau efflux by the isolated RBC of *C. batrachus* under iso- and anisotonic conditions when the osmolarity of the medium was maintained with mannitol. Maximum efflux of Tau 80 ± 7% of the total was obtained within 10 min with no further increase at later stages. Whereas, when DIDS was added along with the hypotonic condition, Tau efflux was greatly reduced to 29 ± 4% of the total within 10 min of incubation. Under hypertonic condition, Tau efflux by the isolated RBC was found to be negligible.

Effect of different osmolarities on the Tau efflux by the isolated RBC of *C. batrachus* pre-incubated with 2 mM Tau for 2 h:

A linear relationship was seen between the osmolarity of the medium and the efflux of Tau from the isolated RBC of *C. batrachus* incubated at a wide range of osmolarity changes in the incubation medium (Table 32; Fig 52). The efflux of Tau, which was 10 ± 2% of the total at 265 mOsmol/l (isotonic), increased linearly to 75 ± 7% of the total at 165 mOsmol/l osmolarity of the external medium. There is a direct correlation with the osmolarity changes and Tau efflux by the isolated RBC of *C. batrachus* ($r = 0.99$, $P < 0.00002$).

Effects of anisotonicity on water content in the isolated RBC pre-incubated with/without Tau:

The water content in the isolated RBC of *C. batrachus* under isotonic condition was recorded to be 30 ± 2%, which under hypotonic condition increased to 34 ± 3%, and under hypertonic condition it decreased to 27 ± 2% (Table 33).

The water content of isolated RBC of *C. batrachus*, pre-incubated with Tau (2 mM) for 2 h under isotonic condition, was found to be 31 ± 2%, which under hypotonic condition increased to 35 ± 3%, and under hypertonic condition decreased to 28 ± 2% (Table 33).

TABLES

Table 1: Effects of anisotonicity on the levels of different FAAs in the perfused liver of *C. batrachus*. Values are expressed as mean ± SEM (n = 5).

Amino acids	Conditions		
	Isotonic	Hypotonic	Hypertonic
Non-essential			
Asp	1.19 ± 0.01	0.59 ± 0.07 (-50)[b]	2.14 ± 0.06 (+80)[b]
Gly	0.83 ± 0.07	0.57 ± 0.05 (-31)[a]	1.32 ± 0.08 (+59)[b]
Ala	1.84 ± 0.04	0.78 ± 0.06 (-58)[b]	3.57 ± 0.08 (+94)[b]
Ser	0.44 ± 0.04	0.20 ± 0.01 (-55)[b]	2.33 ± 0.09 (+430)[c]
Glu	2.00 ± 0.07	0.96 ± 0.04 (-52)[b]	2.12 ± 0.07 (+6)
Gln	1.09 ± 0.03	0.71 ± 0.03 (-35)[a]	2.13 ± 0.02 (+95)[b]
Cit	0.52 ± 0.03	0.23 ± 0.02 (-56)[b]	3.14 ± 0.05 (+504)[c]
Asn	0.48 ± 0.04	0.26 ± 0.05 (-46)[a]	0.56 ± 0.07 (+17)
Tyr	0.61 ± 0.05	0.38 ± 0.04 (-38)[a]	1.12 ± 0.08 (+84)[b]
Pro	1.05 ± 0.03	0.67 ± 0.05 (-36)[a]	0.88 ± 0.07 (-16)
GABA	0.66± 0.08	0.39 ± 0.04 (-41)[a]	0.32 ± 0.01 (-52)[b]
Tau	7.51 ± 0.14	4.61 ± 0.43 (-39)[a]	10.17± 0.03 (+35)[a]
Total	**17.40 ± 0.61**	**10.35 ± 0.87 (-41)[a]**	**29.8 ± 0.675 (+71)[b]**
Essential			
Thr	0.46 ± 0.04	0.30 ± 0.06 (-35)[a]	0.75 ± 0.01 (63)[b]
Val	0.40 ± 0.02	0.19 ± 0.02 (-53)[a]	0.44 ± 0.02 (+10)
Met	0.28 ± 0.03	0.14 ± 0.05 (-50)[a]	0.39 ± 0.05 (+39)[a]
Ile	0.25 ± 0.04	0.09 ± 0.04 (-64)[b]	0.56 ± 0.02 (+124)[c]
Leu	0.41 ± 0.09	0.28 ± 0.04 (-32)[a]	0.59 ± 0.03 (+44)[a]
Trp	0.48 ± 0.04	0.32 ± 0.03 (-33)[a]	0.67 ± 0.06 (+40)[a]
His	0.43 ± 0.05	0.33 ± 0.01 (-23)[a]	0.52 ± 0.01 (+21)
Lys	0.32 ± 0.07	0.14 ± 0.02 (-56)[b]	0.37 ± 0.02 (+16)
Phe	0.30 ± 0.01	0.17 ± 0.02 (-43)[a]	0.30 ± 0.01 (0)
Orn	0.69 ± 0.09	0.40 ± 0.02 (-42)[a]	1.21 ± 0.03 (+75)[b]
Arg	0.93 ± 0.11	0.80 ± 0.05 (-14)	1.68 ± 0.06 (+81)[b]
Total	**4.95 ± 0.57**	**3.16 ± 0.33 (-36)[a]**	**7.48 ± 0.29 (+51)[b]**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the control levels are given in parentheses.

[a, b, c] : *P* values significant at <0.05, <0.01<0.001 levels, respectively (One-way ANOVA).

Table 2: Effects of anisotonicity on the levels of different FAAs in the perfused liver of *C. batrachus* infused with 5 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).

	Conditions		
Amino acids	**Isotonic**	**Hypotonic**	**Hypertonic**
Non-essential			
Asp	1.50 ± 0.16	0.86 ± 0.04 (-43)[a]	2.77 ± 0.52 (+85)[b]
Gly	0.96 ± 0.07	0.83 ± 0.01 (-14)	3.08 ± 0.10 (+221)[c]
Ala	2.05 ± 0.01	0.88 ± 0.06 (-57)[b]	7.00 ± 0.52 (+241)[c]
Ser	0.62 ± 0.12	0.63 ± 0.05 (+2)	2.62 ± 0.33 (+323)[c]
Glu	1.84 ± 0.60	0.95 ± 0.04 (+48)[b]	2.91 ± 0.33 (+58)[b]
Gln	1.98 ± 0.90	1.05 ± 0.02 (-47)[a]	2.57 ± 0.15 (+30)[a]
Cit	1.50 ± 0.08	1.25 ± 0.03 (-17)[a]	4.14 ± 0.55 (+176)[c]
Asn	0.59 ± 0.09	0.62 ± 0.08 (+5)	0.54 ± 0.05 (+8)
Tyr	1.03 ± 0.26	0.67 ± 0.06 (-35)[a]	2.90 ± 0.15 (+182)[c]
Pro	1.54 ± 0.44	0.76 ± 0.04 (-51)[b]	3.38 ± 0.20 (+119)[c]
GABA	0.59 ± 0.17	0.61± 0.02 (+3)	0.29 ± 0.05 (-51)[a]
Tau	10.70 ± 2.53	5.65 ± 0.24 (-47)[b]	12.76 ± 0.21 (+19)[b]
Total	**25.90 ± 5.41**	**14.76 ± 0.61 (-43)[a]**	**44.96 ± 3.14 (+74)[b]**
Essential			
Thr	0.48 ± 0.03	0.36 ± 0.06 (-25)[a]	0.88 ± 0.04 (+83)[b]
Val	0.38 ± 0.04	0.22 ± 0.03 (-42)[a]	0.51 ± 0.07 (+34)[a]
Met	0.23 ± 0.02	0.13 ± 0.03 (-43)[a]	0.56 ± 0.07 (+143)[c]
Ile	0.30 ± 0.02	0.17 ± 0.03 (-43)[a]	0.64 ± 0.05 (+113)[c]
Leu	0.48 ± 0.03	0.39 ± 0.04 (-19)	0.77 ± 0.04 (+60)[b]
Trp	0.45 ± 0.06	0.40 ± 0.02 (-11)	0.70 ± 0.05 (+56)[b]
His	0.45 ± 0.03	0.32 ± 0.03 (-29)[a]	0.61 ± 0.03 (+36)[a]
Lys	0.40 ± 0.05	0.22 ± 0.07(-45)[a]	0.43 ± 0.02 (+8)
Phe	0.32 ± 0.03	0.27 ± 0.03 (-16)	0.30 ± 0.02 (-6)
Orn	0.78 ± 0.04	0.50 ± 0.05 (-36)[a]	1.20 ± 0.02 (+54)[b]
Arg	1.09 ± 0.01	0.68 ± 0.06 (-38)[a]	1.71 ± 0.03 (+57)[b]
Total	**5.36 ± 0.34**	**3.66 ± 0.43 (-32)[a]**	**8.31 ± 0.40 (+55)[b]**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the control levels are given in parentheses

[a, b, c] : *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 3: Effects of anisotonicity on the activity of glutamine synthetase (GSase) and glutamate dehydrogenases (GDH) in the perfused liver of *C. batrachus* infused without/with 5 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).

Enzymes	Conditions	Enzyme Activity	
		Tissue (units/g wet wt)	Specific (units/mg protein)
GSase	Isotonic	76.11 ± 3.56	1.08 ± 0.14
	Hypotonic	79.47 ± 4.21 (+4)	1.16 ± 0.12 (+7)
	Hypertonic	108.69 ± 4.87 (+43)[a]	1.56 ± 0.10 (+44)[a]
With 5 mM NH_4Cl			
GSase	Isotonic	113.62 ± 5.21 [49][b]	1.55 ± 0.13
	Hypotonic	111.21 ± 4.85 (-2)	1.49 ± 0.10 (-4)
	Hypertonic	149.35 ± 6.12 (+31)[a]	2.13 ± 0.11 (+37)[a]
GDH (O.D)	Isotonic	369 ± 13.74	6.79 ± 0.26
	Hypotonic	348 ± 21.63 (-6)	6.29 ± 0.41 (-7)
	Hypertonic	474 ± 15.87 (+28)[a]	8.56 ± 0.24 (+26)[a]
GDH (R.A)	Isotonic	393 ±18.50	7.24 ± 0.28
	Hypotonic	366 ± 21.63 (-7)	6.62 ± 0.41(-9)
	Hypertonic	528 ± 12.00 (+34)[a]	9.55 ± 0.37(+32)[a]
With 5 mM NH_4Cl			
GDH (O.D)	Isotonic	489 ± 17.02 [33][a]	9.00 ± 0.32
	Hypotonic	384 ± 12.00 (-21)[a]	6.94 ± 0.29 (-23)[a]
	Hypertonic	672 ± 21.63 (+37)[a]	12.13 ± 0.16 (+35)[a]
GDH (R.A)	Isotonic	528 ± 12.87 [34][a]	9.72 ± 0.21
	Hypotonic	408 ± 15.87(-23)[a]	7.37 ± 0.31 (-24)[a]
	Hypertonic	864 ± 54.99 (+64)[b]	15.64 ±1.17 (+61)[b]

Percentage increase (+) or decrease (-) of enzyme activity compared to respective controls are given in parentheses. The values in the third bracket represent the percentage increase [+] or decrease [-] of activity in the liver infused with 5 mM NH_4Cl compared to respective controls of without NH_4Cl infusion under isotonic perfusion condition.

[a, b]: *P* values significant at <0.05, <0.01 levels, respectively (One-way ANOVA)

One unit of enzyme activity is defined as the amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

Table 4: Effects of anisotonicity on the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the perfused liver of *C. batrachus* infused without/with 5 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).

Enzymes	Conditions	Enzyme Activity	
		Tissue (units/g wetwt)	**Specific (units/mg protein)**
AST	Isotonic	380 ± 14.44	7.01 ± 0.24
	Hypotonic	324 ± 10.39 (-15)[a]	5.85 ± 0.19 (-17)[a]
	Hypertonic	486 ± 20.79 (+28)[a]	8.80 ± 0.55 (+26)[a]
With 5 mM NH_4Cl			
AST	Isotonic	492 ± 17.80 [29][a]	9.05 ± 0.30
	Hypotonic	354 ± 6.00 (-28)[a]	6.39 ± 0.03 (-29)[a]
	Hypertonic	684 ± 20.79 (+39)[a]	12.38 ± 0.58 (+37)[a]
ALT	Isotonic	411 ± 16.37	7.57± 0.35
	Hypotonic	390 ± 30.00 (-5)	7.07 ± 0.69 (-7)
	Hypertonic	512 ± 7.04 (+25)[a]	8.12 ± 0.73 (+7)
With 5 mM NH_4Cl			
ALT	Isotonic	384 ± 10.04 [-7]	7.10 ± 0.25
	Hypotonic	384 ± 42.00 (0)	6.92 ± 0.67 (-3)
	Hypertonic	622 ± 5.78 (+62)[b]	8.39 ± 0.44 (+18)[a]

Percentage increase (+) or decrease (-) of enzyme activity compared to respective controls are given in parentheses. The values in the third bracket represent the percentage increase [+] or decrease [-] of activity in the liver infused with 5 mM NH_4Cl compared to respective controls of without NH_4Cl infusion under isotonic perfusion condition.

[a, b]: *P* values significant at <0.05, <0.01 levels, respectively (One-way ANOVA)

One unit of enzyme activity is defined as the amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

Table 5: Effect of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine (μmoles/g liver/min) from the perfused liver of *C. batrachus* infused with 1 mM NH_4Cl and 0.5 mM ornithine. Values are expressed as mean ± SEM (n=5).

Conditions	Ammonia-N	Urea-N	Glutamate	Glutamine
Isotonic	3.06 ± 0.17	0.44 ± 0.07	0.08 ± 0.02	0.13 ± 0.03
Hypotonic	3.55 ± 0.10[a] (+16)	0.19 ± 0.05[b] (-57)	0.04 ± 0.02[b] (-50)	0.02 ± 0.04[b] (-85)
Isotonic	2.82 ± 0.25	0.51± 0.1	0.13 ± 0.03	0.09 ± 0.03
Hypertonic	2.31 ± 0.12 (-18)[a]	0.85 ± 0.06[b] (+67)	0.21± 0.06[b] (+62)	0.06 ± 0.01[a] (-33)

Percentage increase (+) or decrease (-) of efflux compared to respective controls are given in parentheses.

[a, b:] *P* values significant at <0.05 and <0.01 levels, respectively (One-way ANOVA).

Table 6: Effect of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine (μmoles/g liver/min) from the perfused liver of *C. batrachus* infused with 0.25 mM glutamine and 0.5 mM ornithine. Values are expressed as mean ± SEM (n=5).

Conditions	Ammonia-N	Urea-N	Glutamate	Glutamine
Isotonic	0.16 ± 0.01	0.12 ± 0.04	0.07 ± 0.01	0.49 ± 0.05
Hypotonic	0.34 ± 0.03[c] (+113)	0.25 ± 0.07[c] (+108)	0.11 ± 0.01[b] (+57)	0.33 ± 0.07[a] (+33)
Isotonic	0.71 ± 0.06	0.14 ± 0.03	0.33 ± 0.02	0.54 ± 0.05
Hypertonic	0.28 ± 0.04[b] (-61)	0.34 ± 0.04[c] (+143)	0.12 ± 0.02[b] (-64)	0.60 ± 0.06[a] (+11)

Percentage increase (+) or decrease (-) of efflux compared to respective controls are given in parentheses.

[a, b, c:] *P* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

Table 7: Effects of anisotonicity on the efflux of ammonia-N, urea-N and total-N (μmoles/g liver/min) from the perfused liver of *C. batrachus* infused with different amino acids (5 mM). Values are expressed as mean ± SEM (n=5).

Amino acids	Conditions	Ammonia-N (μmoles/g/min)	Urea-N (μmoles/g/min)	Total-N (μmoles/g/min)
Glutamine	Isotonic	0.85 ± 0.11	0.55 ± 0.05	1.40 ± 0.15
	Hypotonic	1.44 ± 0.14(+69)^{b}	0.89±0.07(+62)^{b}	2.33 ± 0.14(+66)^{b}
	Hypertonic	0.52 ± 0.06(-39)^{a}	0.26 ± 0.02(-53)^{b}	0.78 ± 0.06 (-44)^{b}
Glutamic acid	Isotonic	0.42 ± 0.09	0.37 ± 0.03	0.79 ± 0.18
	Hypotonic	0.67 ± 0.11(+60)^{b}	0.98±0.13(+165)^{c}	1.65 ± 0.34(+109)^{c}
	Hypertonic	0.22 ± 0.08(-48)^{c}	0.17 ± 0.02(-54)^{c}	0.39 ± 0.06(-51)^{c}
Aspartic acid	Isotonic	0.54 ± 0.03	0.23 ± 0.04	0.77 ± 0.08
	Hypotonic	0.80 ± 0.12(+48)^{b}	0.59 ± 0.12(+157)^{c}	1.39 ± 0.12(+157)^{c}
	Hypertonic	0.35 ± 0.02(-35)^{a}	0.05 ± 0.08(-48)^{c}	0.40 ± 1.21(-48)^{b}
Alanine	Isotonic	0.41± 0.08	0.16 ± 0.08	0.57 ± 0.23
	Hypotonic	0.71± 0.12(+73)^{c}	0.39 ± 0.05(+144)^{c}	1.10 ± 0.45(+93)^{c}
	Hypertonic	0.20 ± 0.02(-51)^{c}	0.06 ± 0.01(-63)^{c}	0.26 ± 0.03(-54)^{c}
Glycine	Isotonic	0.24 ± 0.05	0.15 ± 0.08	0.39 ± 0.08
	Hypotonic	0.38 ± 0.11(+58)^{b}	0.29 ± 0.08(+93)^{c}	0.67 ± 0.15(+72)^{c}
	Hypertonic	0.15 ± 0.04(-38)^{b}	0.08 ± 0.01(-47)^{b}	0.23 ± 0.08(-41)^{b}
Threonine	Isotonic	0.24 ± 0.05	0.10 ± 0.07	0.38 ± 0.05
	Hypotonic	0.40 ± 0.08(+58)^{b}	0.18 ± 0.04(+80)^{c}	0.58 ± 0.10(+53)^{b}
	Hypertonic	0.15 ± 0.05(-38)^{b}	0.15 ± 0.04(-50)^{c}	0.20 ± 0.07(-47)^{c}
Serine	Isotonic	0.21 ± 0.08	0.08 ± 0.01	0.29 ± 0.06
	Hypotonic	0.41± 0.09(+95)^{c}	0.24 ± 0.08(+200)^{c}	0.65 ± 0.14(+124)^{c}
	Hypertonic	0.12 ± 0.06(-43)^{b}	0.04 ± 0.02(-50)^{c}	0.16 ± 0.05(-45)^{c}

Percentage increase (+)/decrease (-) in the efflux of ammonia-N, urea-N and total-N as against the controls are given in parentheses.

$^{a, b, c}$: *P* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

Table 8: Changes in the levels of different FAAs (µmoles/g wet wt) in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

Amino acids	Control	Treated	Control	Treated
Non-essential				
Asp	2.47 ± 0.19	3.63 ± 0.21 (+47)[a]	2.26 ± 0.22	4.21 ± 0.33 (+86)[c]
Gly	1.43 ± 0.18	2.41 ± 1.04 (+69)[b]	1.51 ± 0.02	2.62 ± 0.08 (+74)[b]
Ala	1.45 ± 0.24	1.94 ± 0.02 (+34)[a]	2.05 ± 0.20	4.14 ± 0.33 (+102)[c]
Ser	1.31 ± 0.10	2.15 ± 0.24 (+64)[b]	1.40 ± 0.12	1.88 ± 0.15 (+34)[a]
Asn	0.40 ± 0.21	0.69 ± 0.07 (+73)[b]	0.36 ± 0.02	1.14 ± 0.12 (+217)[c]
Glu	2.43 ± 0.20	6.90 ± 0.34 (+184)[c]	3.15 ± 0.33	5.90 ± 0.38 (+87)[b]
Gln	1.55 ± 0.13	2.87 ± 0.23 (+85)[b]	1.38 ± 0.14	3.21 ± 0.32 (+133)[c]
Cit	0.24 ± 0.04	0.52 ± 0.11 (+117)[c]	0.34 ± 0.05	0.78 ± 0.07 (+129)[c]
Tyr	0.97 ± 0.16	0.72 ± 0.13 (-26)[a]	1.05 ± 0.08	1.37 ± 0.11 (+30)[a]
Pro	1.54 ± 0.26	1.42 ± 0.21 (-8)	1.82 ± 0.28	2.23 ± 0.16 (+23)[a]
Tau	16.98 ± 1.64	28.62 ± 2.21(+69)[b]	15.39 ± 1.07	32.65 ± 0.08 (+112)
Total	**31.41 ± 3.46**	**52.49 ± 8.06 (+67)[b]**	**31.31 ± 2.58**	**60.30 ± 3.65 (+93)[b]**
Essential				
Thr	2.86 ± 0.32	2.91 ± 0.22 (+2)	2.33 ± 0.35	2.50 ± 0.29 (+7)
Val	1.27 ± 0.14	1.49 ± 0.12 (+17)[a]	1.78 ± 0.14	1.88 ± 0.16 (+6)
Met	0.88 ± 0.12	0.50 ± 0.44 (+2)	0.66 ± 0.04	0.85 ± 0.05 (+29)[a]
Ile	0.75 ± 0.07	0.80 ± 0.06 (+7)	1.18 ± 0.11	1.34 ± 0.12 (+14)
Leu	1.52 ± 0.29	1.72 ± 0.18 (+13)	1.58 ± 0.14	1.95 ± 0.21 (+23)[a]
Trp	0.46 ± 0.45	0.48 ± 0.44 (+4)	0.35 ± 0.01	0.47 ± 0.02 (+34)[a]
Phe	0.54 ± 0.05	0.68 ± 0.05 (+26)[a]	0.52 ± 0.07	0.69 ± 0.07 (+33)[a]
His	0.85 ± 0.28	0.95 ± 0.28 (+12)	0.79 ± 0.13	0.93 ± 0.11 (+14)
Orn	1.25 ± 0.15	1.55 ± 0.15 (+24)[a]	1.77 ± 0.17	1.86 ± 0.16 (+5)
Arg	0.69 ± 0.09	0.75 ± 0.03 (+9)	0.78 ± 0.06	0.80 ± 0.13 (+3)
Total	**11.07 ± 1.96**	**12.23 ± 1.97 (+10)[a]**	**11.74 ± 1.22**	**13.27 ± 1.32 (+13)[a]**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the respective controls are given in parentheses

[a, b, c] : *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 9: Changes in the levels of different FAAs (μmoles/g wet wt) in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

Amino acids	Control	Treated	Control	Treated
Non-essential				
Asp	2.94 ± 0.22	4.35 ± 0.37 (+48)[b]	3.09 ± 0.01	5.13 ± 0.41 (+66)[b]
Gly	3.15 ± 0.23	4.27 ± 0.47 (+36)[a]	3.16 ± 0.02	5.17 ± 0.52 (+64)[b]
Ala	6.25 ± 0.45	9.41 ± 0.61 (+51)[a]	6.88 ± 0.06	12.1 ± 0.92 (+76)[b]
Ser	5.24 ± 0.56	5.45 ± 0.36 (+4)	5.90 ± 0.04	7.27 ± 0.83 (+23)[a]
Asn	0.76 ± 0.13	0.95 ± 0.07 (+25)[a]	0.73 ± 0.03	1.30 ± 0.12 (+78)[b]
Glu	6.21 ± 0.61	7.51 ± 1.00 (+21)[a]	6.15 ± 0.03	8.43 ± 1.03 (+37)[a]
Gln	1.35 ± 0.13	3.73 ± 0.29 (+176)[c]	1.70 ± 0.06	4.31 ± 0.32 (+154)[c]
Cit	0.65 ± 0.05	1.14 ± 0.11 (+75)[b]	0.61 ± 0.01	1.26 ± 0.12 (+107)[c]
Tyr	1.13 ± 0.07	1.52 ± 0.13 (+35)[a]	1.06 ± 0.02	2.11 ± 0.32 (+99)[c]
Pro	1.65 ± 0.08	1.73 ± 0.11 (+5)	1.63 ± 0.56	2.88 ± 0.23 (+77)[b]
GABA	0.70 ± 0.05	0.75 ± 0.18 (+7)	0.64 ± 0.11	0.62 ± 0.09 (-3)
Tau	12.45 ± 1.13	17.52 ± 1.89 (+41)[a]	11.21 ± 1.02	19.4 ± 1.62 (+73)[b]
Total	**42.48 ± 3.71**	**58.33 ± 5.59 (+37)[a]**	**42.76 ± 1.97**	**69.98 ± 6.53 (+64)[b]**
Essential				
Thr	3.07 ± 0.41	3.44 ± 0.39 (+12)	3.23 ± 0.22	3.58 ± 0.26 (+11)
Val	3.27 ± 0.33	4.65 ± 0.34 (+42)[a]	3.38 ± 0.34	4.76 ± 0.44 (+41)[a]
Met	1.17 ± 0.12	1.61 ± 0.23 (+38)[a]	1.06 ± 0.08	2.29 ± 0.31 (+116)[c]
Ile	2.28 ± 0.24	3.39 ± 0.34 (+49)[b]	2.08 ± 0.13	3.16 ± 0.34 (+52)[b]
Leu	3.24 ± 0.23	4.45 ± 0.46 (+37)[a]	4.13 ± 0.21	5.17 ± 0.42 (+25)[a]
Trp	0.50 ± 0.05	0.69 ± 0.15 (+38)[a]	0.57 ± 0.05	0.86 ± 0.12 (+51)[a]
Phe	1.14 ± 0.22	1.57 ± 0.08 (+38)[a]	1.07 ± 0.12	1.58 ± 0.13 (+48)[a]
His	1.26 ± 0.13	1.58 ± 0.16 (+25)[a]	1.30 ± 0.12	1.38 ± 0.14 (+6)
Orn	1.13 ± 0.12	1.34 ± 0.18 (+19)[a]	1.39 ± 0.13	1.62 ± 0.14 (+17)[a]
Arg	1.17 ± 0.12	1.32 ± 0.11 (+13)	1.15 ± 0.13	1.39 ± 0.14 (+21)[a]
Total	**18.23 ± 1.97**	**24.04 ± 2.44 (+32)[a]**	**19.36 ± 1.53**	**25.79 ± 2.34 (+33)[a]**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the respective controls are given in parentheses

[a, b, c] : *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 10: Changes in the levels of different FAAs (µmoles/g wet wt) in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

Amino acids	Control	Treated	Control	Treated
Non-essential				
Asp	0.59 ± 0.08	1.59 ± 0.19 (+169)^{c}	0.44 ± 0.05	0.80 ± 0.04 (+82)^{c}
Gly	12.41 ± 0.65	15.72 ± 1.20 (+27)^{a}	13.23 ± 1.02	16.21 ± 2.03 (+23)^{a}
Ala	6.47 ± 0.53	9.75 ± 1.21 (+51)^{a}	7.26 ± 0.92	12.1 ± 1.22 (+67)^{b}
Ser	0.57 ± 0.06	0.91 ± 0.11 (+60)^{b}	0.60 ± 0.06	0.67 ± 0.03 (+12)
Asn	0.26 ± 0.02	0.36 ± 0.08 (+38)^{a}	0.30 ± 0.03	0.73 ± 0.05 (+143)^{c}
Glu	0.42 ± 0.05	1.17 ± 0.11 (+179)^{c}	0.44 ± 0.04	1.94 ± 0.25 (+341)^{c}.
Gln	0.38 ± 0.06	0.80 ± 0.08 (+111)^{c}	0.44 ± 0.03	2.31 ± 0.24 (+425)^{c}
Cit	0.21 ± 0.02	0.52 ± 0.13 (+148)^{c}	0.17 ± 0.02	0.18 ± 0.02 (+6)
Tyr	0.37 ± 0.03	0.44 ± 0.07 (+19)	0.45 ± 0.03	0.59 ± 0.05 (+31)^{a}
Pro	0.51 ± 0.08	0.77 ± 0.19 (+51)^{b}	0.32 ± 0.04	0.35 ± 0.04 (+9)
GABA	0.65 ± 0.08	0.64 ± 0.15 (-2)	0.65 ± 0.08	0.64 ± 0.15 (-2)
Tau	13.34 ± 2.14	16.65 ± 2.15 (+25)^{a}	14.29 ±1.44	17.34 ± 2.05 (+21)^{a}
Total	**36.18 ± 3.80**	**49.32 ± 5.73 (+36)^{a}**	**38.59 ± 3.76**	**53.86 ± 6.17 (+40)^{b}**
Essential				
Thr	0.32 ± 0.10	0.48 ± 0.10 (+50)^{b}	0.58 ± 0.04	0.95 ± 0.03 (+64)^{b}
Val	0.17 ± 0.02	0.26 ± 0.05 (+53)^{b}	0.17 ± 0.01	0.29 ± 0.04 (+71)^{b}
Met	0.44 ± 0.04	0.31 ± 0.09 (-30)^{a}	0.26 ± 0.02	0.37 ± 0.02 (+42)^{a}
Ile	0.65 ± 0.02	0.46 ± 0.04 (-30)^{a}	0.16 ± 0.03	0.22 ± 0.02 (+38)^{a}
Leu	0.47 ± 0.09	0.40 ± 0.06 (-15)	0.73 ± 0.04	1.17 ± 0.02 (+60)^{b}
Trp	0.89 ± 0.04	0.25 ± 0.01 (-72)	0.17 ± 0.02	0.20 ± 0.01 (+18)
Phe	0.17 ± 0.02	0.19 ± 0.02 (+12)	0.16 ± 0.03	0.23 ± 0.02 (+44)^{a}
His	0.12 ± 0.02	0.40 ± 0.05 (+233)^{c}	0.13 ± 0.02	0.18 ± 0.02 (+38)^{a}
Orn	0.29 ± 0.07	0.46 ± 0.05 (+59)^{b}	0.24 ± 0.02	0.30 ± 0.04 (+25)^{a}
Arg	0.13 ± 0.03	0.46 ± 0.07 (+254)^{c}	0.33 ± 0.02	0.44 ± 0.02 (+33)^{a}
Total	**3.79 ± 0.93**	**4.09 ± 0.58 (+8)**	**2.93 ± 0.27**	**4.35 ± 0.24 (+48)^{b}**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the respective controls are given in parentheses

$^{a, b, c}$: *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 11: Changes in the levels of different FAAs (µmoles/g wet wt) in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

Amino acids	Control	Treated	Control	Treated
Non-essential				
Asp	0.50 ± 0.08	0.62 ± 0.29 (+24)[a]	0.56 ± 0.04	1.39 ± 0.17 (+148)[c]
Gly	1.12 ± 0.03	1.17 ± 0.02 (+4)[a]	1.11 ± 0.12	1.27 ± 0.11 (+14)
Ala	0.56 ± 0.05	0.64 ± 0.18 (+14)	0.84 ± 1.13	0.64 ± 0.08 (-24)[a]
Ser	0.32 ± 0.08	0.33 ± 0.04 (+3)	0.44 ± 0.02	0.53 ± 0.04 (+20)[a]
Asn	0.27 ± 0.05	0.32 ± 0.11 (+19)[a]	0.17 ± 0.02	0.32 ± 0.11(+88)[b]
Glu	3.21 ± 0.05	4.42 ± 0.08 (+38)[a]	3.47 ± 0.25	5.42 ± 0.38 (+56)[b]
Gln	2.39 ± 0.24	3.61 ± 0.33 (+51)[b]	2.41 ± 0.17	3.61 ± 0.33 (+50)[a]
Cit	0.29 ± 0.14	0.60 ± 0.11 (+107)[c]	0.27 ± 0.02	0.60 ± 0.11 (+122)[c]
Tyr	0.16 ± 0.02	0.45 ± 0.11 (+181)[c]	0.17 ± 0.03	0.45 ± 0.06 (+165)[c]
Pro	0.14 ± 0.02	0.51 ± 0.05 (+5)	0.15 ± 0.03	0.51 ± 0.04 (+240)[c]
Tau	11.30 ± 1.02	14.00 ± 1.24 (+41)[a]	10.18 ± 1.89	15.28 ± 2.06 (+24)[a]
Total	**23.26 ± 1.78**	**26.67 ± 2.56 (+32)[a]**	**19.77 ± 1.89**	**30.02 ± 1.43 (+52)[b]**
Essential				
Thr	0.20 ± 0.02	0.27 ± 0.05 (+35)[a]	0.24 ± 0.03	0.31 ± 0.02 (+29)[a]
Val	0.30 ± 0.10	0.53 ± 0.34 (+77)[b]	0.28 ± 0.02	0.38 ± 0.03 (+36)[a]
Met	0.27 ± 0.06	0.37 ± 0.07 (+37)[a]	0.15 ± 0.02	0.17 ± 0.02 (+13)
Ile	0.25 ± 0.01	0.41 ± 0.04 (+64)[b]	0.16 ± 0.02	0.12 ± 0.02 (-25)
Leu	0.66 ± 0.04	0.43 ± 0.05 (-35)	0.63 ± 0.04	0.77 ± 0.06 (+22)[a]
Trp	0.15 ± 0.02	0.50 ± 0.05 (+233)[c]	0.16 ± 0.02	0.19 ± 0.04 (+19)
Phe	0.14 ± 0.05	0.43 ± 0.26 (+207)[c]	0.16 ± 0.04	0.56 ± 0.07 (+250)[c]
His	0.39 ± 0.05	0.87 ± 0.06 (+123)[c]	0.33 ± 0.02	0.70 ± 0.05 (+112)[c]
Orn	0.25 ± 0.01	0.81 ± 0.09 (+224)[c]	0.25 ± 0.03	0.66 ± 0.05 (+164)[c]
Arg	0.59 ± 0.17	0.92 ± 0.03 (+56)[b]	0.54 ± 0.03	0.74 ± 0.13 (+37)[a]
Total	**3.20 ± 0.53**	**5.54 ± 1.04 (+73)[b]**	**2.9 ± 0.27**	**4.60 ± 0.49 (+59)[b]**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the respective controls are given in parentheses

[a, b, c] : *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 12: Changes in the levels of different FAAs (µmoles/g wet wt) in the plasma of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

Amino acids	Control	Treated	Control	Treated
Non-essential				
Asp	0.28 ± 0.05	0.74 ± 0.07 (+164)^{c}	0.24 ± 0.04	0.77 ± 0.06 (+221)^{c}
Gly	0.26 ± 0.02	0.66 ± 0.02 (+154)^{c}	0.23 ± 0.04	0.73 ± 0.05 (+217)^{c}
Ala	0.23 ± 0.04	0.98 ± 0.07 (+326)^{c}	0.28 ± 0.03	0.81 ± 0.07 (+189)^{c}
Ser	0.20 ± 0.02	0.34 ± 0.07 (+70)^{b}	0.24 ± 0.05	0.39 ± 0.04 (+63)^{b}
Asn	0.11 ± 0.02	0.04 ± 0.02 (-64)^{b}	0.18 ± 0.03	0.19 ± 0.02 (+6)
Glu	0.19 ± 0.02	0.80 ± 0.05 (+321)^{c}	0.17 ± 0.05	0.60 ± 0.08 (+253)^{c}
Gln	0.16 ± 0.02	0.38 ± 0.11 (+46)^{a}	0.14 ± 0.03	0.36 ± 0.07 (+157)^{c}
Cit	0.05 ± 0.02	0.20 ± 0.07 (+300)^{c}	0.06 ± 0.05	0.23 ± 0.05 (+283)^{c}
Tyr	0.14 ± 0.02	0.40 ± 0.05 (+186)^{c}	0.17 ± 0.04	0.49 ± 0.09 (+188)^{c}
Pro	0.09 ± 0.02	0.03 ± 0.01 (-67)^{b}	0.12 ± 0.01	0.14 ± 0.03 (+17)^{a}
Tau	1.27 ± 0.17	2.47 ± 0.34 (+94)^{c}	1.22 ± 0.24	4.79 ± 0.33 (+293)^{c}
Total	**2.98 ± 0.42**	**7.04 ± 0.88 (+136)^{c}**	**3.05 ± 0.61**	**10.13 ± 0.90 (+232)^{c}**
Essential				
Thr	0.13 ± 0.02	0.56 ± 0.04 (+331)^{c}	0.15 ± 0.05	0.62 ± 0.07 (+313)^{c}
Val	0.07 ± 0.03	0.15 ± 0.01 (+114)^{c}	0.08 ± 0.05	0.18 ± 0.05 (+125)^{b}
Met	0.06 ± 0.03	0.15 ± 0.01 (+150)^{c}	0.05 ± 0.02	0.17 ± 0.02 (+240)^{c}
Ile	0.17 ± 0.07	0.28 ± 0.04 (+65)^{b}	0.18 ± 0.04	0.31 ± 0.09 (+72)^{b}
Leu	0.06 ± 0.03	0.35 ± 0.05 (+483)^{c}	0.06 ± 0.03	0.28 ± 0.04 (+367)^{c}
Trp	0.21 ± 0.02	0.34 ± 0.06 (+62)^{b}	0.27 ± 0.05	0.30 ± 0.08 (+11)
Phe	0.04 ± 0.02	0.10 ± 0.02 (+150)^{c}	0.04 ± 0.04	0.15 ± 0.04 (+275)^{c}
His	0.07 ± 0.04	0.11 ± 0.01(+57)^{a}	0.08 ± 0.03	0.07 ± 0.05 (-13)
Orn	0.11 ± 0.02	0.36 ± 0.06 (+227)^{c}	0.13 ± 0.02	0.39 ± 0.10 (+200)^{c}
Arg	0.13 ± 0.06	0.14 ± 0.02 (+8)	0.16 ± 0.04	0.20 ± 0.04 (+25)^{a}
Total	**1.05 ± 0.34**	**2.54 ± 0.6 (+142)^{c}**	**1.2 ± 0.45**	**2.87 ± 0.58 (+139)^{c}**

Percentage increase (+)/decrease (-) in the levels of FAAs as against the respective controls are given in parentheses

$^{a, b, c}$: *P* values significant at <0.05, <0.01, <0.001 levels, respectively (One-way ANOVA).

Table 13: Changes in the tissue (µmoles/g wet wt) and specific (units/mg protein) activities of different amino acid metabolism-related enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± S.E.M. (n=5).

Enzymes	Days of exposure							
	3				7			
	Control		Treated		Control		Treated	
	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
GSase	133 ± 6.2	2.46 ± 0.14	161.8 ± 14.4 (+21)[a]	2.92 ± 0.22 (+19)[a]	135.8 ± 14.3	3.13 ± 0.31	185.4 ± 4.8 (+37)[a]	4.30 ± 0.19 (+37)[a]
GDH (R.A)	672 ± 27.7	7.44 ± 0.93	1328 ± 55.9 (+98)[c]	18.06 ± 2.07 (+143)[c]	708 ± 55.4	7.77 ± 0.53	1104 ± 83.1 (+56)[b]	17.58 ± 1.44 (+126)[c]
GDH (O.D)	218 ± 10.6	2.52 ± 0.25	362.7 ± 37.2 (+66)[b]	4.86 ± 0.49 (+93)[b]	228 ± 27.7	6.64 ± 0.64	304 ± 9.2 (+33)[a]	9.84 ± 0.58 (+48)[b]
AST	348 ± 12.0	6.73 ± 1.25	372 ± 15.9 (+7)	6.95 ± 0.75 (+3)	332 ± 27.4	9.99 ± 0.80	338 ± 33.4 (+2)	8.98 ± 0.56 (-10)
ALT	294 ± 15.9	3.32 ± 0.35	396 ± 10.4 (+35)[a]	5.42 ± 0.81 (+63)[b]	310 ± 28.13	11.76 ± 2.06	606 ± 53.33 (+95)[c]	22.31±1.24 (+90)[c]

Percentage increase (+) of enzyme activity compared to the respective controls are given in parentheses.

One unit of enzyme activity is defined as the amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

[a, b, c]: *P* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

GSase - glutamine synthetase; GDH (O.D.) - glutamate dehydrogenase (oxidative deamination); GDH (R.A.) - glutamate dehydrogenase (reductive amination); AST - aspartate aminotransferase; ALT - alanine aminotransferase.

Table 14: Changes in the tissue (µmoles/g wet wt) and specific (units/mg protein) activities of different amino acid metabolism-related enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± S.E.M. (n=5).

Enzymes	Days of exposure							
	3				7			
	Control		Treated		Control		Treated	
	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
GSase	107.2 ± 9.3	2.51 ± 0.10	130 ± 5.7 (+21)[a]	2.95 ± 0.23 (+18)[a]	103.9 ± 8.6	2.43 ± 0.17	136.5 ± 9.1 (+31)[a]	2.92 ± 0.32 (+20)[a]
GDH (R.A)	592 ± 15.9	10.26 ± 1.59	1072 ± 15.9 (+81)[c]	13.74 ± 1.23 (+34)[a]	704 ± 84.6	12.74 ± 1.65	1180 ± 15.9 (+68)[b]	19.37± 0.78 (+52)[b]
GDH (O.D)	192 ± 18.5	3.47 ± 0.29	336 ± 9.2 (+75)[b]	4.29 ± 0.28 (+24)[a]	220 ± 46.2	6.25 ± 0.91	314.6 ± 14.1 (+43)[b]	9.13 ± 0.31 (+46)[b]
AST	264 ± 6.0	4.66 ± 0.42	336 ± 15.8 (+28)[a]	7.33 ± 0.52 (+57)[b]	254 ± 22.0	5.83 ± 0.90	414 ± 20.8 (+63)[b]	9.08 ± 0.39 (+56)[b]
ALT	288 ± 10.4	5.04 ± 0.22	366 ± 31.8 (+27)[a]	6.62 ± 0.13 (+31)[a]	290 ± 26.2	7.61 ± 0.50	432 ± 27.5 (+49)[b]	17.03 ± 0.44 (+124)[c]

Percentage increase (+) in the enzyme activity compared to the respective controls are given in parentheses.

[a, b, c]: *P* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

Table 15: Changes in the tissue (µmoles/g wet wt) and specific (units/mg protein) activities of different amino acid metabolism-related enzymes in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± S.E.M. (n=5).

Enzymes	Days of exposure							
	3				7			
	Control		Treated		Control		Treated	
	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
GSase	21.4 ± 2.5	0.83 ± 0.05	26.4 ± 2.8 (+23)[a]	1.07 ± 0.10 (+29) [a]	21.1 ± 5.0	0.78 ± 0.13	27.3 ± 4.7 (+29)[a]	1.06 ± 0.12 (+36)[a]
GDH (R.A)	213.3 ± 5.3	5.47 ± 1.63	362.7 ± 5.3 (+70)[b]	7.78 ± 0.39 (+42)[a]	207.3 ± 37.3	6.96 ± 1.01	330.7 ± 68.0 (+59)[b]	17.49 ± 1.20 (+151)[c]
GDH (O.D)	186.6 ± 10.6	4.77 ± 1.36	194 ± 16.0 (+4)	5.24 ± 0.60 (+10)	205 ± 19.2	6.28 ± 0.55	296.7 ± 19.2 (+44)[a]	8.32 ± 0.59 (+32)[a]
AST	246.0 ± 15.9	6.58 ± 2.40	372 ± 15.9 (+51)[b]	9.73 ± 0.82 (+48)[b]	256 ± 22.0	7.37 ± 1.11	396.0 ± 20.7 (+55)[b]	9.82 ± 0.50 (+33)[a]
ALT	264 ± 6.0	6.72 ± 1.91	350 ± 21.6 (+33)[a]	9.07 ± 0.54 (+35)[a]	260 ± 27.5	9.02 ± 0.75	492.0 ± 22.0 (+89)[b]	11.33 ± 0.32 (+26)[a]

Percentage increase (+) in the enzyme activity compared to the respective controls are given in parentheses.

[a, b, c]: *P* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

Table 16: Changes in the tissue (µmoles/g wet wt) and specific (units/mg protein) activities of different amino acid metabolism-related enzymes in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± S.E.M. (n=5).

Enzymes	Days of exposure							
	3				7			
	Control		Treated		Control		Treated	
	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
GSase	364.5 ± 4.9	10.56 ± 0.84	395.6 ± 9.6 (+9)	10.96 ± 0.47 (+4)	329.1 ± 6.4	10 ± 0.53	398.7 ± 12.3 (+21)[a]	14.40 ± 1.28 (+44)[a]
GDH (R.A)	2224 ± 100	41.13 ± 3.49	2341.3 ± 85.3 (+5)	45.23 ± 3.43 (+10)	2303.3 ± 98.7	49.90 ± 4.03	2485.3 ± 104.8 (+8)	46.51± 3.09 (-7)
GDH (O.D)	218.7 ± 14.1	4.29 ± 0.68	222 ± 18.5 (+2)	5.35 ± 0.23 (+25)[a]	266.7 ± 19.2	5.79 ± 0.64	282.6 ± 29.7 (+6)	5.90 ± 0.45 (+2)
AST	64 ± 3.6	2.97 ± 0.30	73 ± 8.0 (+14)	3.22 ± 0.51 (+8)	72 ± 4.8	4.12 ± 0.86	76 ± 7.5 (+6)	4.18 ± 0.56 (+2)
ALT	58 ± 6.0	3.15 ± 0.34	94 ± 5.4 (+62)[b]	3.95 ± 0.52 (+25)[a]	66 ± 46.8	4.28 ± 5.87	117 ± 5.7 (+77)[b]	6.48 ± 0.92 (+51)[b]

Percentage increase (+) or decrease (-) in the enzyme activity compared to the respective controls are given in parentheses.

[a, b,] : *P* values significant at <0.05, and <0.01 levels, respectively (One-way ANOVA).

Table 17: Pattern of excretion of ammonia and urea-N (µmoles/kg body wt/h) by *C. batrachus* during exposure to 250 mOsmol/l mannitol for 7 days. Values are expressed as mean ± SEM (n=5).

	Ammonia-N		Urea-N	
Days	**Control**	**Treated**	**Control**	**Treated**
1	303.11 ± 16.3	141.78 ± 8.28* (-54)	85.11 ± 4.38	125 ± 6.51* (+47)
2	343.5 ± 21.4	147.17 ± 12.34* (-57)	79 ± 4.93	123.5 ± 8.05* (+56)
3	349.33 ± 21.6	105.33 ± 7.69* (-70)	94.83 ± 6.49	158.33 ± 10.53* (+67)
4	294 ± 18.58	94.33 ± 5.36* (-68)	97.67 ± 6.57	143.67 ± 9.24* (+47)
5	309.33 ± 26.44	93.33 ± 6.33* (-70)	89.33 ± 9.26	141 ± 9.29* (+58)
6	315.33 ± 27.84	100 ± 5.69* (-68)	79.33 ± 4.98	143.33 ± 8.69* (+81)
7	324.67 ± 23.24	106.33 ± 11.14* (-67)	75.33 ± 12.41	138 ± 6.35* (+83)

Percentage increase (+) or decrease (-) in ammonia-N and urea-N excretion by the treated fish compared to respective controls are given in parentheses.

*: *P* value significant at <0.001 level (One-way ANOVA).

Table 18: Changes in the concentration of ammonia in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* following exposure to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Tissue	Conditions	Days of exposure	
		3	7
Liver	Control	10.7 ± 0.62	11.1 ± 0.72
	Mannitol	12.9 ± 1.22[a]	13.4 ± 1.15[a]
		(+21)	(+21)
Kidney	Control	9.7 ± 0.68	10.4 ± 0.78
	Mannitol	12.2 ±1.11[a]	12.9 ± 1.16[a]
		(+26)	(+24)
Muscle	Control	4.8 ± 0.72	5.12 ± 0.37
	Mannitol	5.2 ± 0.31	5.6 ± 0.35
		(-9)	(+15)
Brain	Control	3.5 ± 0.28	3.4 ± 0.22
	Mannitol	3.2 ± 0.32	3.9 ± 0.25
		(-9)	(+15)
Plasma	Control	0.69 ± 0.08	0.68 ± 0.07
	Mannitol	0.75 ± 0.12	0.78 ± 0.11
		(+9)	(+15)

Percentage increase (+)/decrease (-) in the levels of ammonia as against the respective controls are given in parentheses

[a] : *P* value significant at <0.05 level, (One-way ANOVA).

Table 19: Changes in the concentration of urea-N in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* following exposure to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Tissue	Conditions	Days of exposure	
		3	7
Liver	Control	8.2 ± 0.65	8.4 ± 0.75
	Mannitol	12.8 ± 0.75[b] (+56)	13.4 ± 1.25[b] (+61)
Kidney	Control	7.2 ± 0.45	7.6 ± 0.65
	Mannitol	9.5 ± 0.75[a] (+32)	11.4 ± 0.85[b] (+50)
Muscle	Control	3.2 ± 0.27	3.6 ± 0.25
	Mannitol	5.6 ± 0.37[b] (+75)	6.2 ± 0.41[b] (+72)
Brain	Control	2.5 ± 0.12	2.6 ± 0.16
	Mannitol	3.1 ± 0.25[a] (+24)	3.4 ± 0.21[a] (+31)
Plasma	Control	0.81 ± 0.08	0.82 ± 0.06
	Mannitol	1.68 ± 0.12[c] (+107)	1.94 ± 0.14[c] (+137)

Percentage increase (+) in the levels of urea-N as against the respective controls are given in parentheses

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively (One-way ANOVA).

Table 20: Changes in tissue activity (units/g wet wt) of urea cycle enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Days of exposure	Conditions	Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	3.15 ± 0.15	105.4 ± 5.7	44.2 ± 3.9	51.4 ± 4.6	3785 ± 115
	Treated	4.05 ± 0.19[a] (+29)	115.3 ± 6.2 (+9)	57.4 ± 4.5[a] (+30)	68.3 ± 4.9[a] (+33)	3815 ± 127[a] (+1)
7	Control	3.21 ± 0.21	109.2 ± 7.1	47.3 ± 4.2	49.7 ± 3.8	3791 ± 138
	Treated	4.38 ± 0.25[a] (+36)	119.5 ± 7.5 (+9)	63.4 ± 4.6[a] (+34)	71.2 ± 4.5[b] (+43)	3821 ± 141[b] (+1)

Percentage increase (+) of enzyme activity compared to the respective controls are given in parentheses

One unit of enzyme activity is defined as that amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

[a, b,] : *P* values significant at <0.05 and <0.01 levels, respectively (One-way ANOVA).

CPS- carbamyl phosphate synthetase; OTC- ornithine transcarbomylase; ASS- argininosuccinate synthetase; ASL- argininosuccinate lyase and ARG- arginase.

Table 21: Changes in specific activity (units/mg protein) of urea cycle enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Days of exposure	Conditions	Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	0.06 ± 0.001	1.95 ± 0.12	0.82 ± 0.05	0.95 ± 0.10	70.1 ± 5.7
	Treated	0.08 ± 0.002[a] (+33)	2.21 ± 0.15 (+13)	1.10 ± 0.07[a] (+34)	1.31 ± 0.09[a] (+38)	73.4 ± 6.2 (+5)
7	Control	0.06 ± 0.002	2.06 ± 0.03	0.89 ± 0.09	0.94 ± 0.07	71.5 ± 7.2
	Treated	0.09 ± 0.003[b] (+50)	2.29 ± 0.11 (+11)	1.22 ± 0.04[a] (+37)	1.37 ± 0.11[b] (+46)	73.5 ± 6.5 (+3)

Percentage increase (+) of enzyme activity compared to the respective controls are given in parentheses

One unit of enzyme activity is defined as that amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

[a, b,] :*P* values significant at <0.05 and <0.01 levels, respectively (One-way ANOVA).

Table 22: Changes in tissue activity (units/g wet wt) of urea cycle enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Days of exposure	Conditions	Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	2.45 ± 0.11	55.4 ± 3.4	41.5 ± 3.8	38.9 ± 4.3	850 ± 55
	Treated	3.05 ± 0.12[a] (+24)	57.2 ± 4.1 (+3)	52.4 ± 4.2[a] (+26)	53.1 ± 4.5[a] (+37	864 ± 57 (+2)
7	Control	2.51 ± 0.12	57.2 ± 4.2	40.7 ± 3.5	39.4 ± 2.8	874 ± 61
	Treated	3.55 ± 0.14[b] (+41)	59.6 ± 5.2 (+4)	56.9 ± 4.3[b] (+40)	55.7 ± 3.5[a] (+41)	889 ± 58 (+2)

Percentage increase (+) of enzyme activity compared to the respective controls are given in parentheses

One unit of enzyme activity is defined as that amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

[a, b,] : *P* values significant at <0.05 and <0.01 levels, respectively (One-way ANOVA).

Table 23: Changes in specific activity (units/mg protein) of urea cycle enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

Days of exposure	Conditions	Enzymes				
		CPS	OTC	ASS	ASL	ARG
3	Control	0.05 ± 0.001	1.15 ± 0.05	0.82 ± 0.05	0.81 ± 0.05	17.7 ± 1.5
	Treated	0.06 ± 0.002[a] (+20)	1.22 ± 0.06 (+6)	1.11 ± 0.07[a] (+29)	1.31 ± 0.06[a] (+40)	18.2 ± 1.5 (+3)
7	Control	0.05 ± 0.002	1.22 ± 0.07	0.87 ± 0.05	0.84 ± 0.04	18.6 ± 1.7
	Treated	0.08 ± 0.003[b] (+60)	1.32 ± 0.05 (+8)	1.28 ± 0.07[b] (+47)	1.24 ± 0.07[b] (+2)	19.8 ± 1.9 (+6)

Percentage increase (+) of enzyme activity compared to the respective controls are given in parentheses

One unit of enzyme activity is defined as that amount of enzyme that catalyzed 1 µmole of product formed or substrate used per h at 30 °C.

[a, b,] : *P* values significant at <0.05 and <0.01 levels, respectively (One-way ANOVA).

Table 24: Changes in the osmolarity of blood plasma (mOsmol/l) of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± S.E.M. (n=5).

Conditions	Days of exposure	
	3	7
Control	265 ± 1.45	267 ± 3.18
Treated	317 ± 9.21 (+20)[a]	347 ± 2.52 (+30)[a]

Percentage increase (+) in the osmolarity compared to the respective controls are given in parentheses.

[a]: *P* value significant at <0.05 level, (One-way ANOVA).

Table 25: Taurine concentration in the isolated RBC of *C. batrachus* under iso- and anisotonic conditions pre-incubated with/without 2 mM taurine.

Values are expressed as mean ± SEM (n = 5).

	Conditions	Taurine concentration (µmoles/g dry wt/2h)
Without taurine	**Isotonic**	0.787 ± 0.09
	Hypotonic	0.254 ± 0.05(-68)[c]
	Hypertonic	0.769 ± 0.10 (-2)
With taurine	**Isotonic**	4.63 ± 0.28
	Hypotonic	1.25 ± 0.12 (-73)[c]
	Hypertonic	4.37 ± 0.59 (-6)

Percentage decrease (-) in the taurine concentration compared to the respective controls are given in parentheses.

[c]: *P* value significant at <0.001 level (One-way ANOVA).

Table 26: Taurine uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentrations of taurine for 2 h both under iso- and anisotonic conditions while maintaining the osmolarity of the media with NaCl.

Values are expressed as mean ± SEM (n = 5).

Taurine concentration (mM)	Conditions	Taurine uptake (µmoles/g dry wt/2 h)
0.1	Isotonic	0.25 ± 0.20
	Hypotonic	0.011 ± 0.002
	Hypertonic	0.67 ± 0.21
0.2	Isotonic	0.55 ± 0.21
	Hypotonic	0.021 ± 0.003
	Hypertonic	1.85 ± 0.12
0.3	Isotonic	0.87 ± 0.23
	Hypotonic	0.025 ± 0.003
	Hypertonic	3.25 ± 0.21
0.4	Isotonic	1.32 ± 0.26
	Hypotonic	0.023 ± 0.001
	Hypertonic	4.36 ± 0.32
0.5	Isotonic	2.14 ± 0.23
	Hypotonic	0.027 ± 0.002
	Hypertonic	6.89 ± 0.39
0.7	Isotonic	3.05 ± 0.28
	Hypotonic	0.029 ± 0.003
	Hypertonic	8.55 ± 0.51
1	Isotonic	0.41 ± 0.29
	Hypotonic	0.031 ± 0.002
	Hypertonic	9.41 ± 0.59
1.5	Isotonic	4.47 ± 0.25
	Hypotonic	0.029 ± 0.001
	Hypertonic	9.62 ± 0.47
2.0	Isotonic	4.51 ± 0.24
	Hypotonic	0.029 ± 0.003
	Hypertonic	9.85 ± 0.55

Table 27: Taurine uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentration of taurine for 2 h both under iso- and anisotonic conditions while maintaining the osmolarity of the media with mannitol.

Values are expressed as mean ± SEM (n = 5).

Taurine concentration (mM)	Conditions	Taurine uptake (µmoles/g dry wt/2 h)
0.1	Isotonic	0.05 ± 0.06
	Hypotonic	0.011± 0.001
	Hypertonic	0.15 ± 0.09
0.2	Isotonic	0.18 ± 0.07
	Hypotonic	0.021 ± 0.002
	Hypertonic	0.45 ± 0.12
0.3	Isotonic	0.35 ± 0.05
	Hypotonic	0.035 ± 0.001
	Hypertonic	0.75 ± 0.06
0.4	Isotonic	0.61 ± 0.05
	Hypotonic	0.027 ± 0.002
	Hypertonic	1.12 ± 0.10
0.5	Isotonic	0.79 ± 0.06
	Hypotonic	0.03 ± 0.001
	Hypertonic	1.65 ± 0.15
0.7	Isotonic	0.89 ± 0.07
	Hypotonic	0.029 ± 0.001
	Hypertonic	2.12 ± 0.18
1	Isotonic	1.05 ± 0.06
	Hypotonic	0.025 ± 0.002
	Hypertonic	2.51 ± 0.21
1.5	Isotonic	1.15 ± 0.08
	Hypotonic	0.036 ± 0.002
	Hypertonic	2.61 ± 0.25
2.0	Isotonic	1.21 ± 0.05
	Hypotonic	0.045 ± 0.001
	Hypertonic	2.55 ± 0.22

Table 28: Maximum taurine uptake (µmoles/g dry wt/2 h) by the isolated RBC of *C. batrachus* under iso- and anisotonic conditions.
Values are expressed as mean ± SEM (n = 5).

Conditions	Taurine uptake (µmoles/g dry wt/2 h)	
	With NaCl	With Mannitol
Isotonic (265 mOsmol/l)	4.51 ± 0.24	1.21 ± 0.05
Hypotonic (185 mOsmol/l)	0.029 ± 0.003	0.045 ± 0.002
Hypertonic (305 mOsmol/l)	9.85 ± 0.55	2.55 ± 0.22

Table 29: Effect of time on taurine efflux by the isolated RBC of *C. batrachus* under hypo and hypertonic conditions while maintaining the osmolarity of the media with NaCl. Values are expressed as mean ± SEM (n = 5).

Time of incubation (min)	Conditions	Taurine efflux (% of total)
2	Hypotonic	55 ± 5
	Hypotonic + DIDS	10 ± 2
	Hypertonic	2 ± 2
5	Hypotonic	75 ± 7
	Hypotonic + DIDS	22 ± 3
	Hypertonic	3 ± 3
10	Hypotonic	81 ± 8
	Hypotonic + DIDS	26 ± 4
	Hypertonic	2 ± 3
20	Hypotonic	79 ± 9
	Hypotonic + DIDS	23 ± 3
	Hypertonic	2 ± 3
30	Hypotonic	78 ± 7
	Hypotonic + DIDS	24 ± 4
	Hypertonic	2 ± 3

Table 30: Effect of time on taurine efflux by the isolated RBC of *C. batrachus* under hypo- and hypertonic conditions while maintaining the osmolarity of the media with mannitol. Values are expressed as mean ± SEM (n =5).

Time of incubation (min)	Conditions	Taurine efflux (% of total)
2	Hypotonic	47 ± 5
	Hypotonic + DIDS	12 ± 2
	Hypertonic	3 ± 2
5	Hypotonic	66 ± 7
	Hypotonic + DIDS	24 ± 3
	Hypertonic	4 ± 2
10	Hypotonic	75 ± 8
	Hypotonic + DIDS	29 ± 4
	Hypertonic	3 ± 2
20	Hypotonic	78 ± 9
	Hypotonic + DIDS	31 ± 3
	Hypertonic	4 ± 2
30	Hypotonic	80 ± 7
	Hypotonic + DIDS	32 ± 4
	Hypertonic	5 ± 3

Table 31: **Maximum taurine efflux (% of total) by the isolated RBC of *C. batrachus* under hypo-, hypo + DIDS (0.2 mM) and hypertonic conditions. Values are expressed as mean ± SEM (n = 5).**

Conditions	Taurine efflux (% of total)	
	With NaCl	With Mannitol
Hypotonic (185 mOsmol/l)	78 ± 7	80 ± 7
Hypotonic + DIDS (185 mOsmol/l)	24 ± 4	32 ± 4
Hypertonic (305 mOsmol/l)	2 ± 3	5 ± 2.5

Table 32: Effect of different osmolarities on the taurine efflux by the isolated RBC of *C. batrachus*. Values are expressed as mean ± SEM (n = 5).

Osmolarities (mOsmol/l)	Taurine efflux (% of total)
265	10 ± 2
245	24 ± 3
225	40 ± 5
205	52 ± 6
185	60 ± 8
165	75 ± 7

Table 33: Effects of anisotonicity on water content in the isolated RBC preincubated with/without taurine. Values are expressed as mean ± SEM (n = 5).

Osmolarities	Water content (%)	
	Without taurine	With taurine
Isotonic (265 mOsmol/l)	30 ± 2	31 ± 2
Hypotonic (185 mOsmol/l)	34 ± 3 (+13)	35 ± 3 (+13)
Hypertonic (305 mOsmol/l)	27 ± 2 (-10)	28 ± 2 (-10)

Percentage increase (+) or decrease (-) of water content (%) compared to the control are given in parentheses.

FIGURES

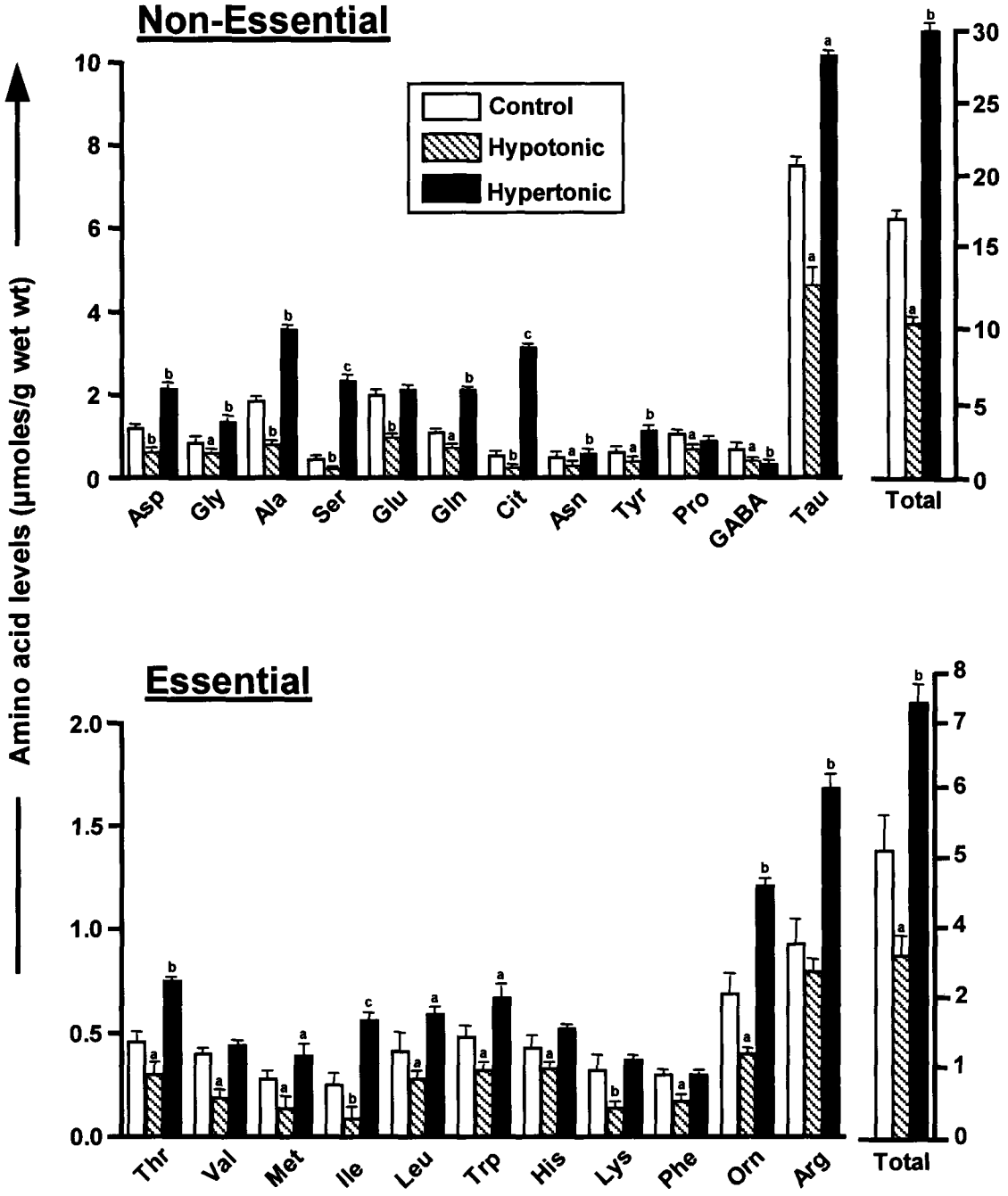


Fig.3 **Effects of anisotinicity on the levels of different FAAs in the perfused liver of *C. batrachus*. Values are plotted as mean ± SEM (n = 5).**

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

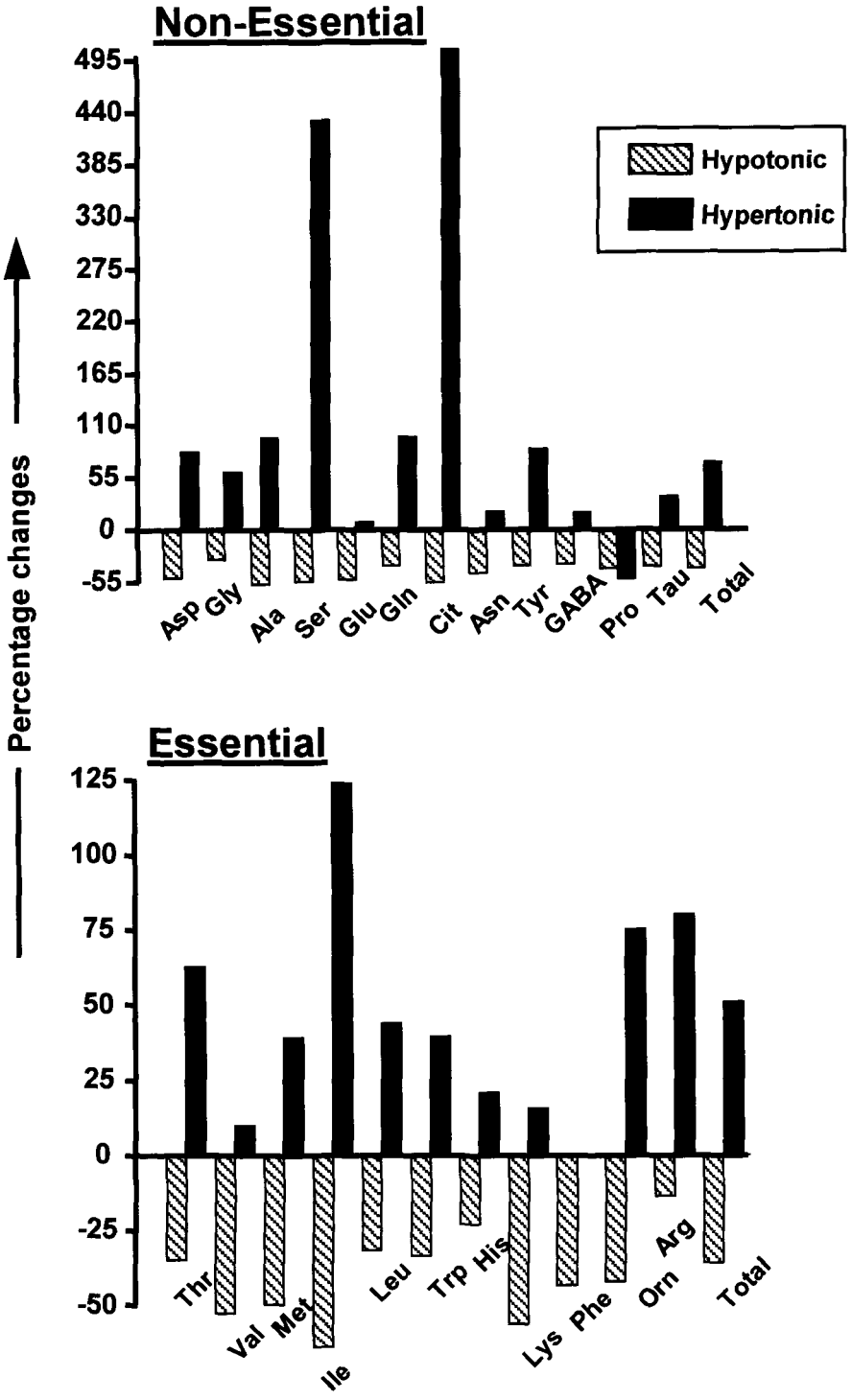


Fig. 4 **Percentage changes in amino acid levels under anisotonic conditions in the perfused liver of *C. batrachus*.**

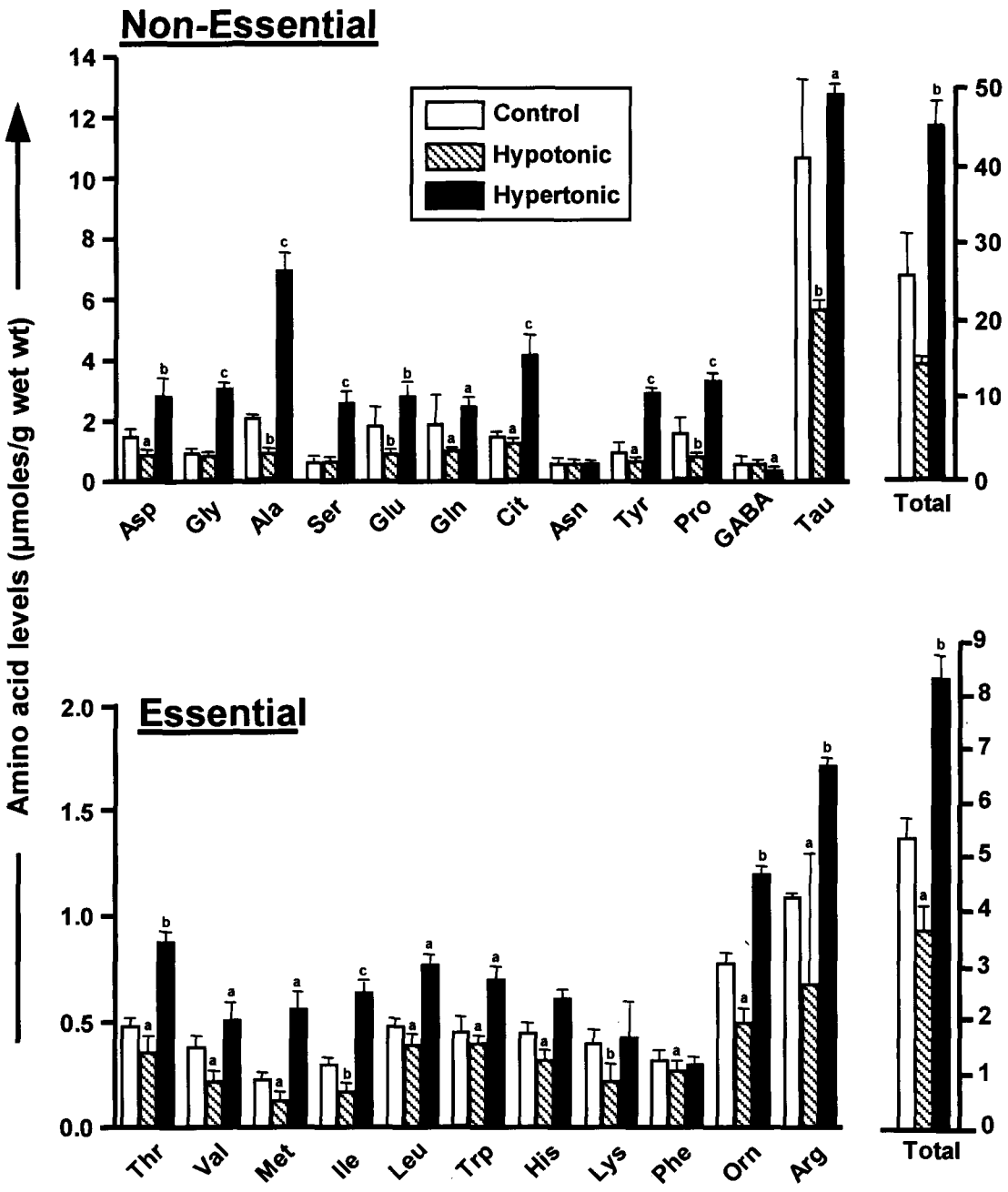


Fig. 5 **Effects of anisotinicity on the levels of different FAAs in the perfused liver of *C. batrachus* infused with 5 mM NH_4Cl. Values are plotted as mean ± SEM (n = 5).**

^{a,b,c} : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

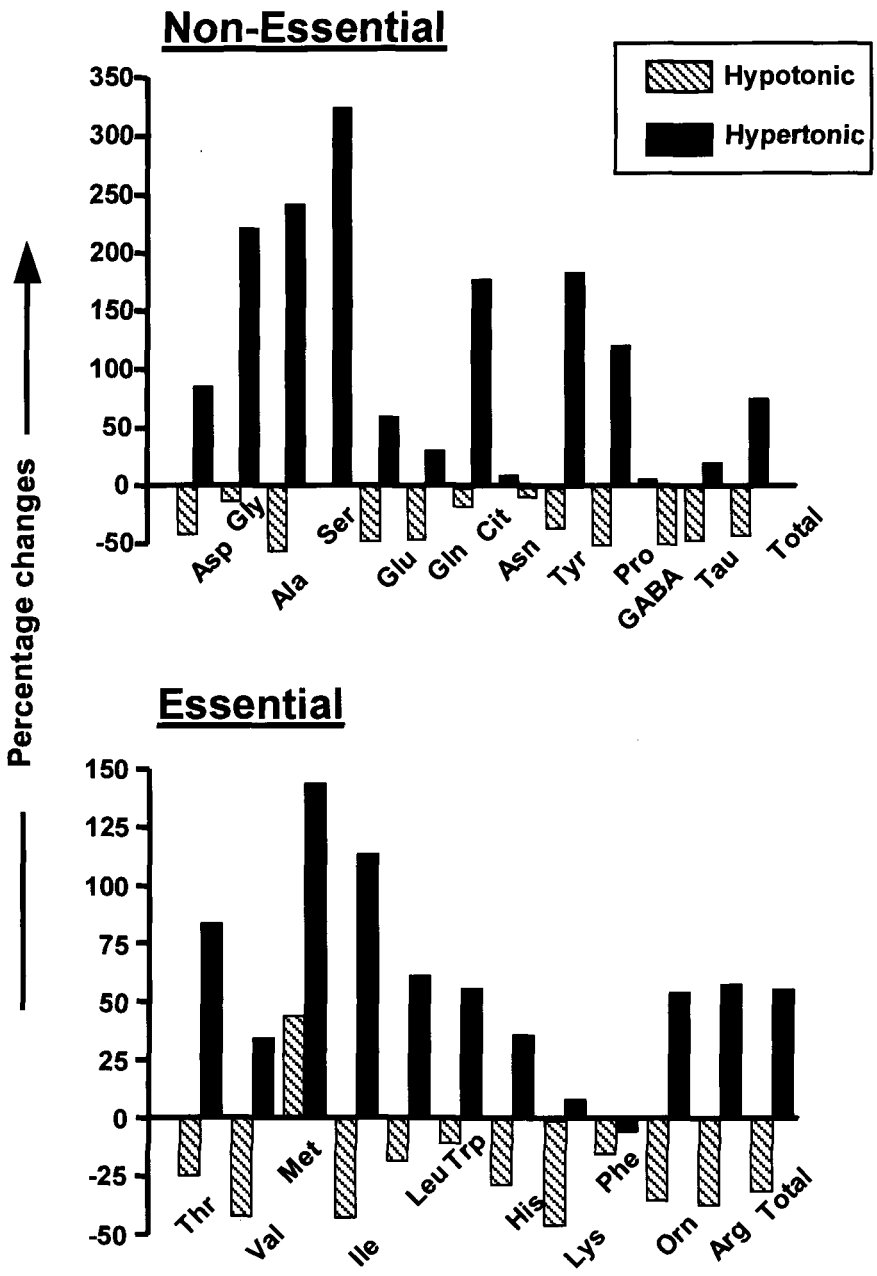


Fig. 6 Percentage changes in amino acid levels under anisotonic conditions in the perfused liver of *C. batrachus* infused with 5 mM NH_4Cl.

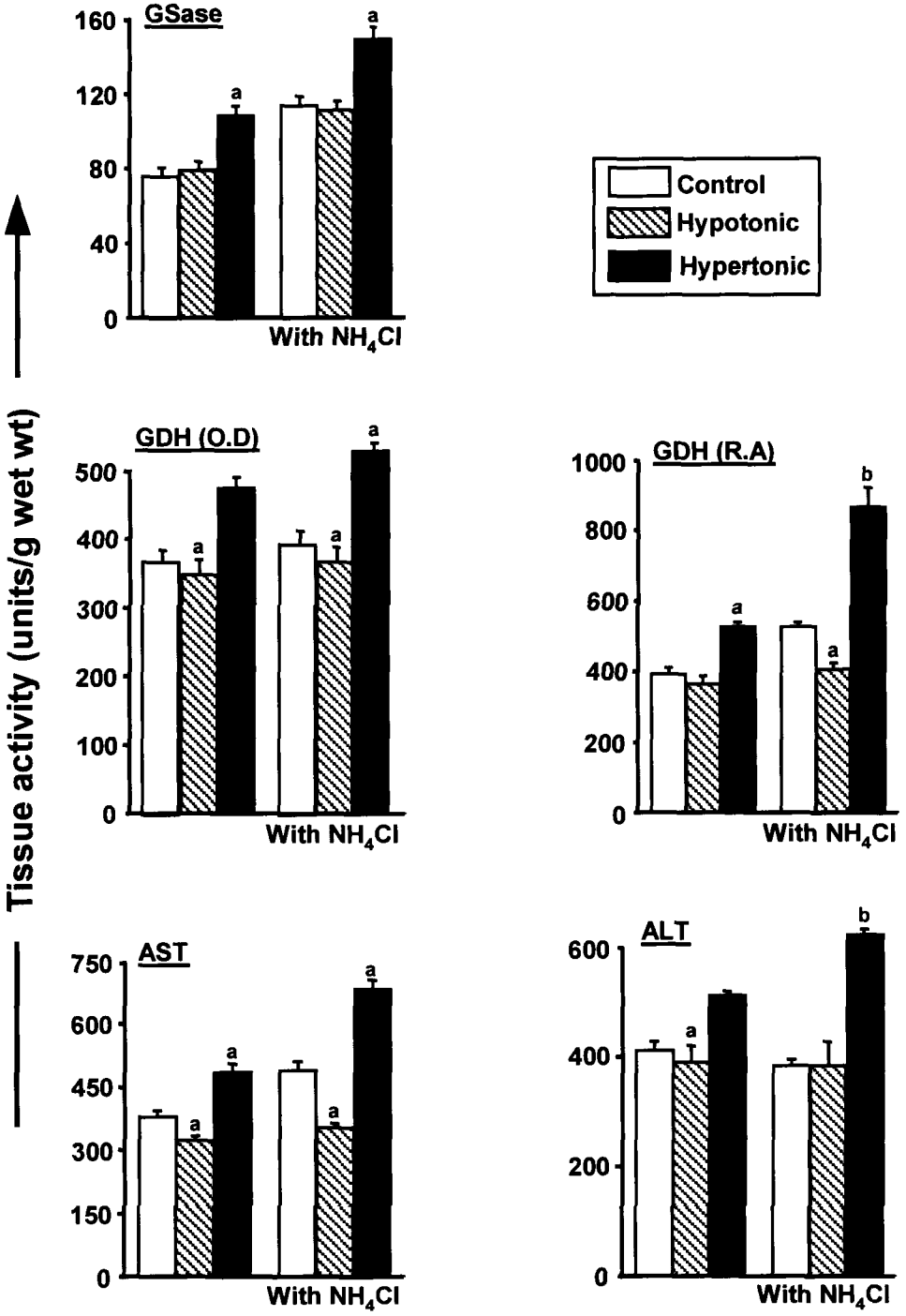


Fig. 7 Effects of anisotonicity on the tissue activity (units/g wet wt) of different enzymes related to amino acid metabolism in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl. Values are plotted as mean ± SEM (n = 5). ^{a,b} : *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

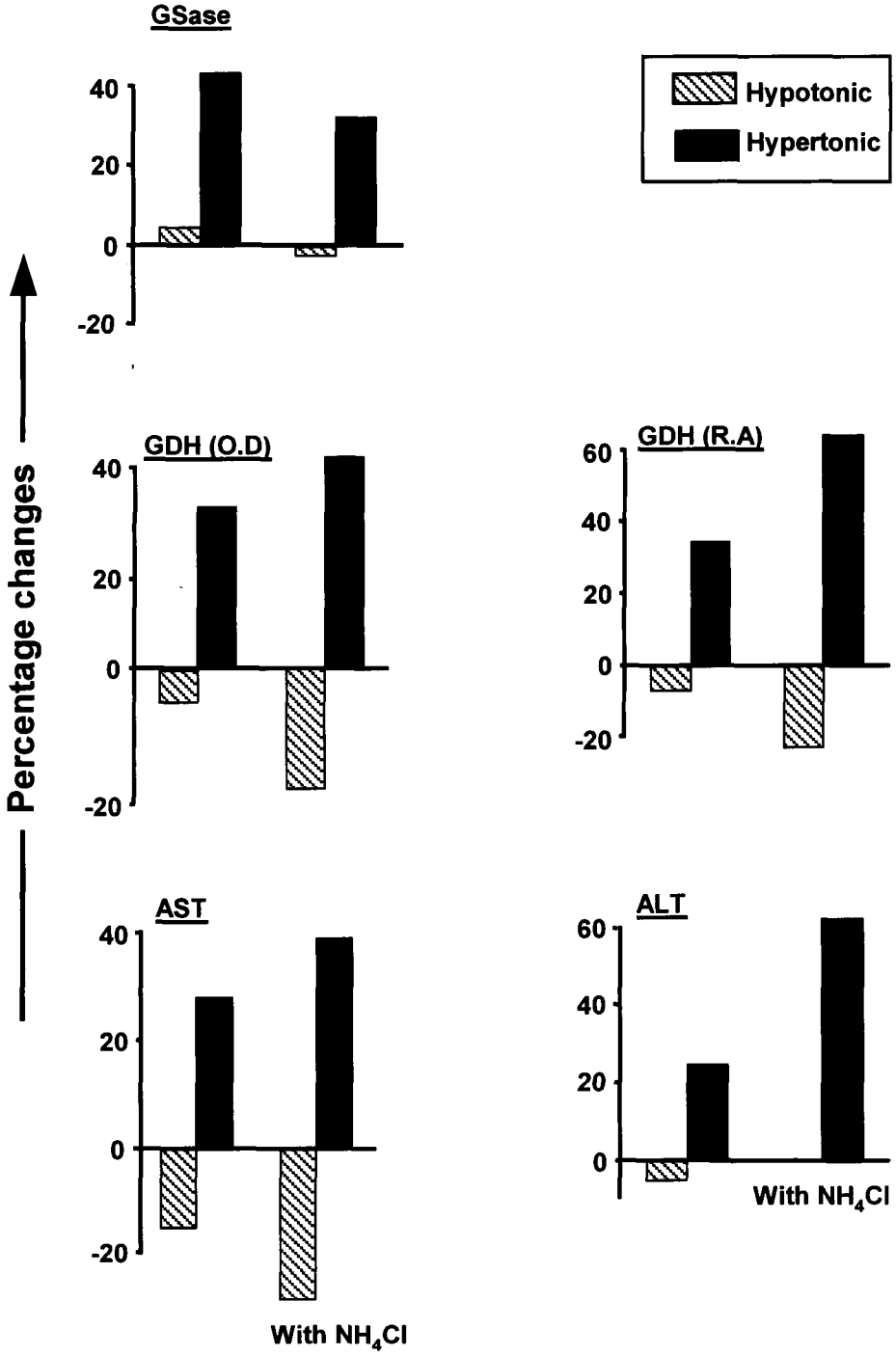


Fig. 8 Percentage changes of tissue activity of different enzymes related to amino acid metabolism under anisotonic conditions in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl.

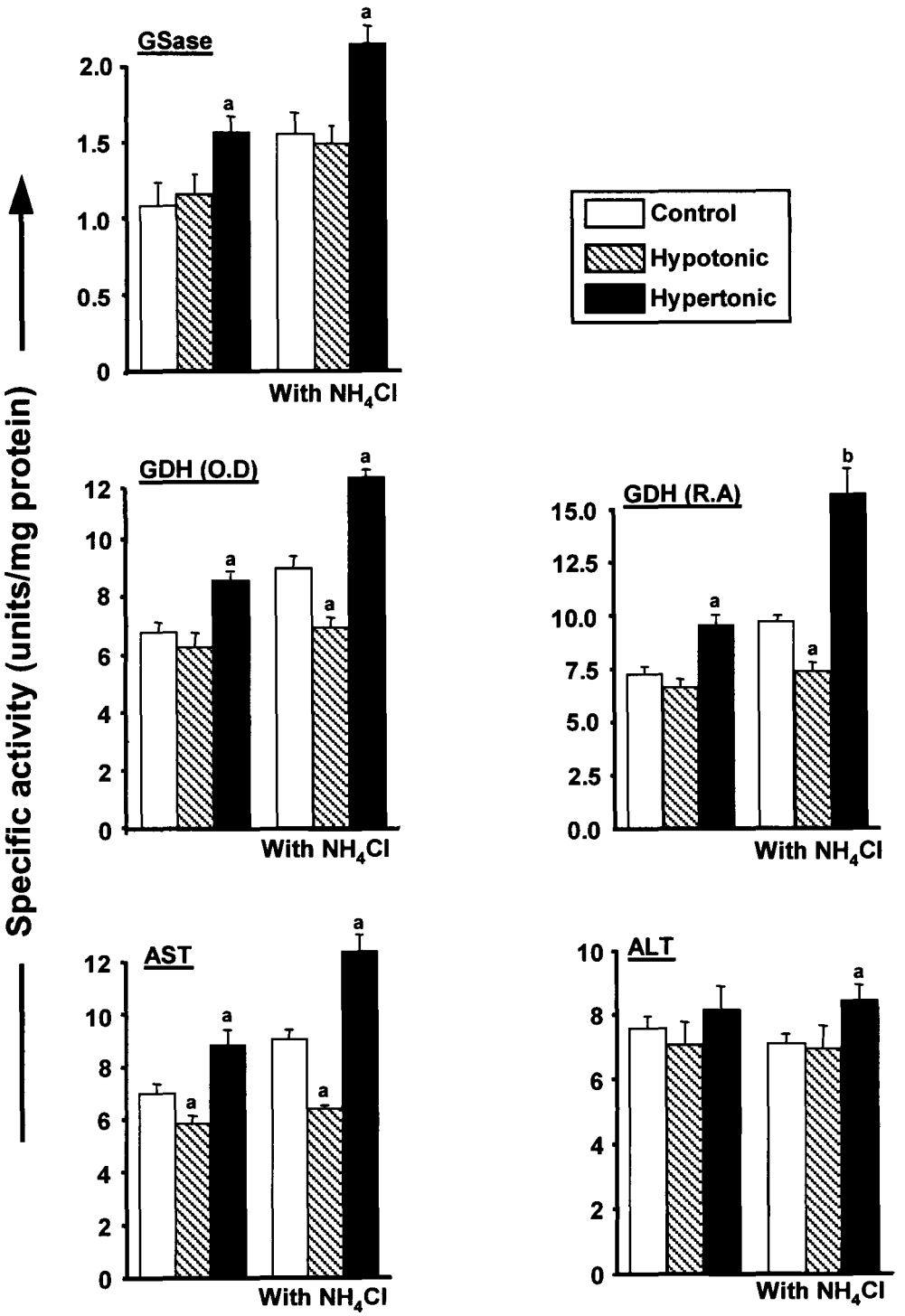


Fig. 9 Effects of anisotonicity on the specific activity (units/mg protein) of different enzymes related to amino acid metabolism in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl. Values are plotted as mean ± SEM (n = 5). ^{a,b} : *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

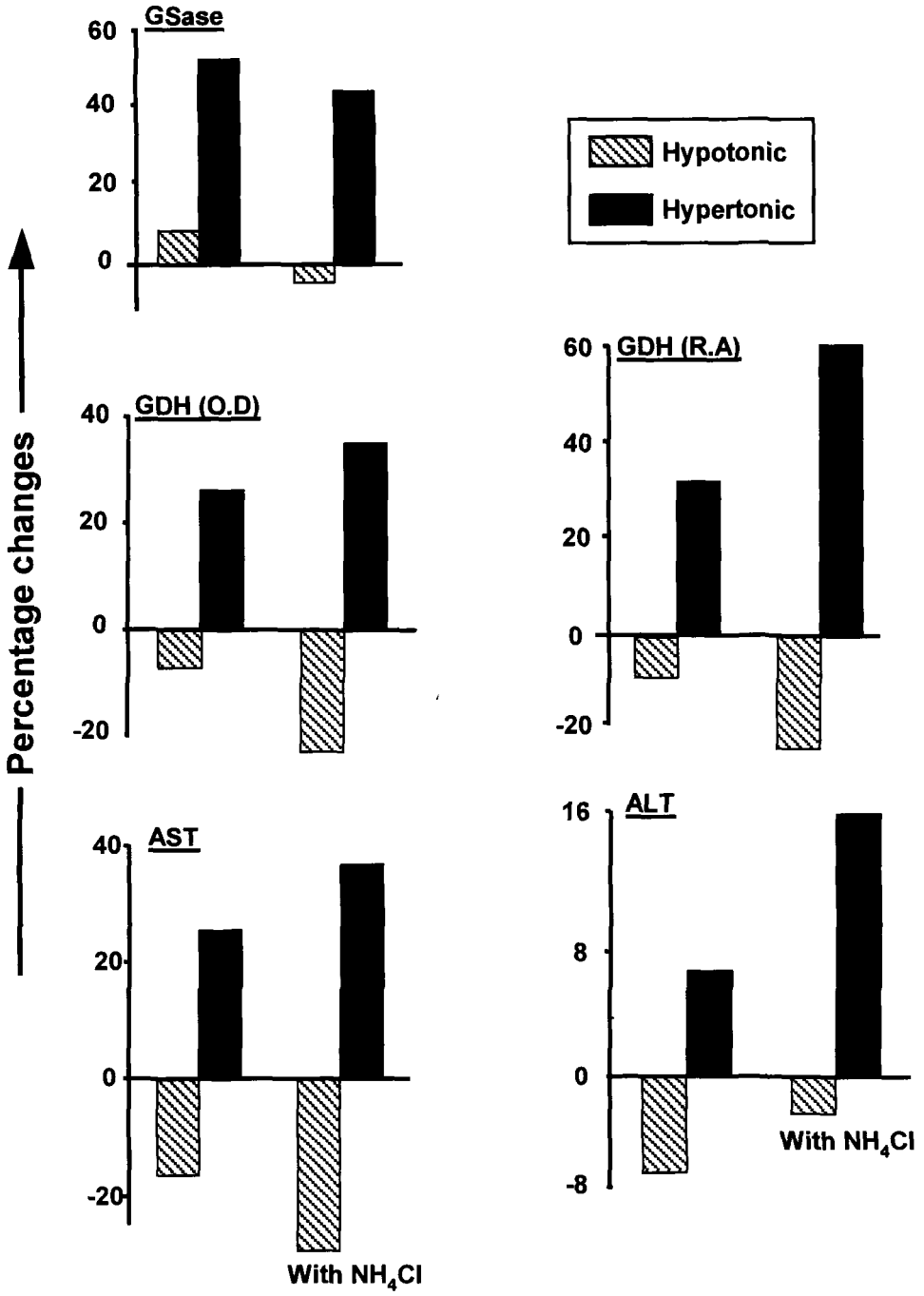


Fig. 10 **Percentage changes of specific activity of different enzymes related to amino acid metabolism under anisotonic conditions in the perfused liver of *C. batrachus* infused with/without 5 mM NH_4Cl.**

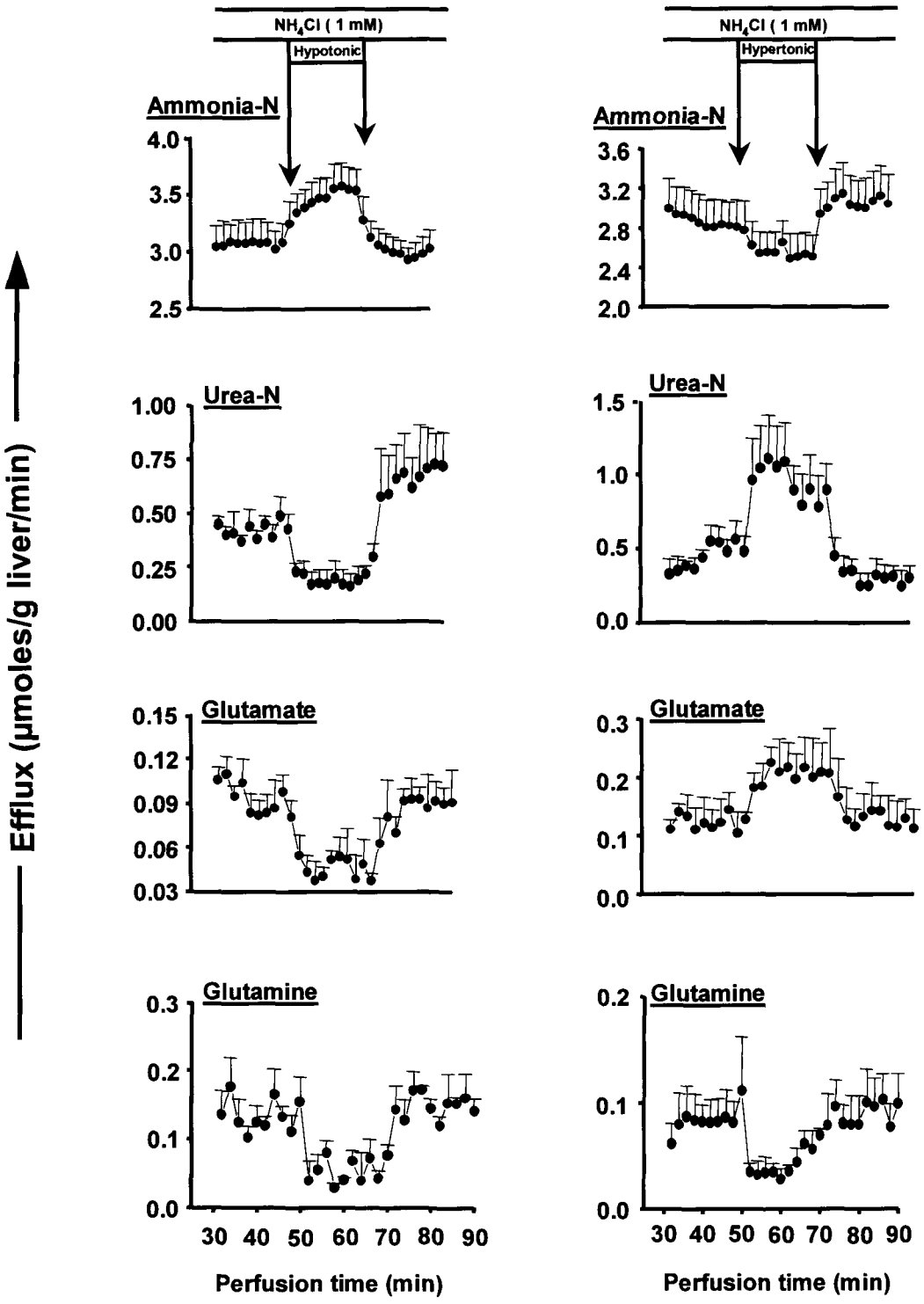


Fig. 11 **Effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine (µmoles/g liver/min) from the perfused liver of *C. batrachus* infused with 1 mM NH_4Cl. Values are expressed as mean ± SEM (n = 5).**

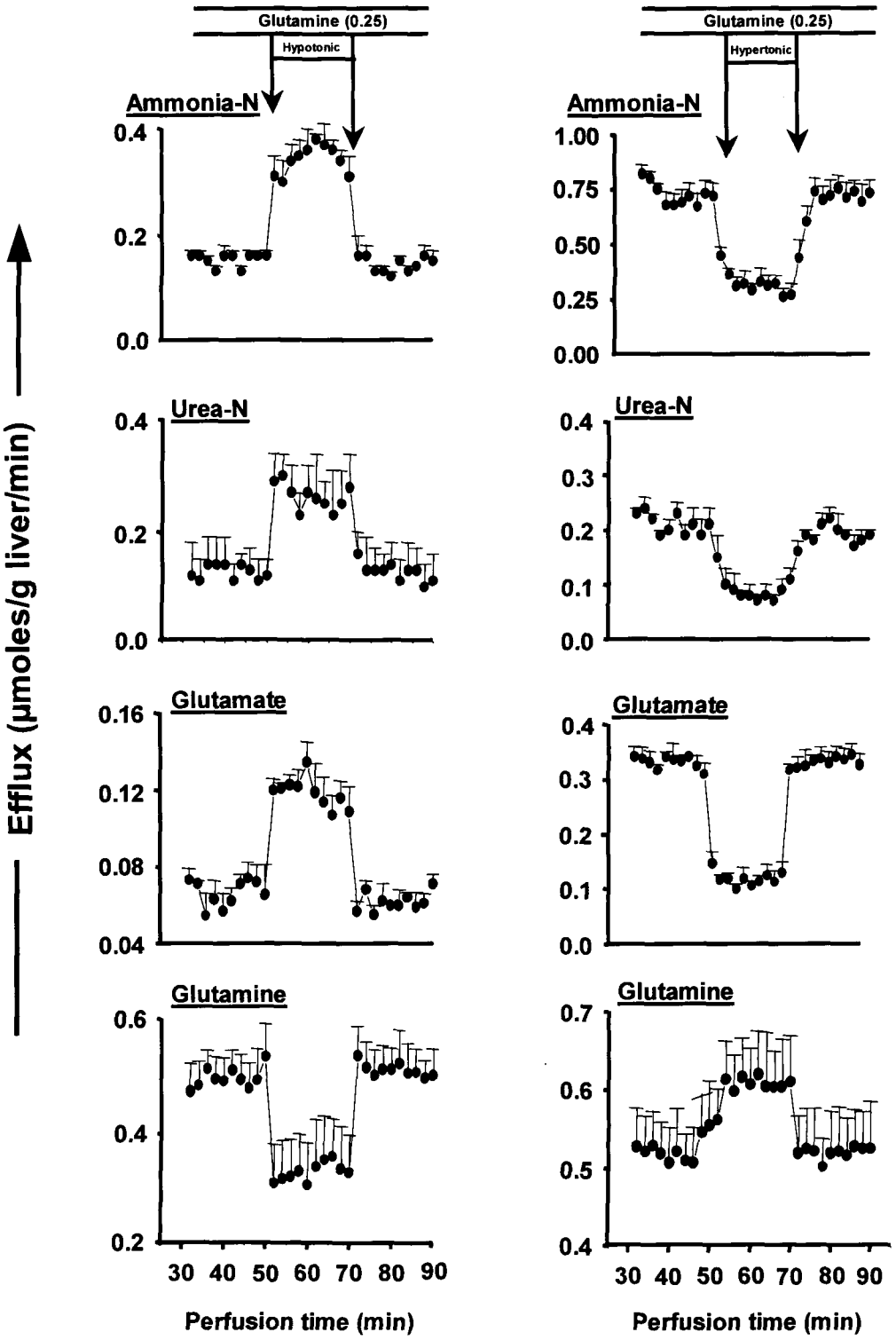


Fig. 12 Effects of anisotonicity on the efflux of ammonia-N, urea-N, glutamate and glutamine (µmoles/g liver/min) from the perfused liver of *C. batrachus* infused with 0.25 mM glutamine. Values are expressed as mean ± SEM (n = 5).

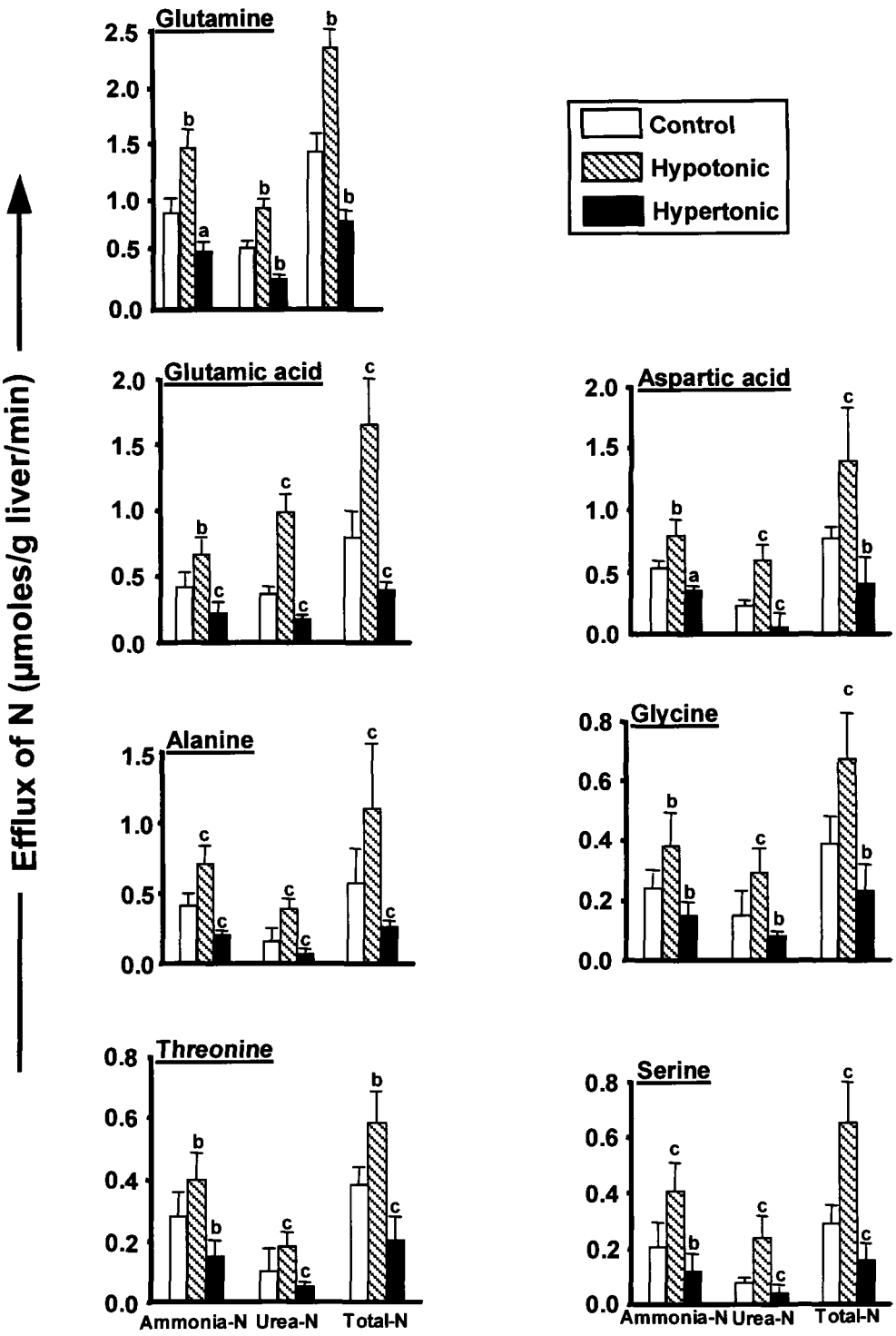


Fig. 13 Effects of anisotonicity on the efflux of ammonia-N, urea-N and total-N (µmoles/g liver/min) from the perfused liver of *C. batrachus* infused with different amino acids (5 mM). Values are plotted as mean ± SEM (n = 5). $^{a, b, c}$: *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

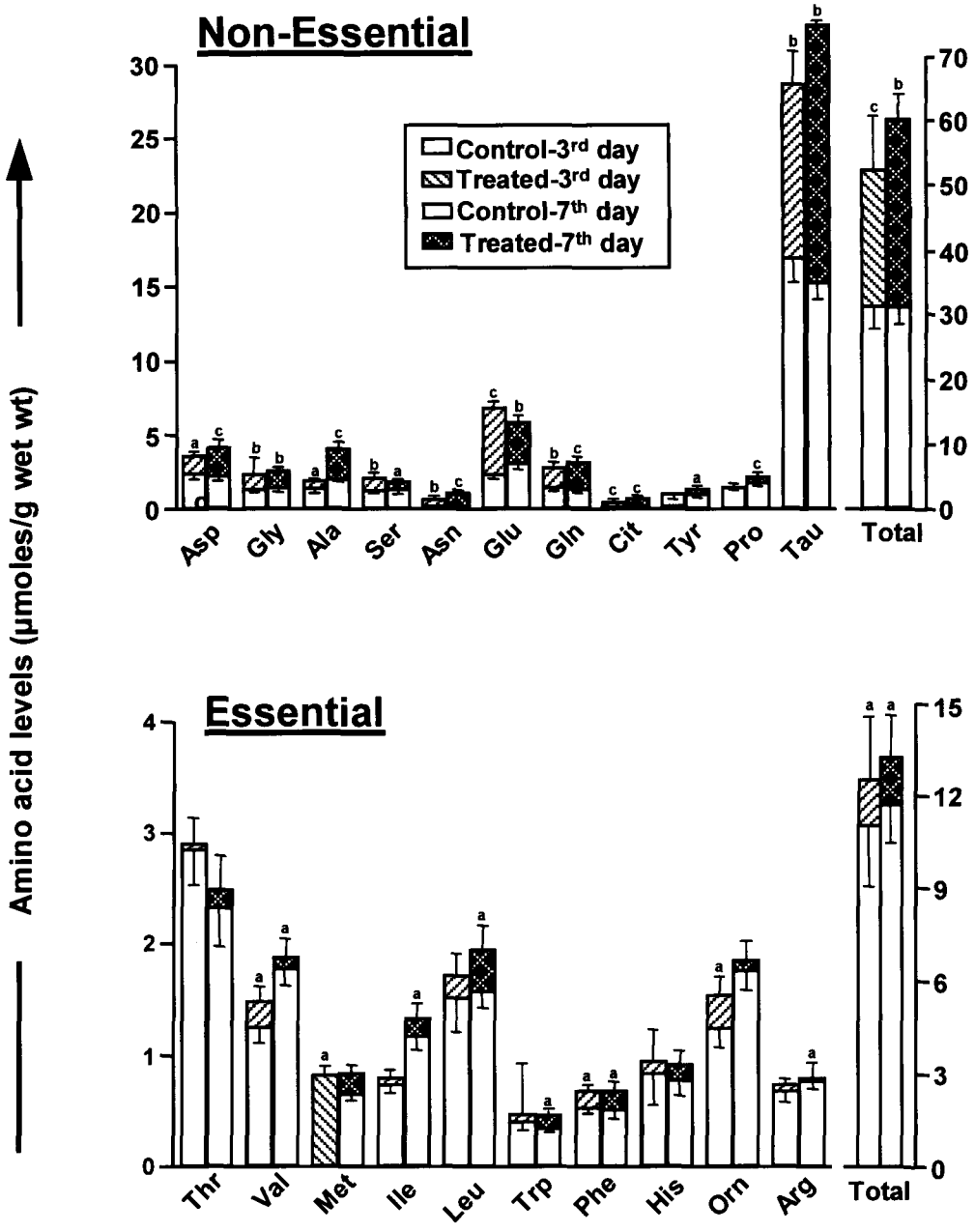


Fig. 14 **Alteration in levels of various FAAs in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are plotted as mean ± SEM (n = 5).**

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

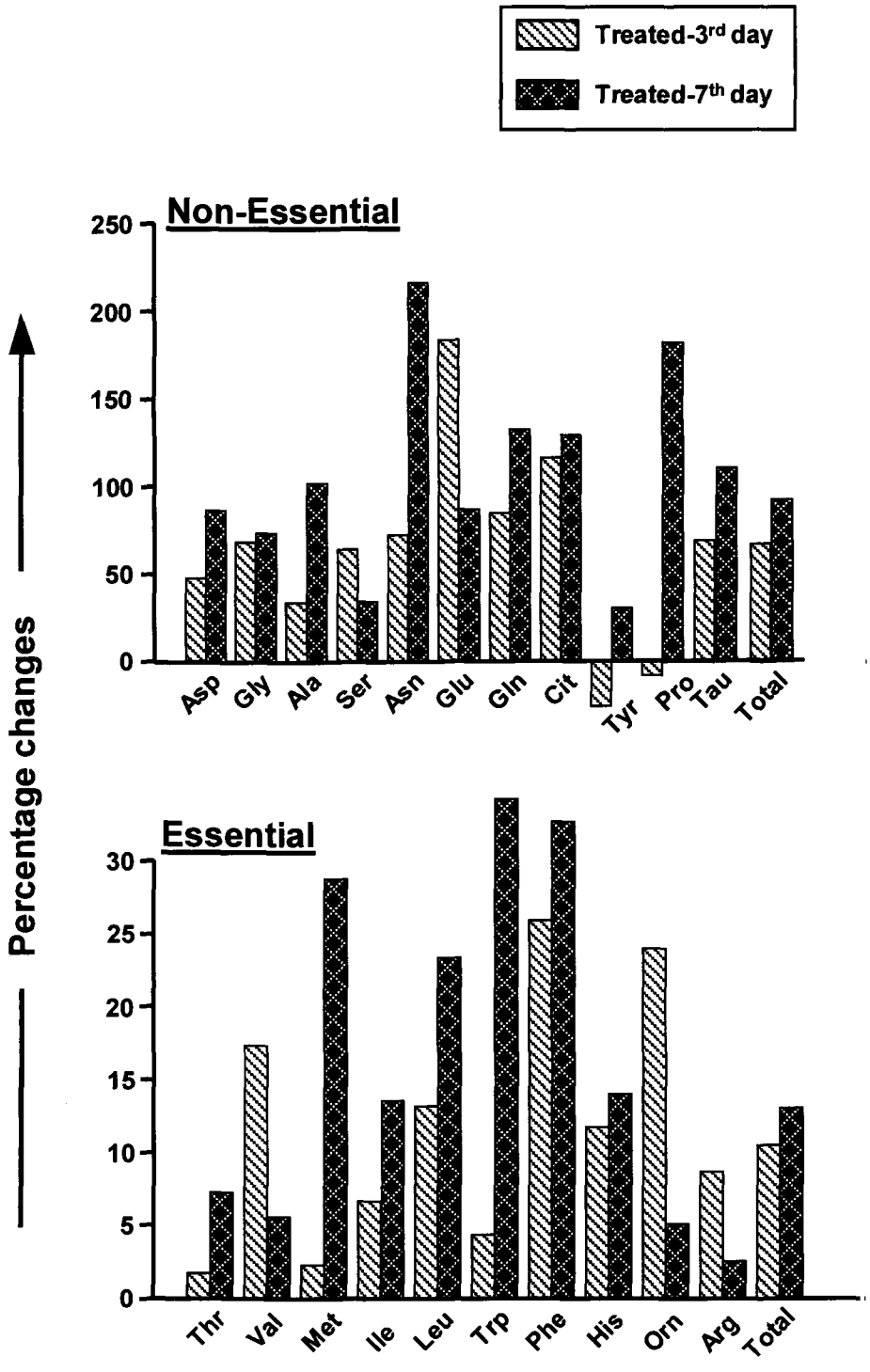


Fig: 15 Percentage changes in amino acid levels in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol.

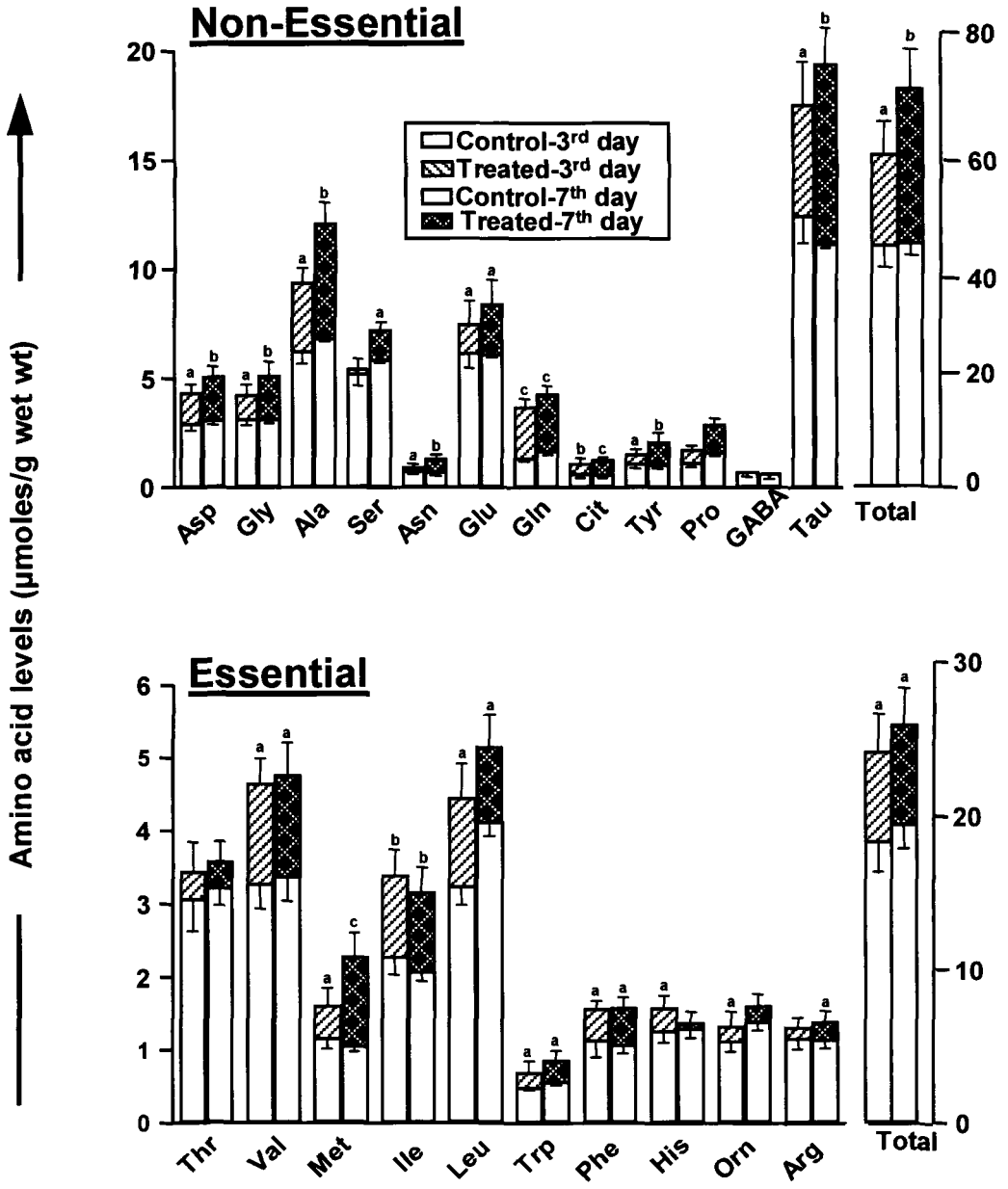


Fig.16 **Alteration in levels of various FAAs in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are plotted as mean ± SEM (*n* = 5).**

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

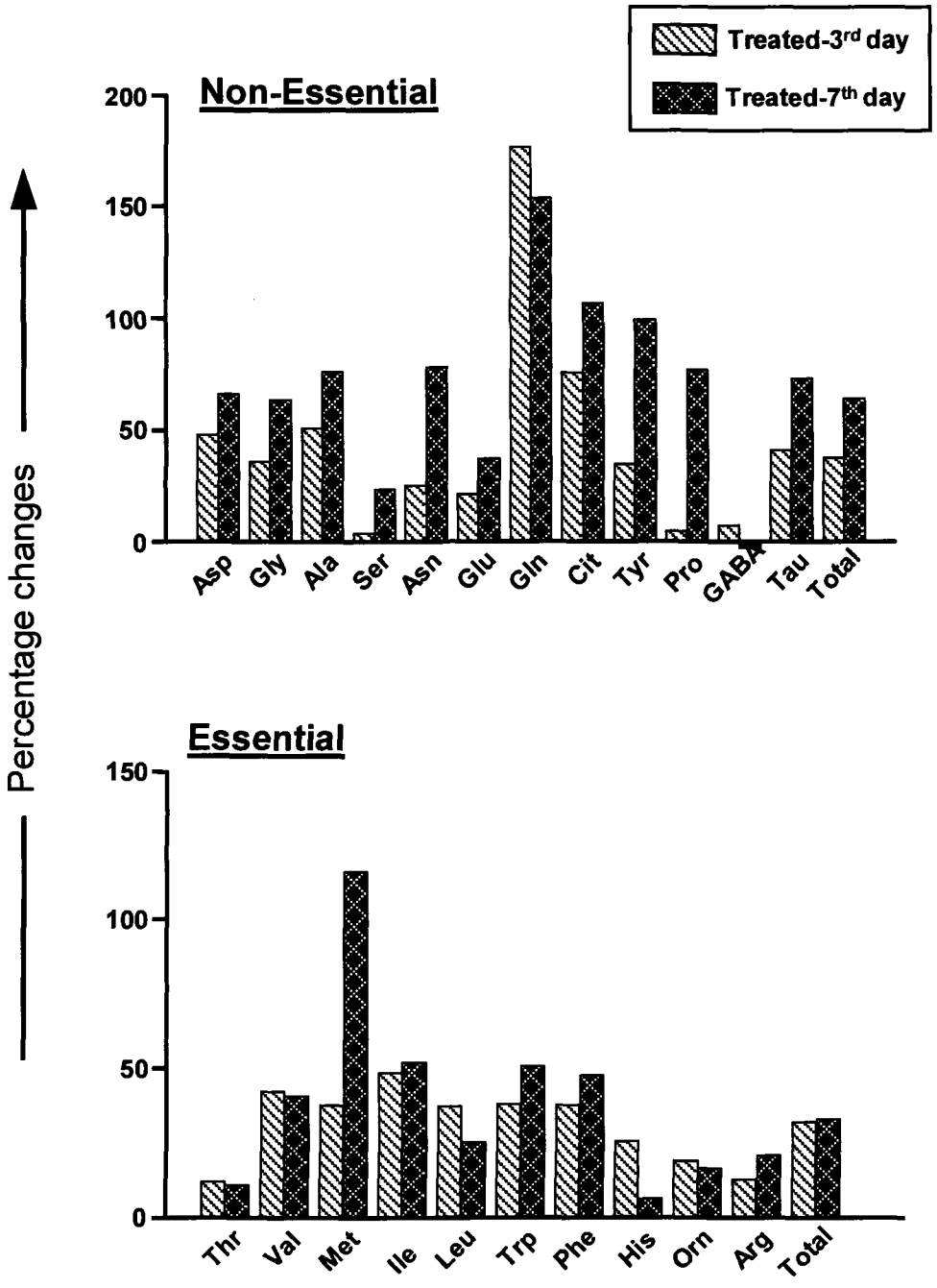


Fig: 17 **Percentage changes in amino acid levels in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol.**

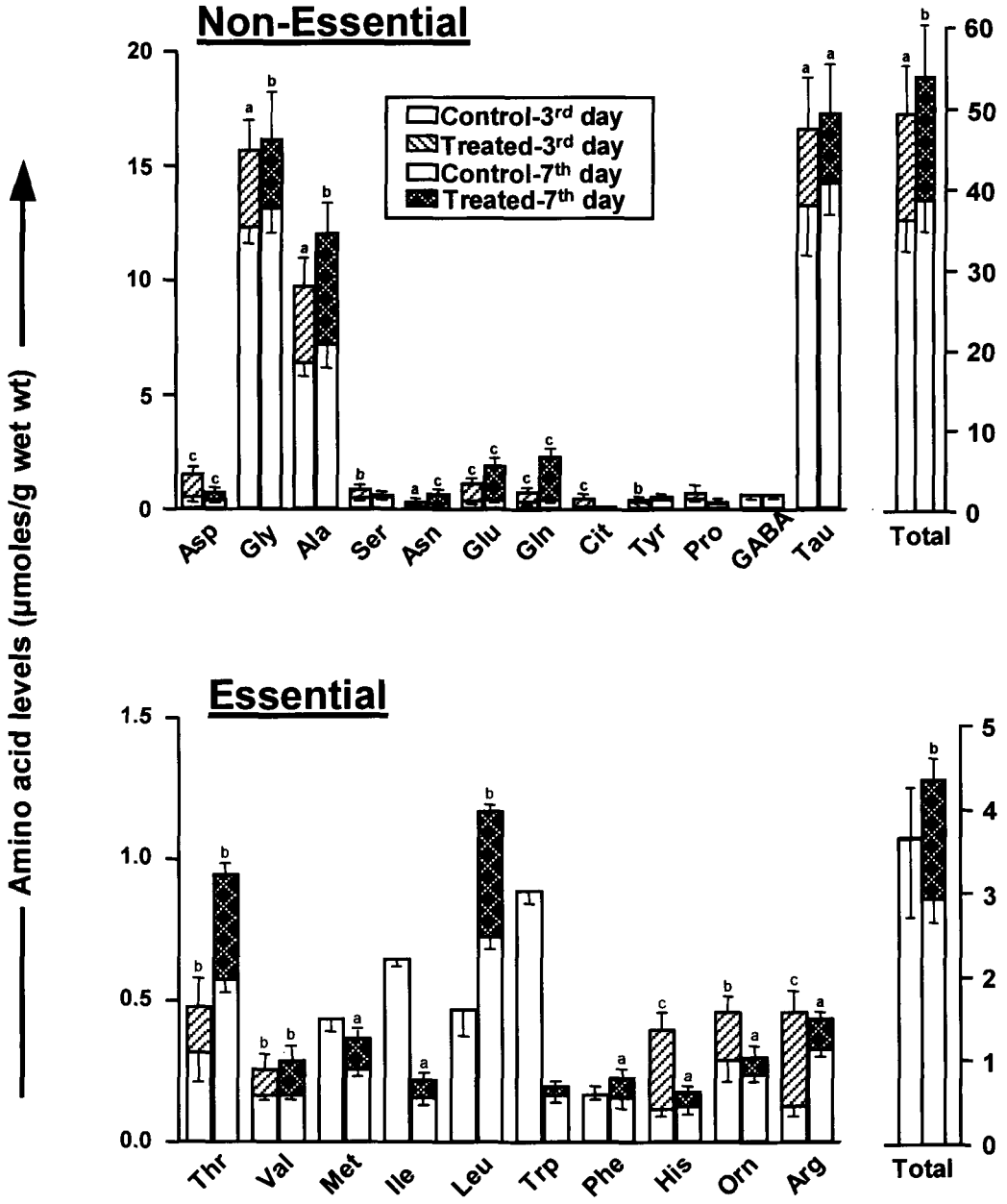


Fig. 18 Alteration in levels of various FAAs in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are plotted as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

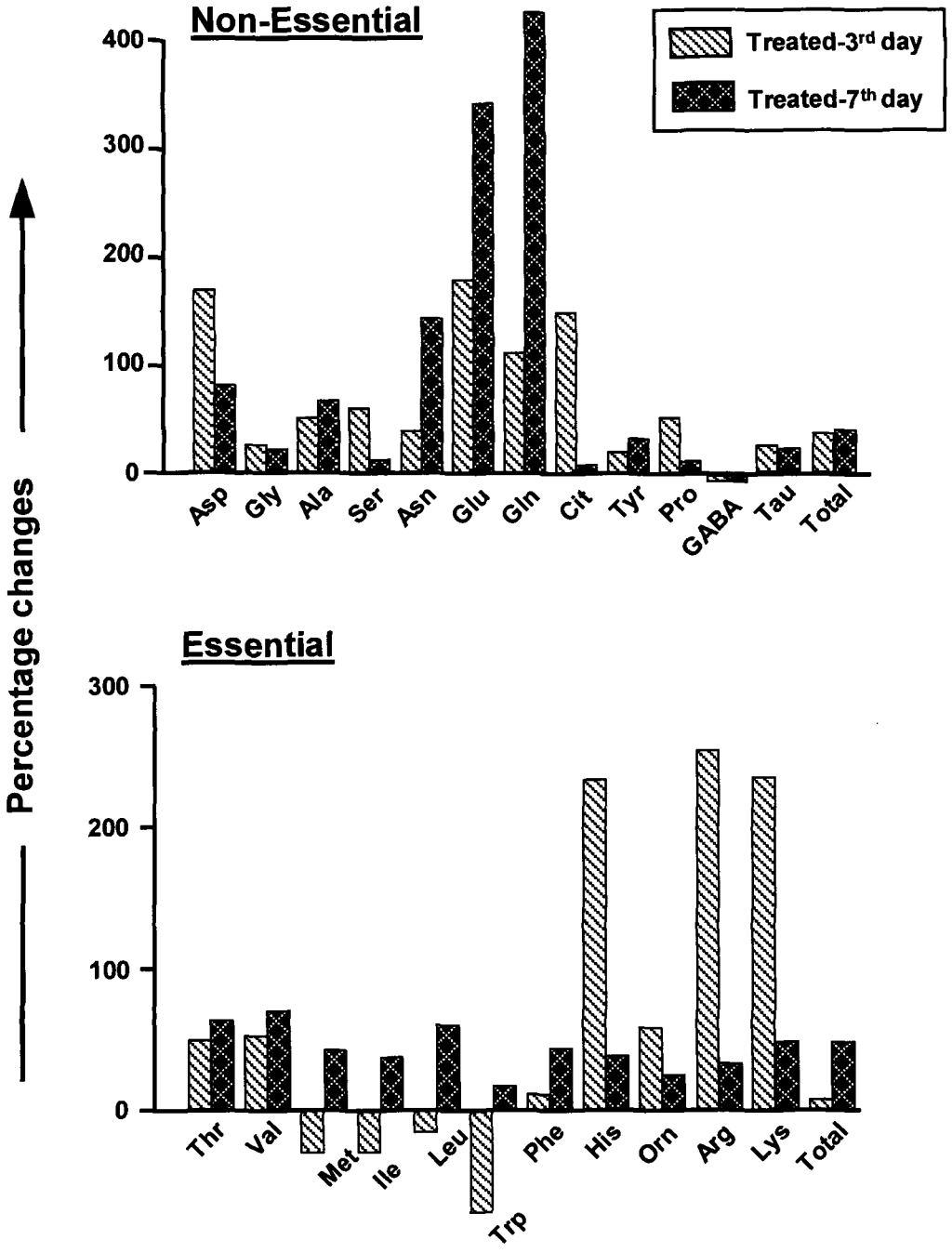


Fig: 19 Percentage changes in amino acid levels in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol.

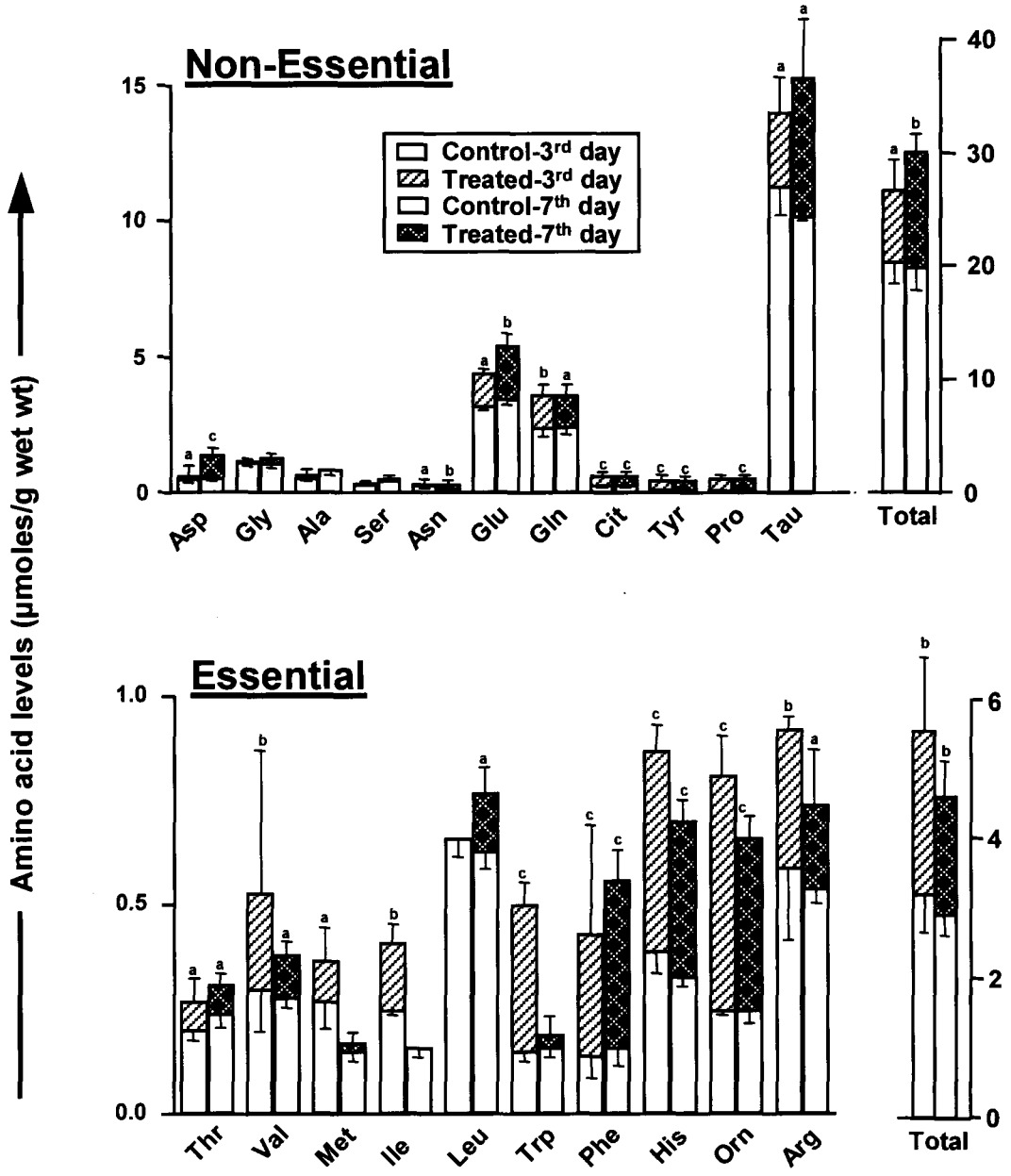


Fig. 20 **Alteration in levels of various FAAs in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are plotted as mean ± SEM (n = 5).**

$^{a, b, c}$: *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

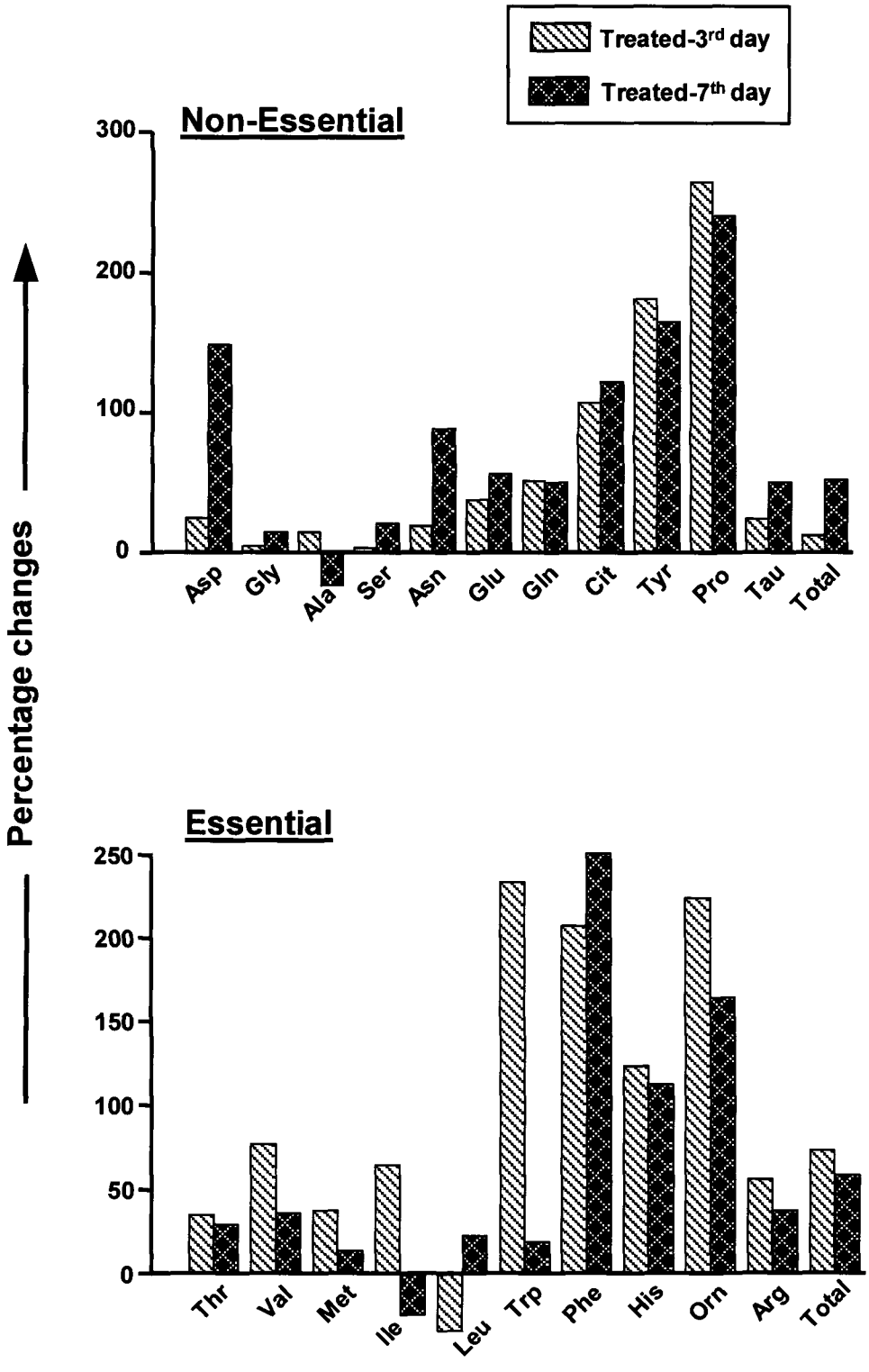


Fig: 21 Percentage changes in amino acid levels in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol.

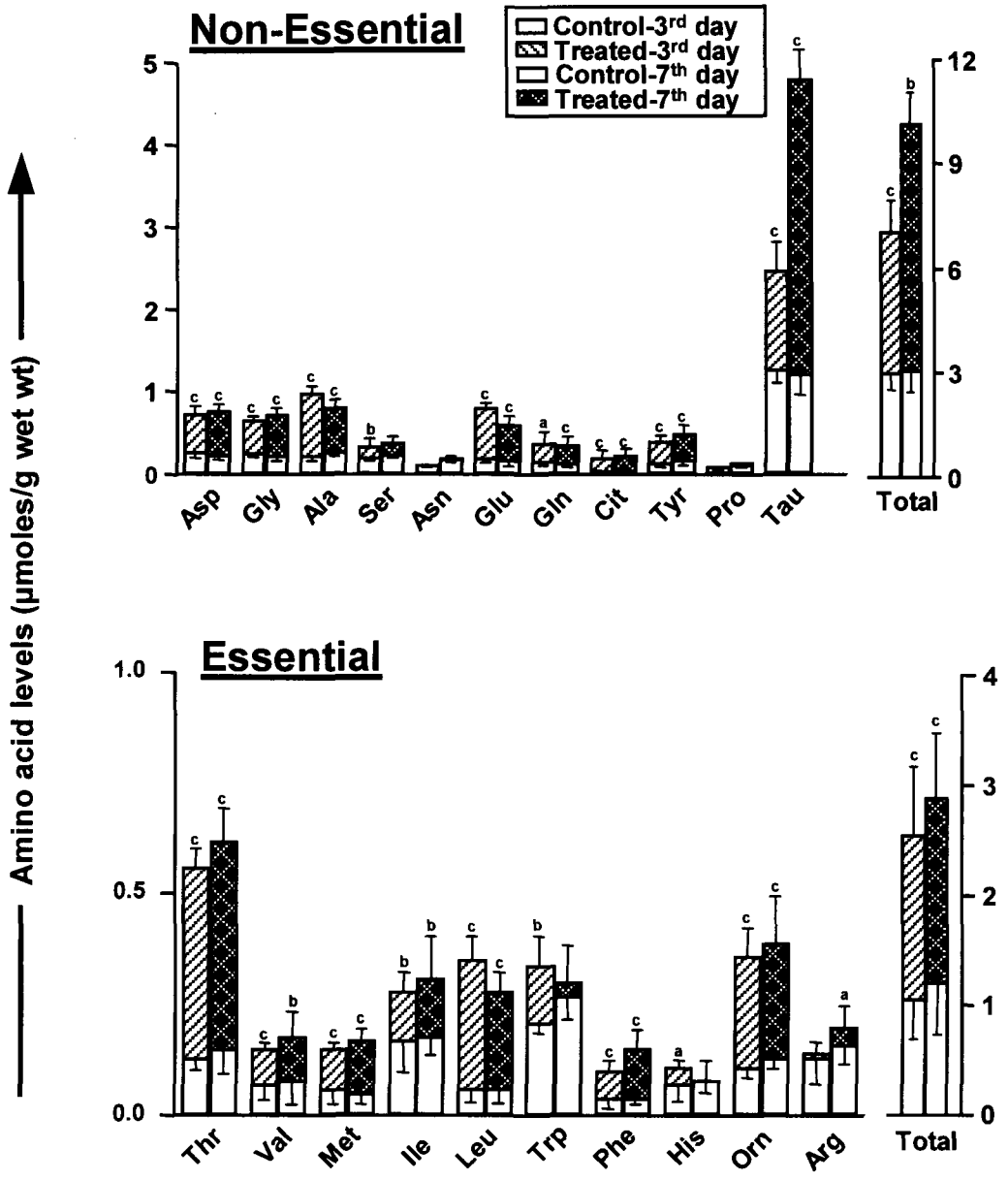


Fig. 22 **Alteration in levels of various FAAs in the plasma of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are plotted as mean ± SEM (n = 5).**

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

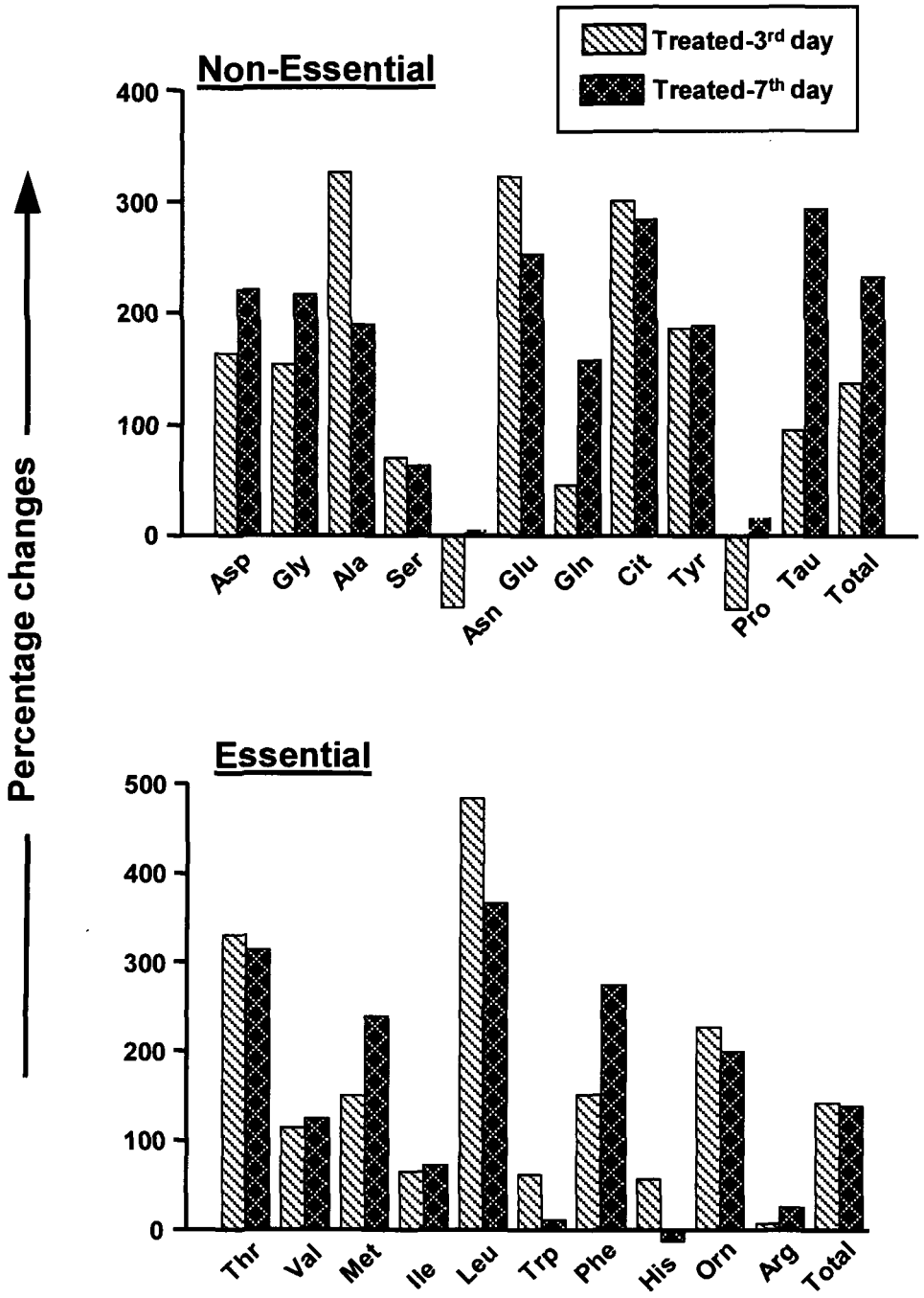


Fig: 23 Percentage changes in amino acid levels in the plasma of *C. batrachus* exposed to 250 mOsmol/l mannitol.

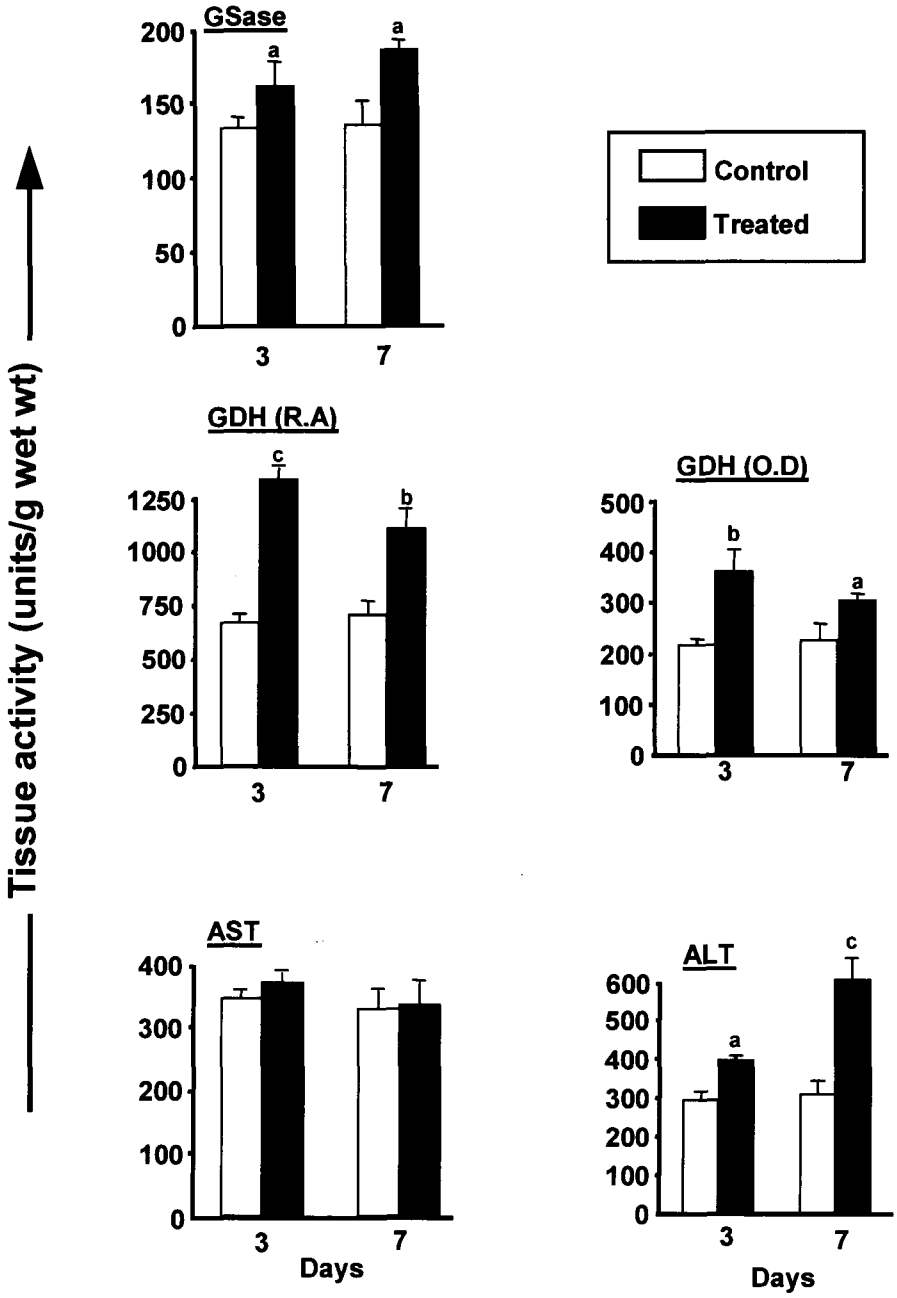


Fig. 24 Changes of tissue activity (units/g wet wt) of different enzymes related to amino acid metabolism in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

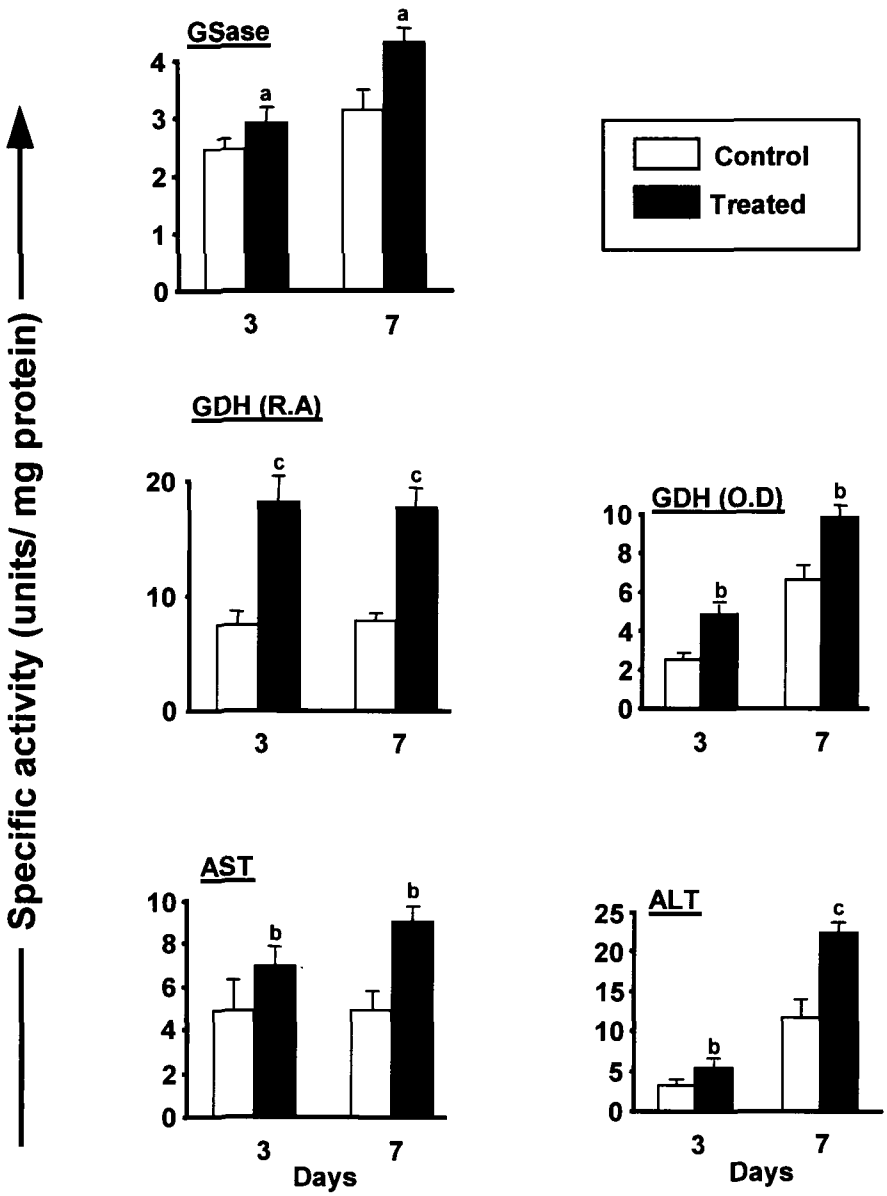


Fig. 25 Changes in the specific activity (units/mg protein) of different enzymes related to amino acid metabolism in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

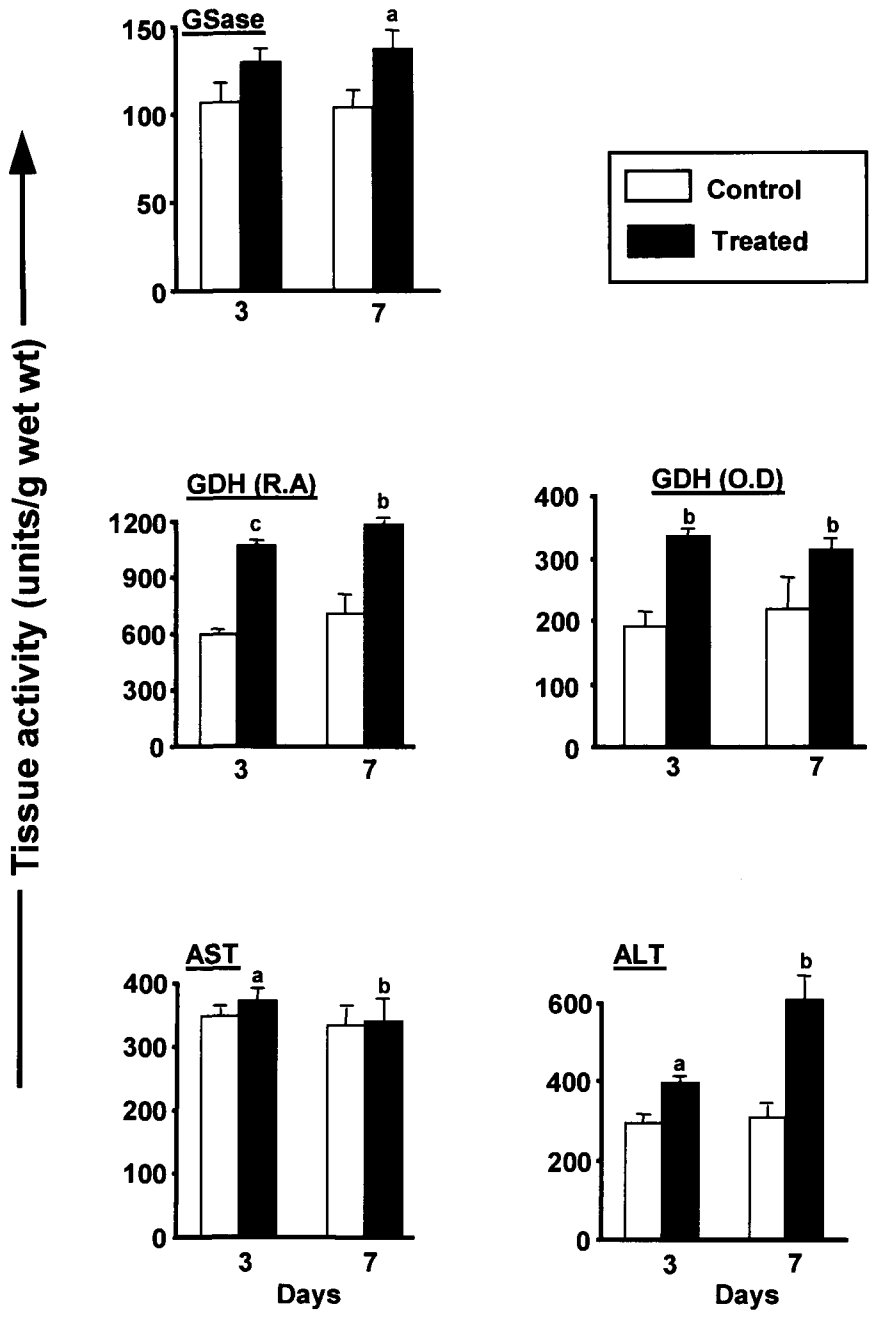


Fig. 26 Changes of tissue activity (units/g wet wt) of different enzymes related to amino acid metabolism in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

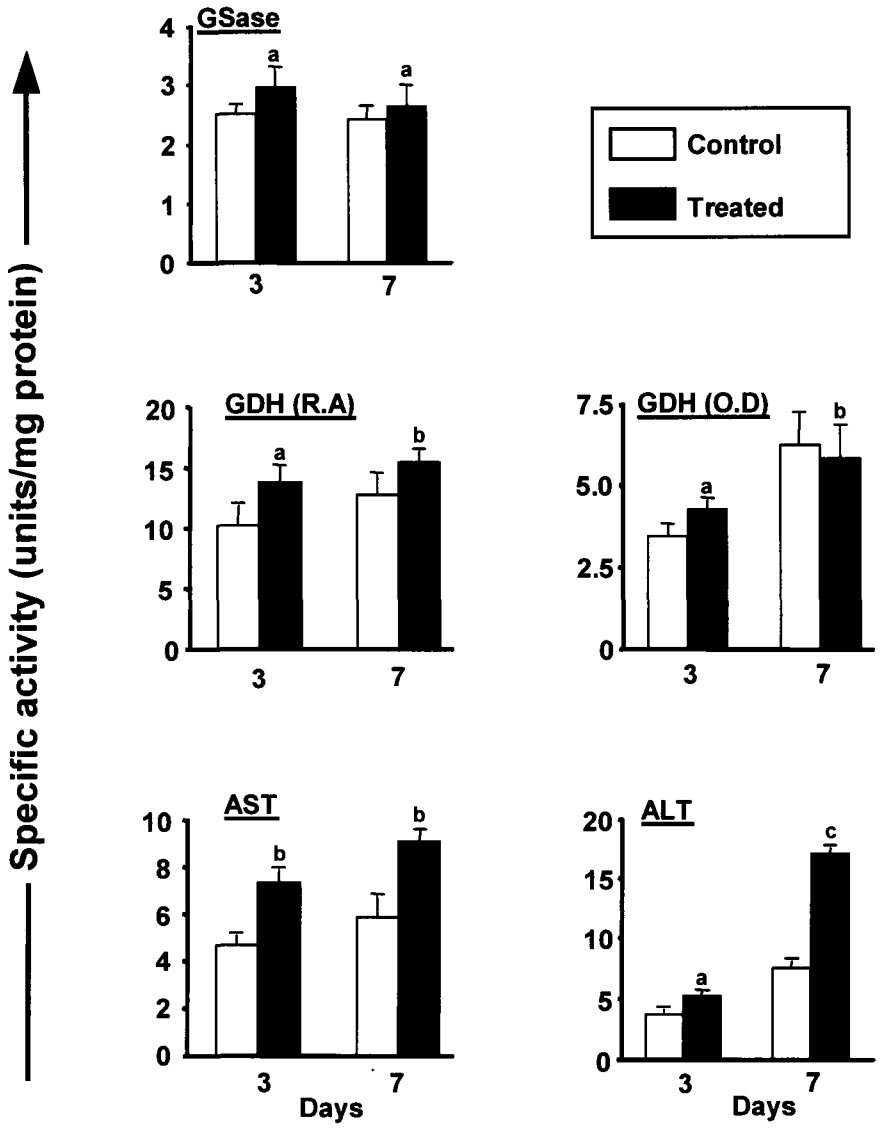


Fig. 27 Changes of specific activity (units/g protein) of different enzymes related to amino acid metabolism in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

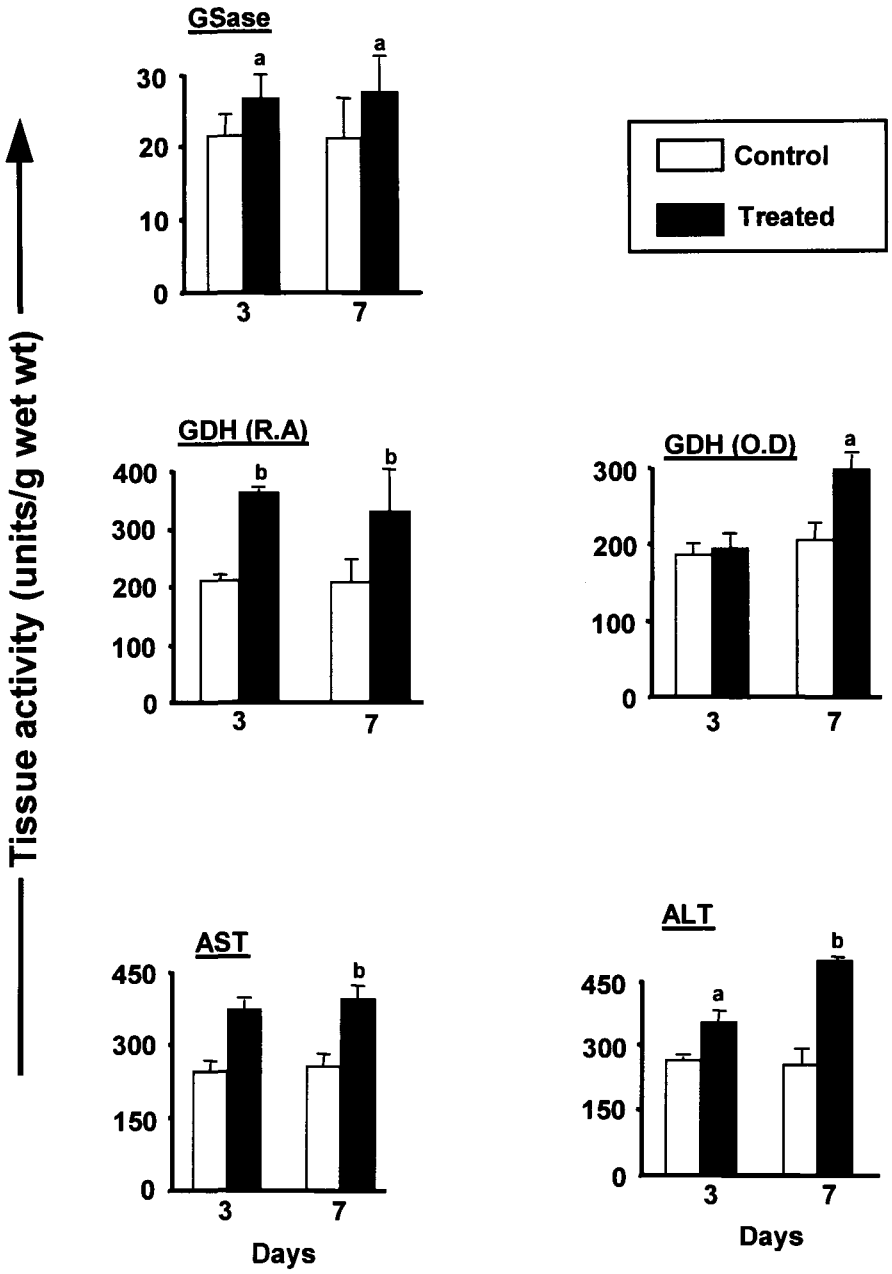


Fig. 28 Changes of tissue activity (units/g wet wt) of different enzymes related to amino acid metabolism in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

$^{a, b, c}$: *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

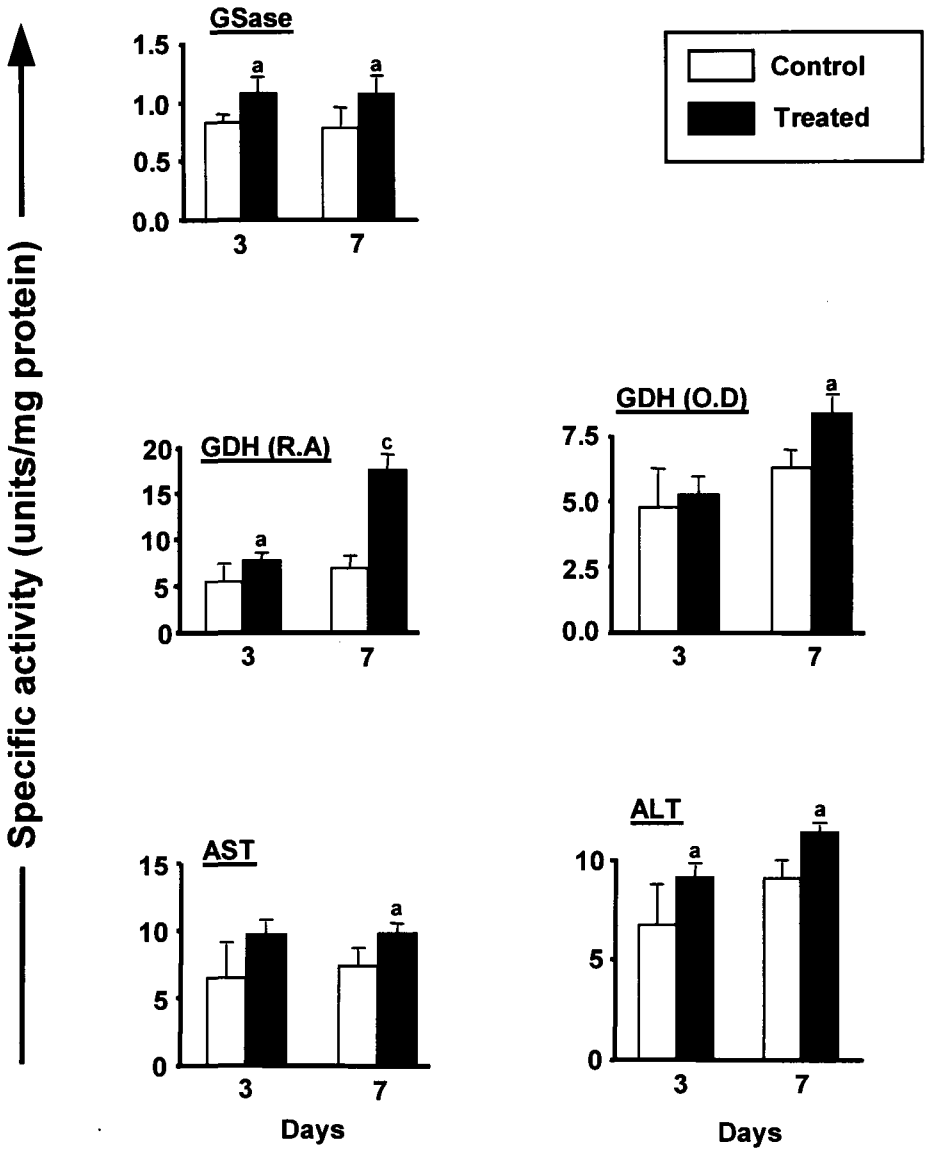


Fig. 29 Changes of specific activity (units/mg protein) of different enzymes related to amino acid metabolism in the muscle of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

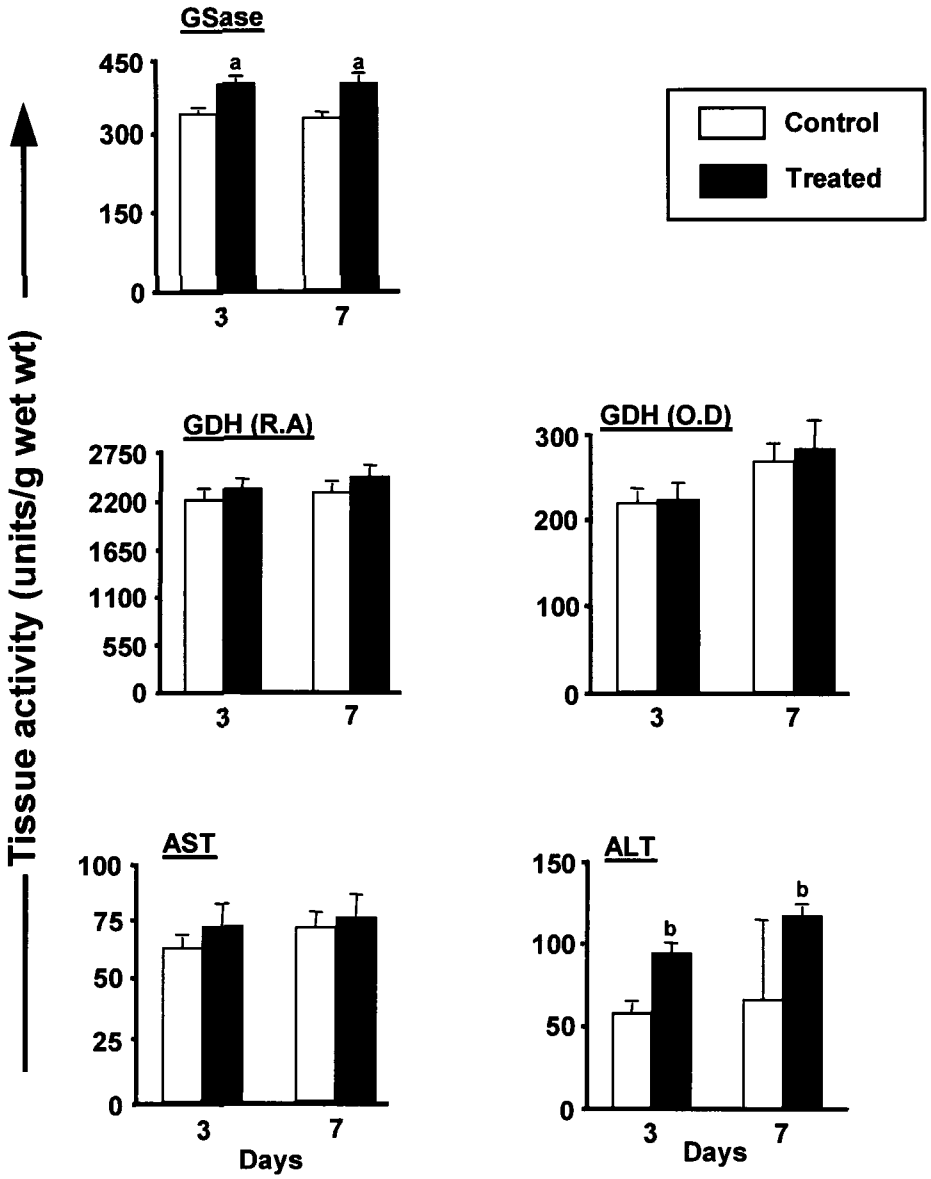


Fig. 30 Changes of tissue activity (units/g wet wt) of different enzymes related to amino acid metabolism in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

a, b, c : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

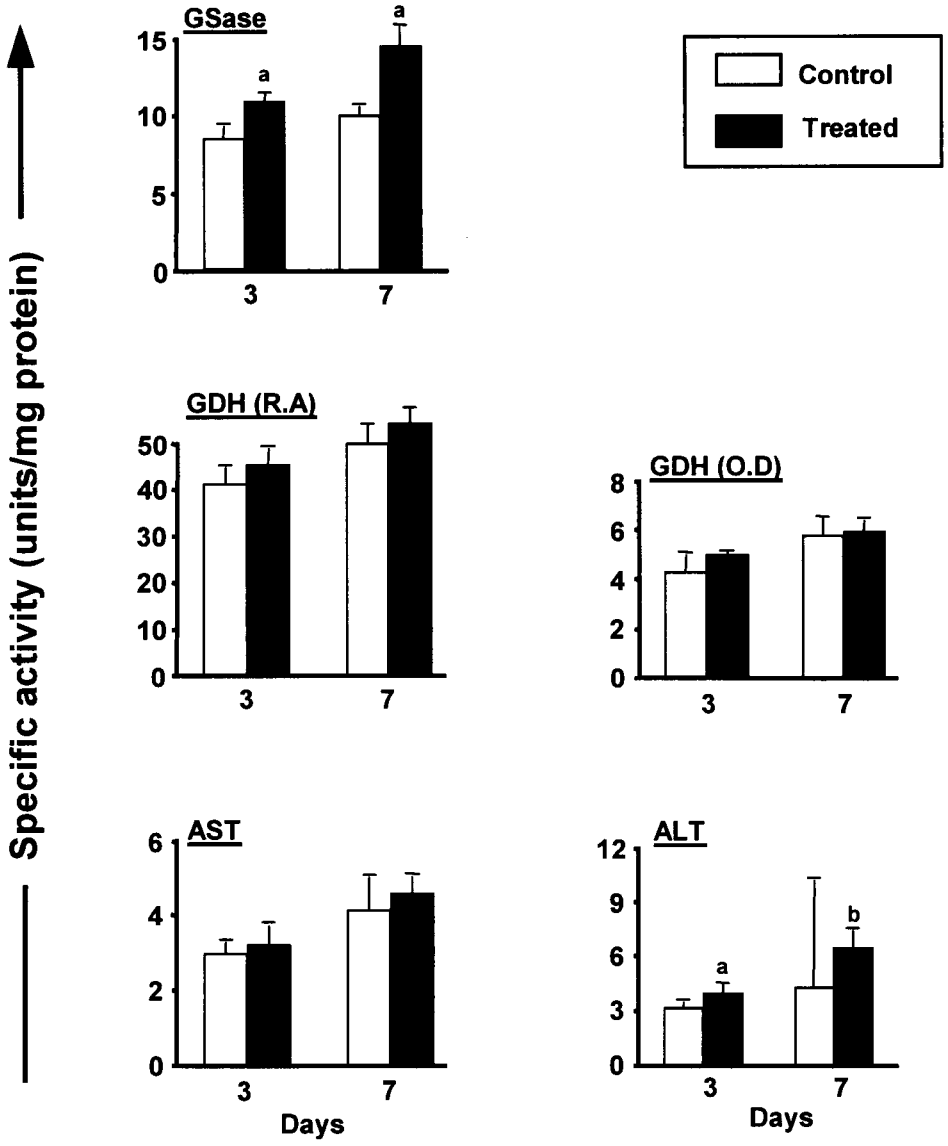


Fig. 31 Changes of specific activity (units/mg protein) of different enzymes related to amino acid metabolism in the brain of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b, c] : *p* values significant at <0.05, <0.01 and <0.001 levels, respectively, (One-way ANOVA).

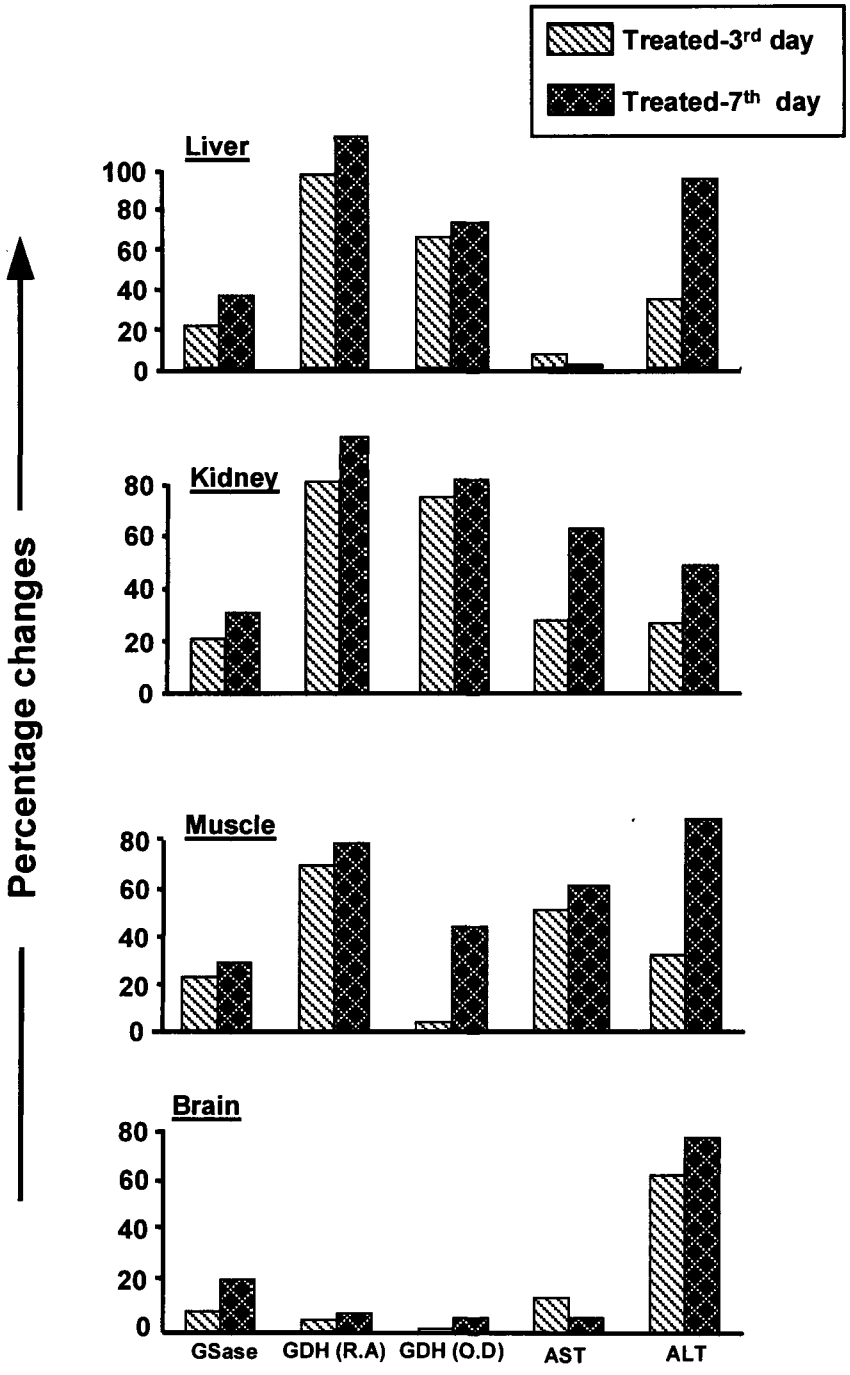


Fig. 32 Percentage changes in the tissue activity of different enzymes related to amino acid metabolism of *C. batrachus* exposed to 250 mOsmol/l mannitol.

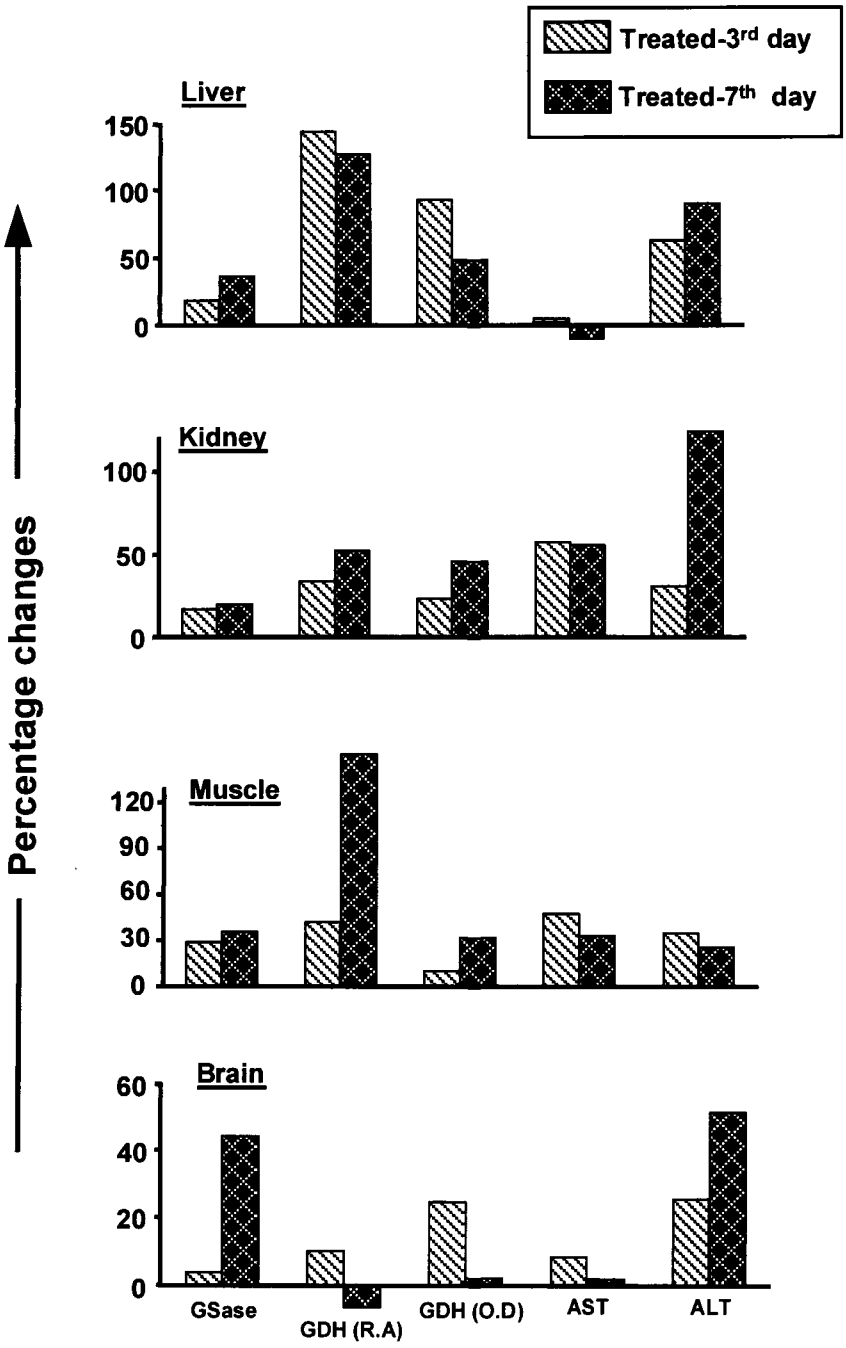


Fig. 33 Percentage changes in the specific activity different enzymes related to amino acid metabolism of *C. batrachus* exposed to 250 mOsmol/l mannitol.

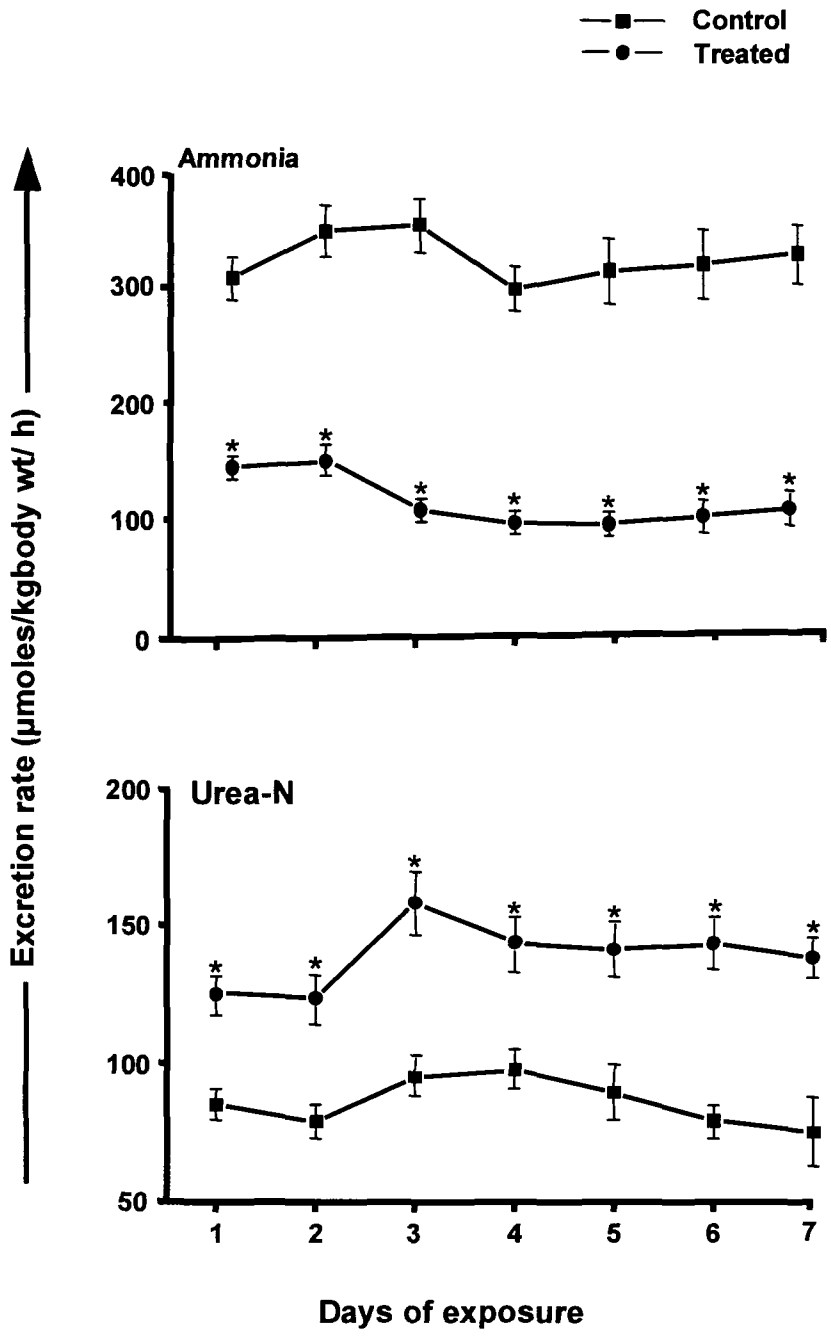


Fig: 34 Rate of excretion of ammonia and urea-N (µmoles/kg body wt/h) of *C. batrachus* exposed to 250 mOsmol/l mannitol for 7 days. Values are plotted as mean ± SEM ($n = 5$).

*Significantly different from respective control values ($P < 0.001$). (One-way ANOVA)

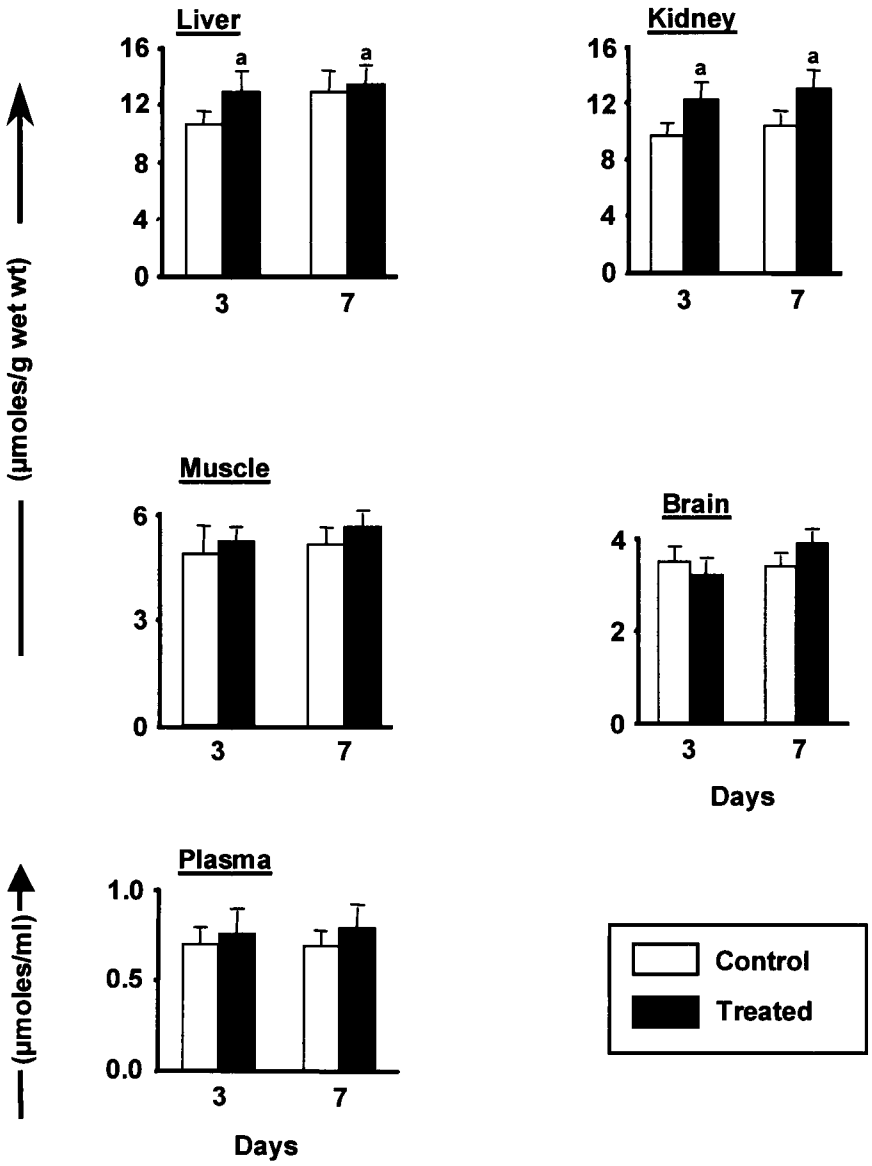


Fig: 35 Changes in the concentration of ammonia in different tissues (µmoles/g wet wt) and in plasma (µmoles/ml) of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b]: *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

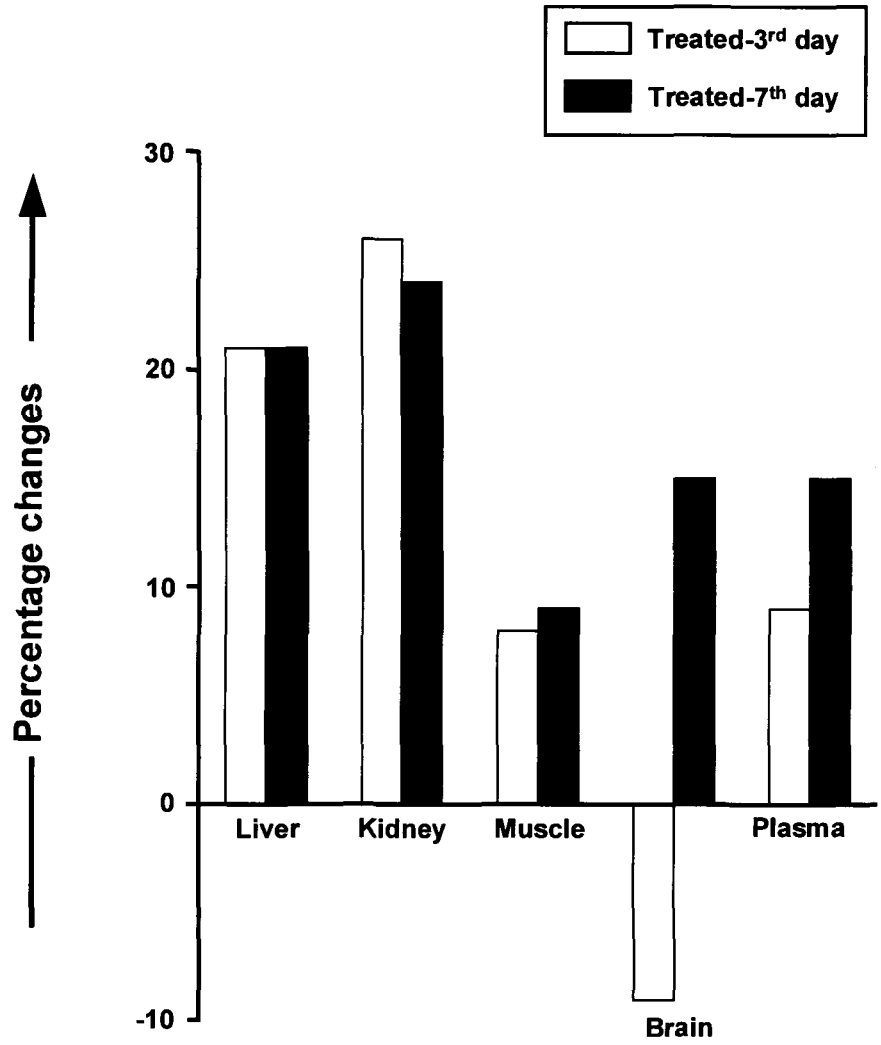


Fig: 36 **Percentage changes in the concentration of ammonia in different tissues and in the plasma of of *C. batrachus* exposed to 250 mOsmol/l mannitol.**

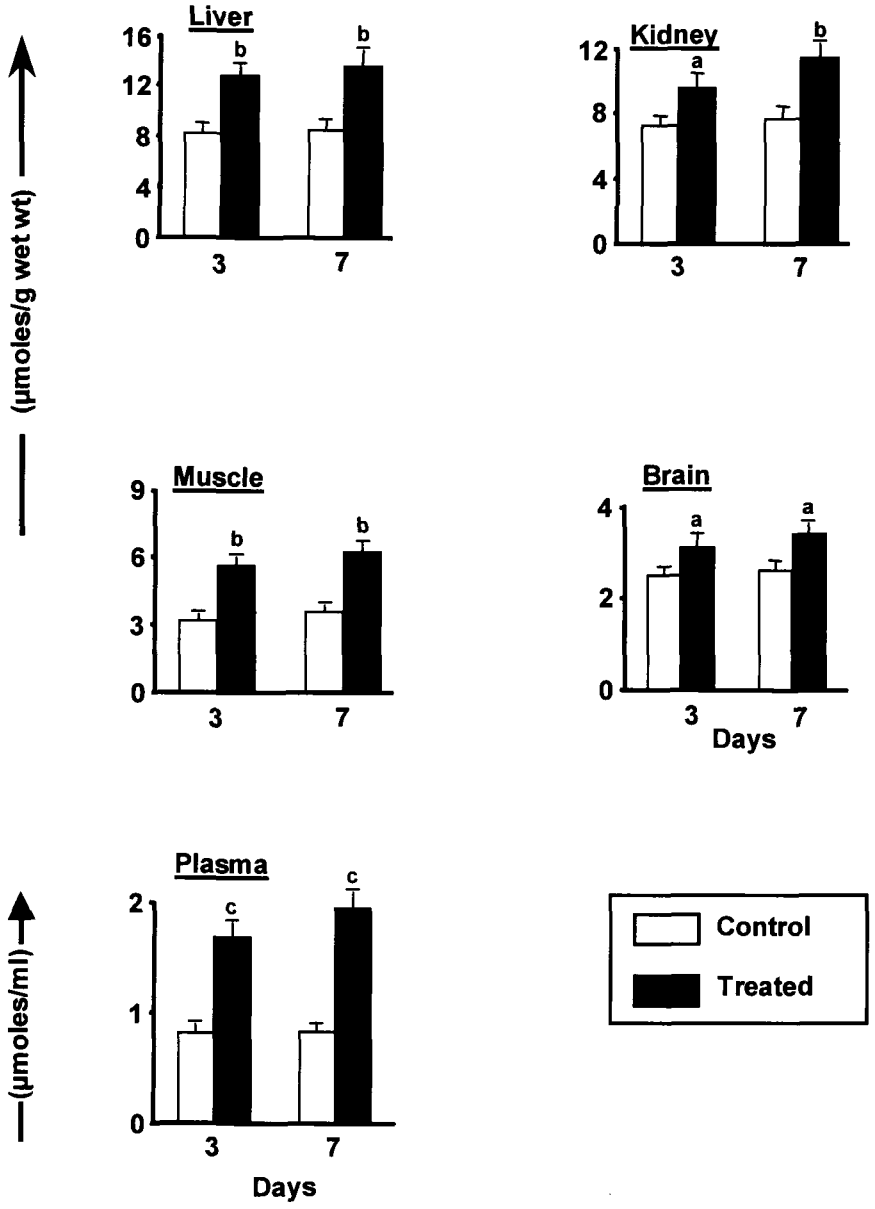


Fig: 37 **Changes in the concentration of urea-N in different tissues (µmoles/g wet wt) and in the plasma (µmoles/ml) of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).**

[a, b, c] : *p* values significant at <0.05 ,<0.01 and <0.001 levels, respectively, (One-way ANOVA).

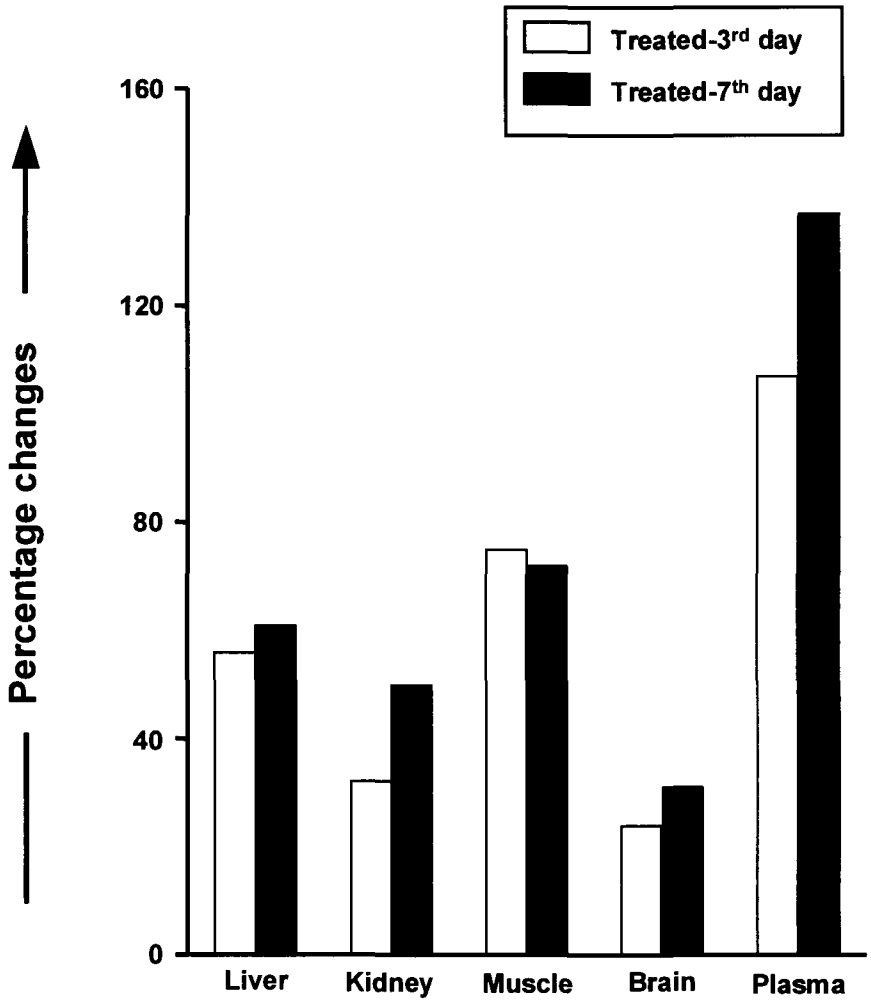


Fig: 38 **Percentage changes in the concentration of urea-N in different tissues and in the plasma of of *C. batrachus* exposed to 250 mOsmol/l mannitol.**

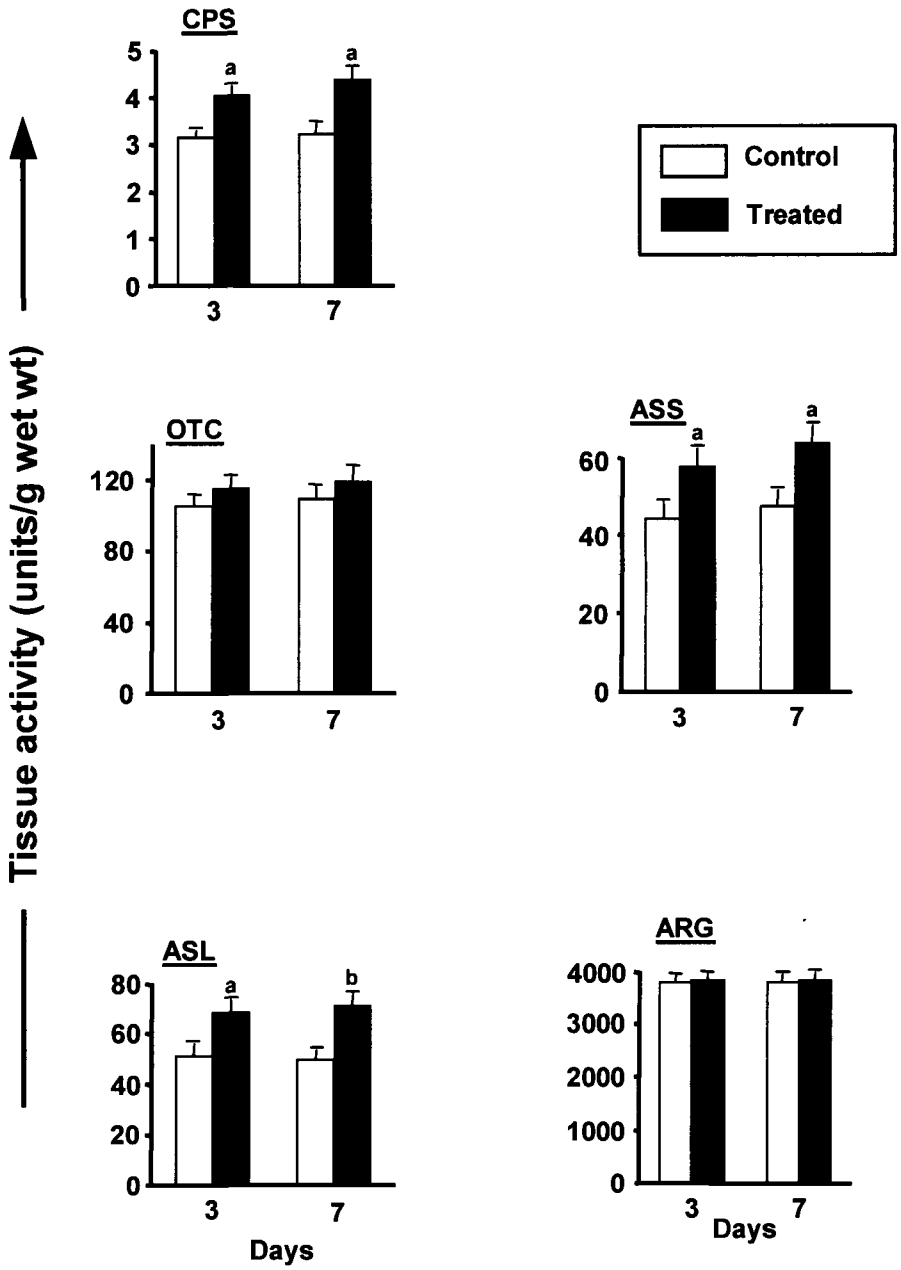


Fig: 39 Changes of tissue activity (units/g wet wt) of OUC enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b]: *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

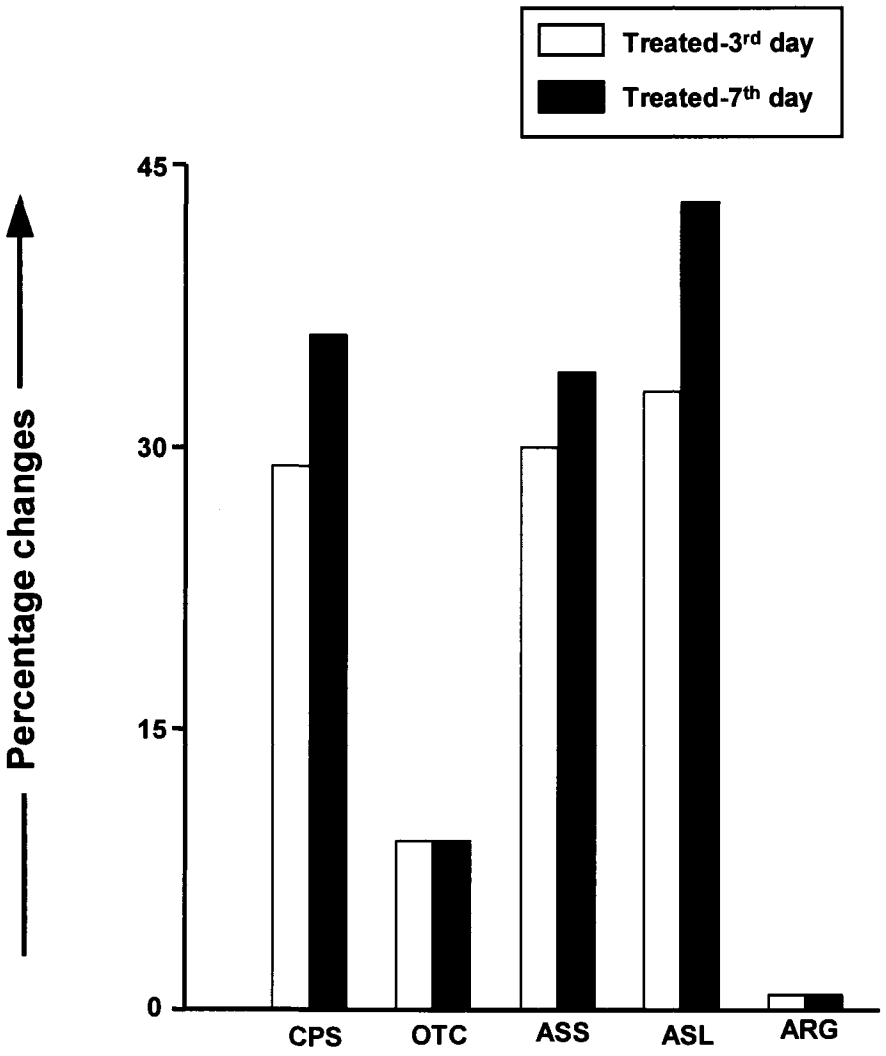


Fig: 40 **Percentage changes in tissue activity of OUC enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol.**

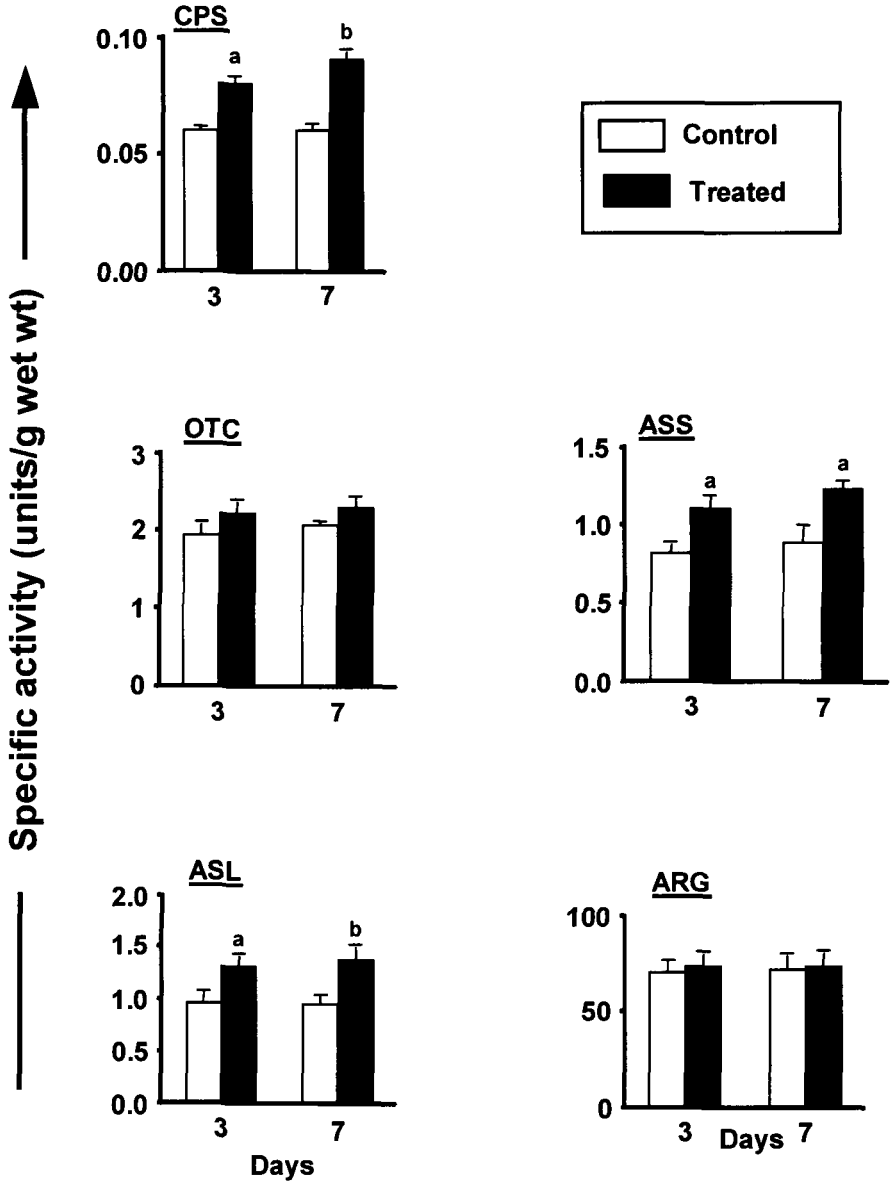


Fig: 41 Changes of specific activity (units/mg protein) of OUC enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b]: *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

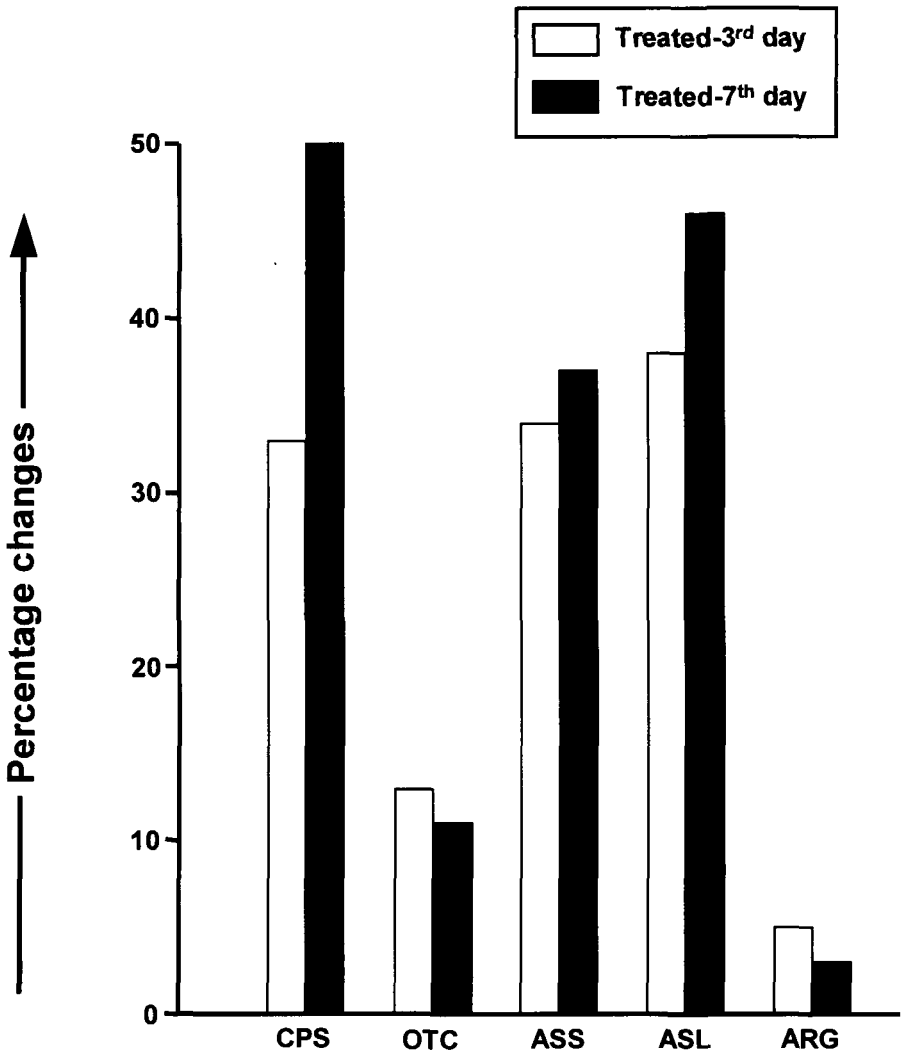


Fig: 42 Percentage changes in specific activity of OUC enzymes in the liver of *C. batrachus* exposed to 250 mOsmol/l mannitol.

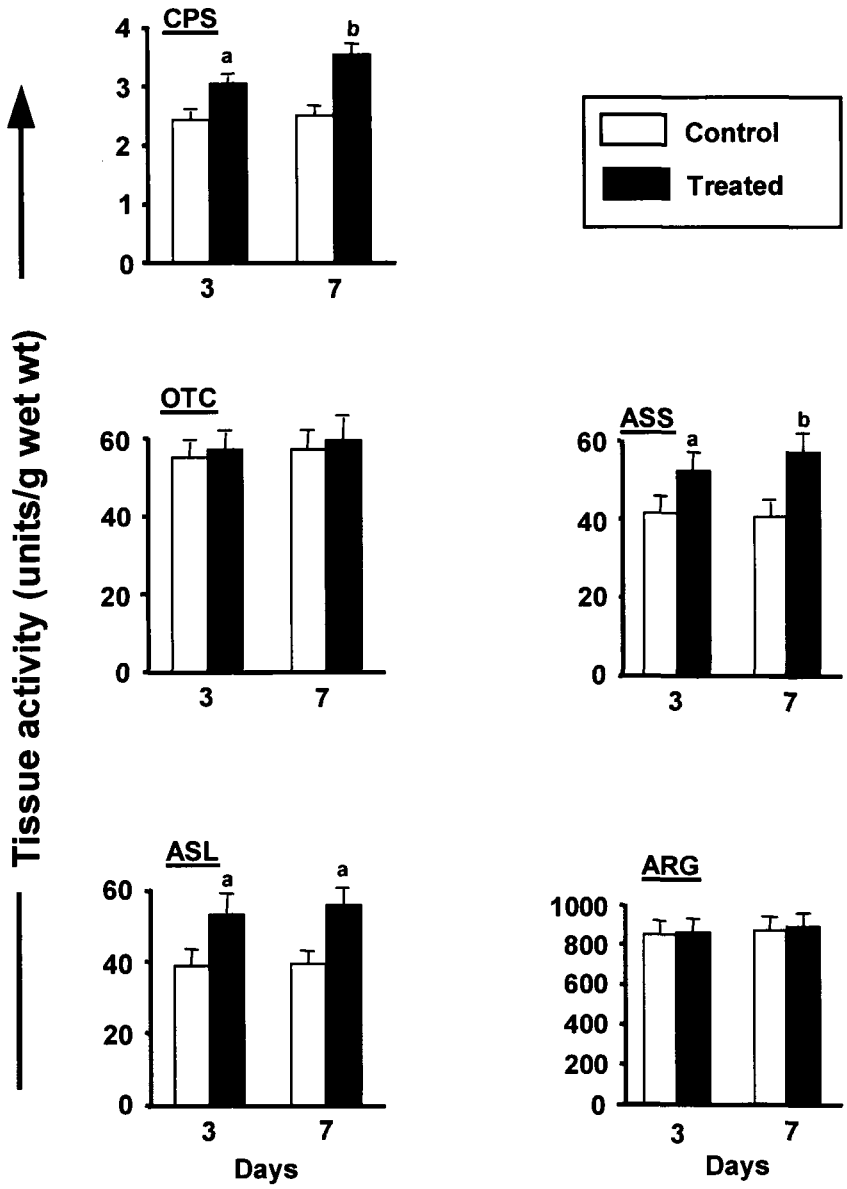


Fig: 43 Changes of tissue activity (units/g wet wt) of OUC enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b] : *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

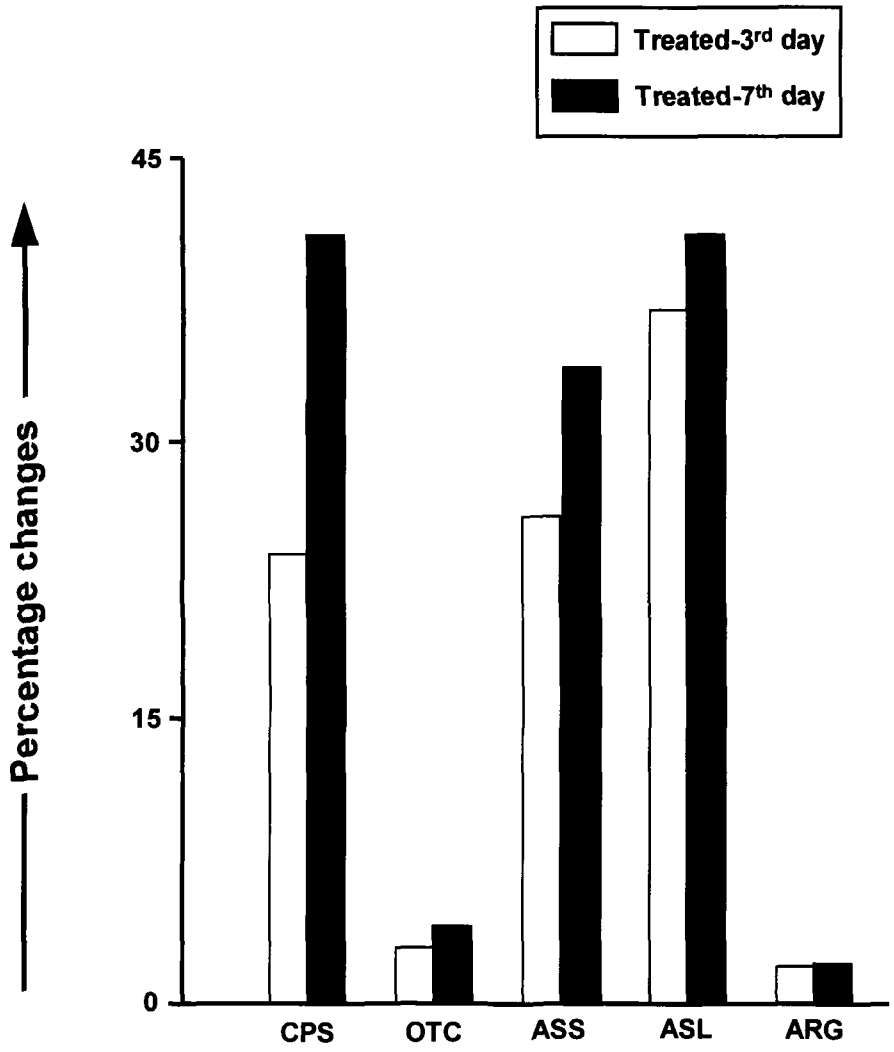


Fig: 44 Percentage changes in tissue activity of OUC enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol.

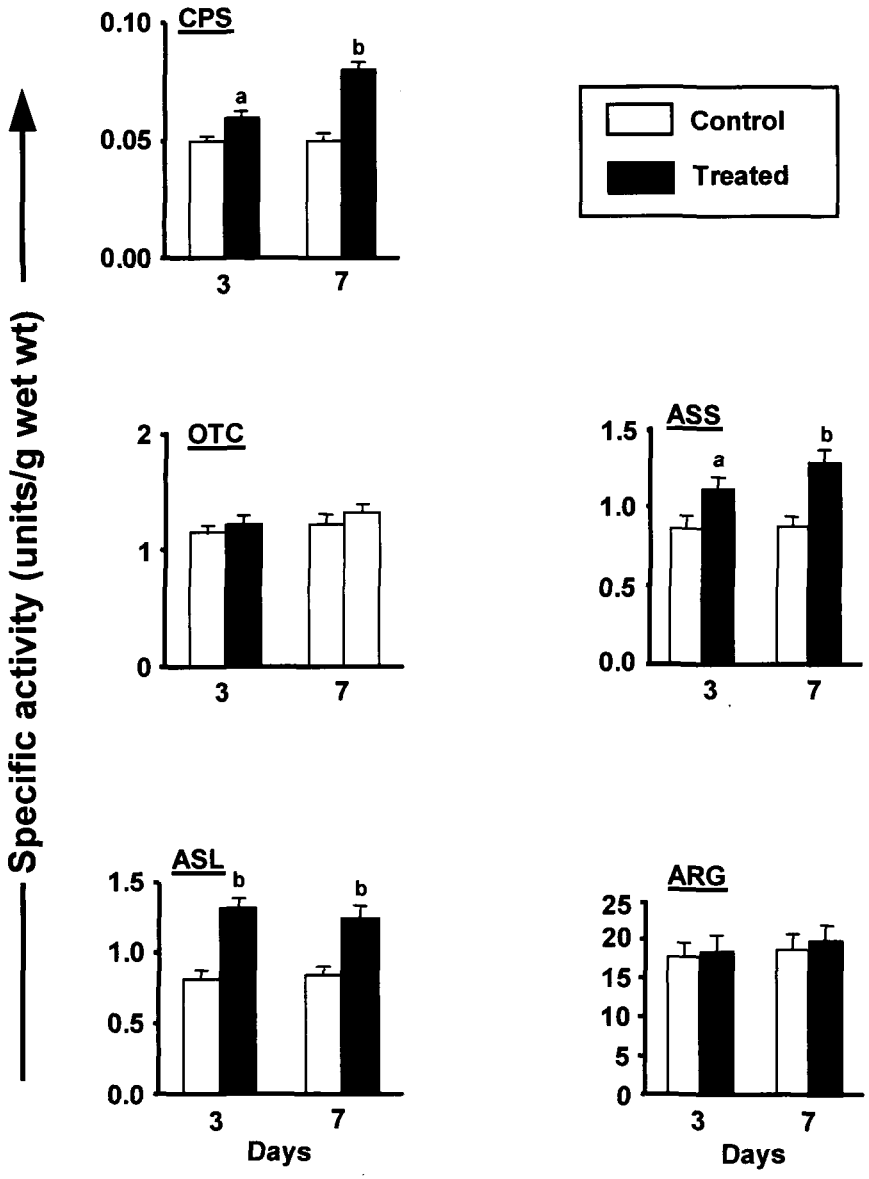


Fig: 45 Changes of specific activity (units/mg protein) of OUC enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol. Values are expressed as mean ± SEM (n = 5).

[a, b] : *p* values significant at <0.05 and <0.01 levels, respectively, (One-way ANOVA).

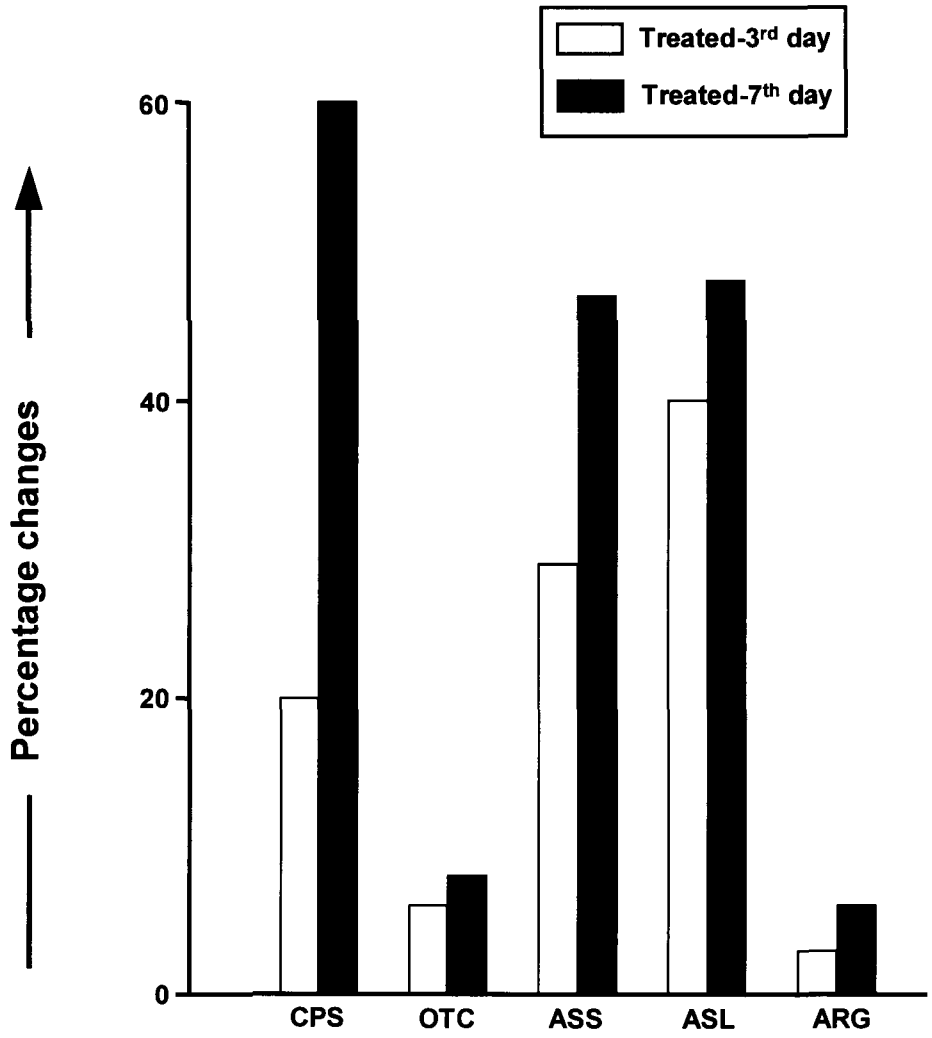


Fig: 46 Percentage changes in specific activity of OUC enzymes in the kidney of *C. batrachus* exposed to 250 mOsmol/l mannitol.

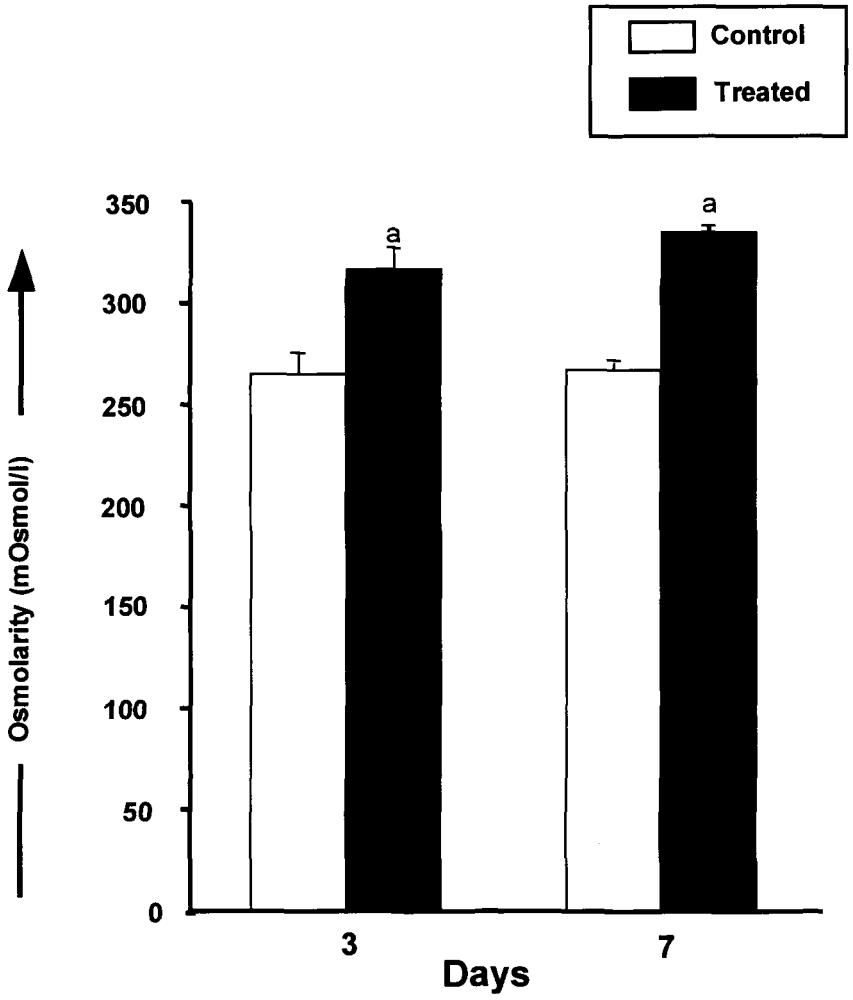


Fig: 47 Changes in the osmolarity of blood plasma (mOsmol/l) of *C. batrachus* exposed to 250 mOsmol/l mannitol.
[a] : *p* values significant at <0.05 level (One-way ANOVA).

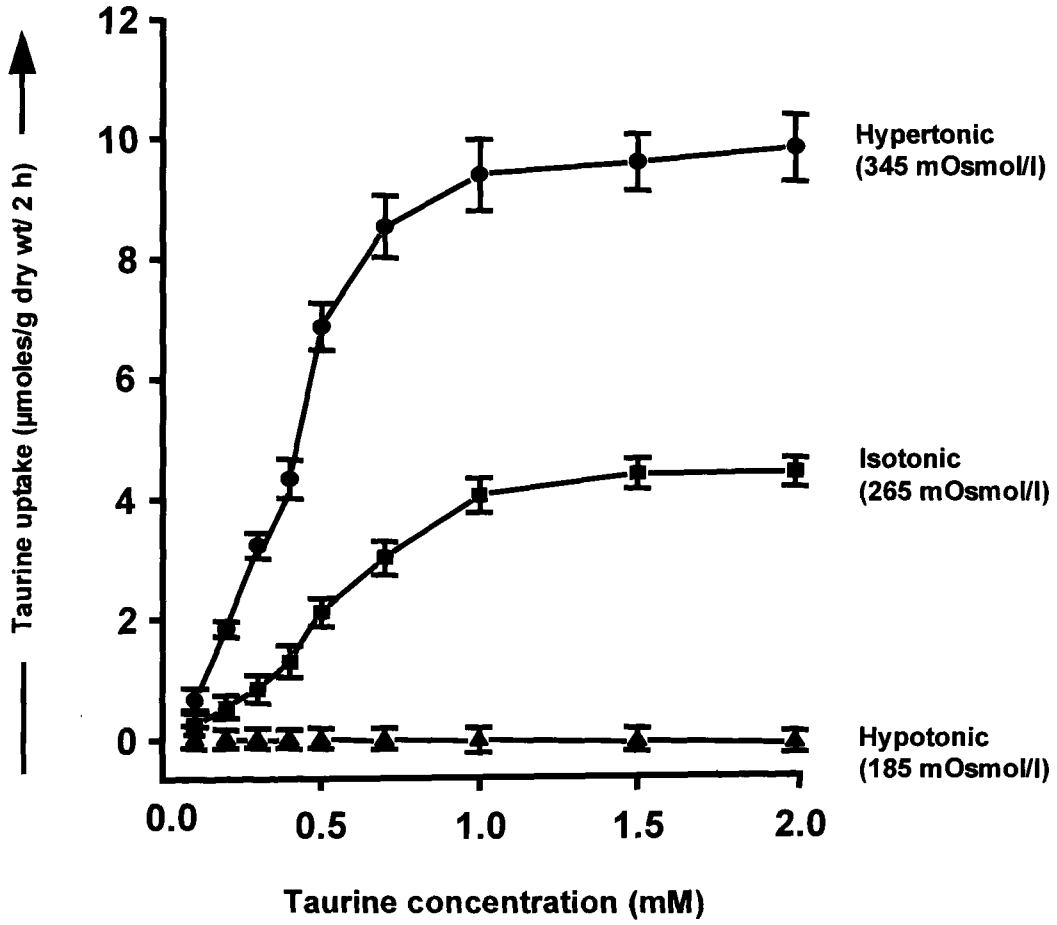


Fig: 48 **Taurine uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentrations of taurine for 2 h both under iso- and aniso-tonic conditions while maintaining the osmolarity of the media with NaCl. Values are expressed as mean ± SEM (n = 5).**

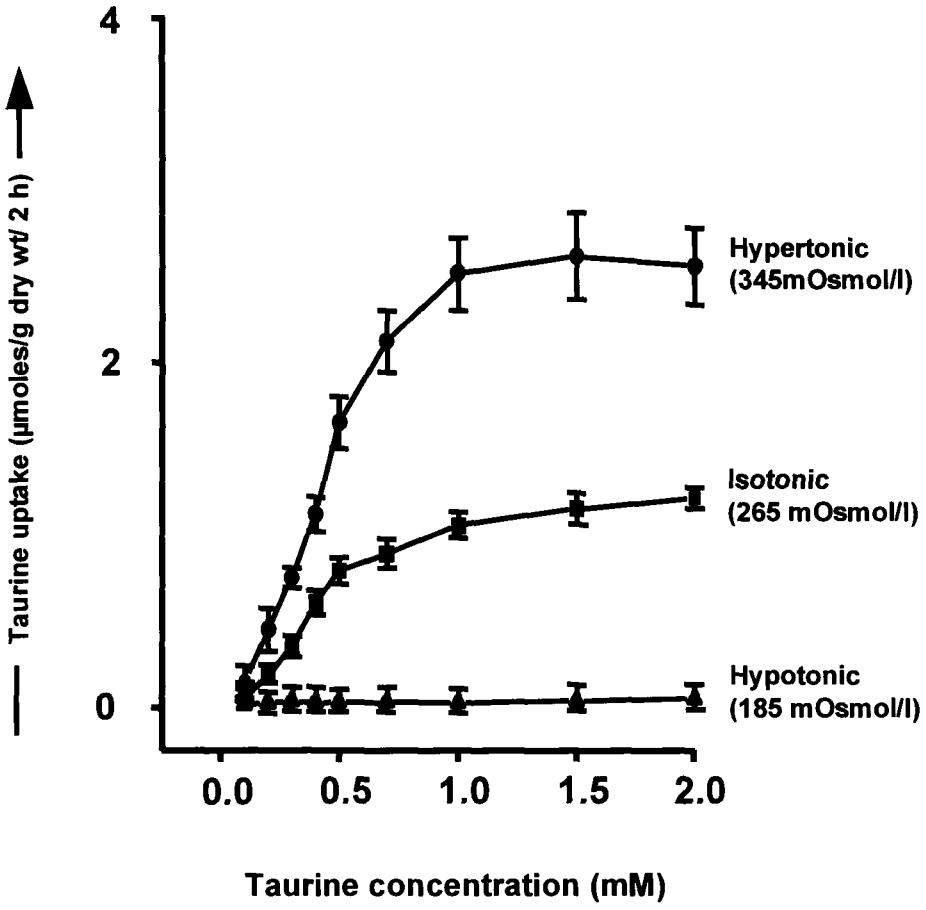


Fig: 49 Taurine uptake by the isolated RBC of *C. batrachus* pre-incubated at different concentrations of taurine for 2 h both under iso- and aniso-tonic conditions while maintaining the osmolarity of the media with mannitol. Values are expressed as mean ± SEM (n = 5).

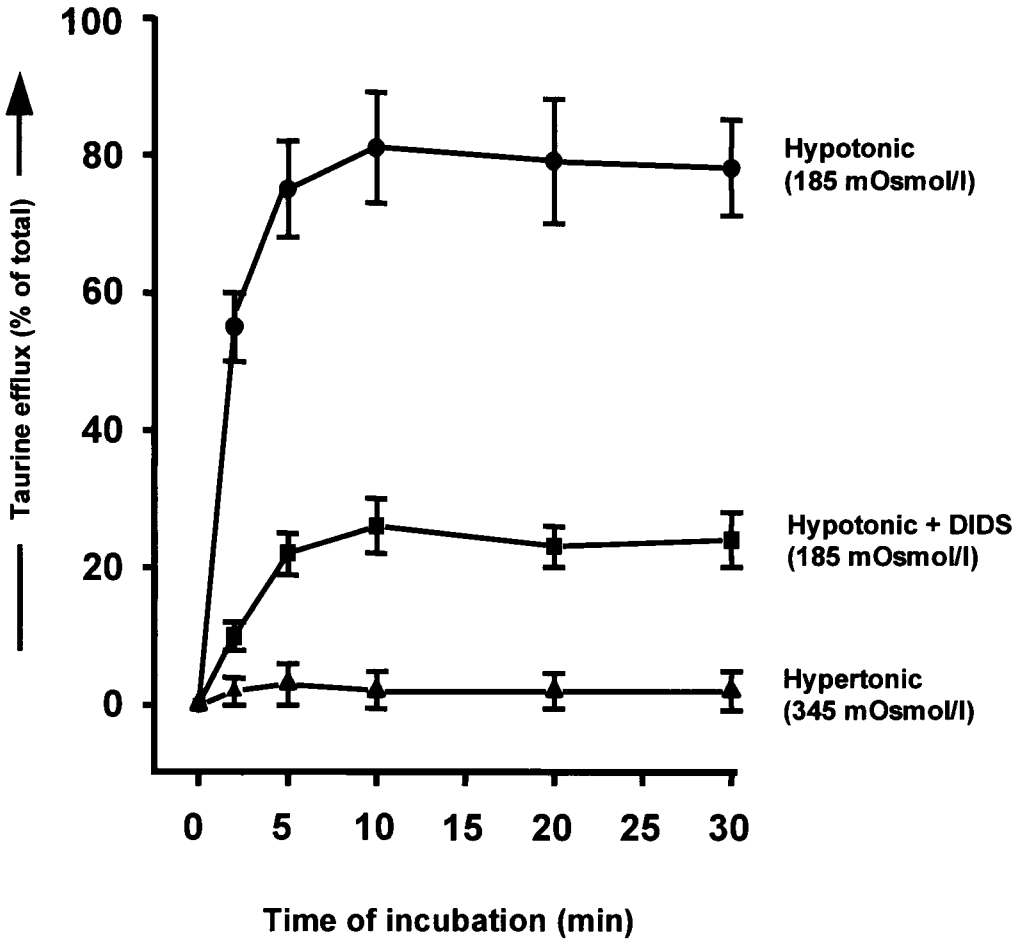


Fig: 50 **Effect of time on taurine efflux by the isolated RBC of *C. batrachus* under hypo- and hypertonic conditions while maintaining the osmolarity of the media with NaCl. Values are expressed as mean ± SEM (n = 5).**

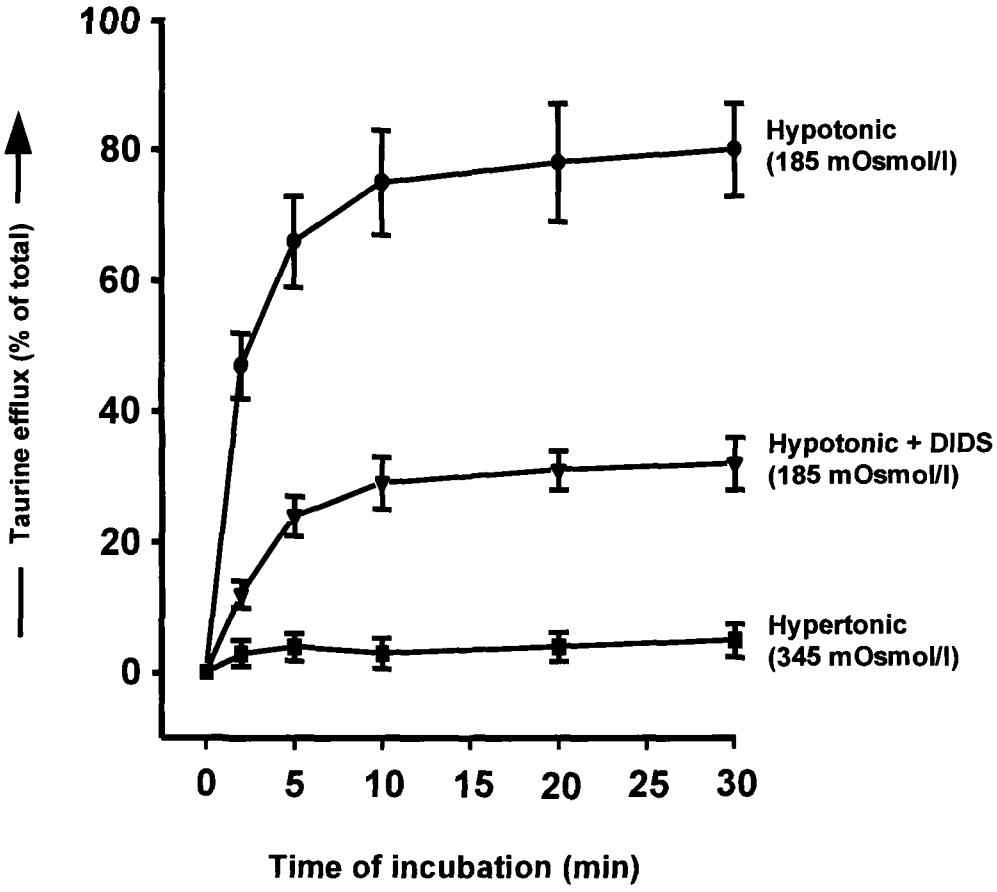


Fig: 51 Effect of time on taurine efflux by the isolated RBC of *C. batrachus* under hypo- and hypertonic conditions while maintaining the osmolarity of the media with mannitol. Values are expressed as mean ± SEM (n = 5).

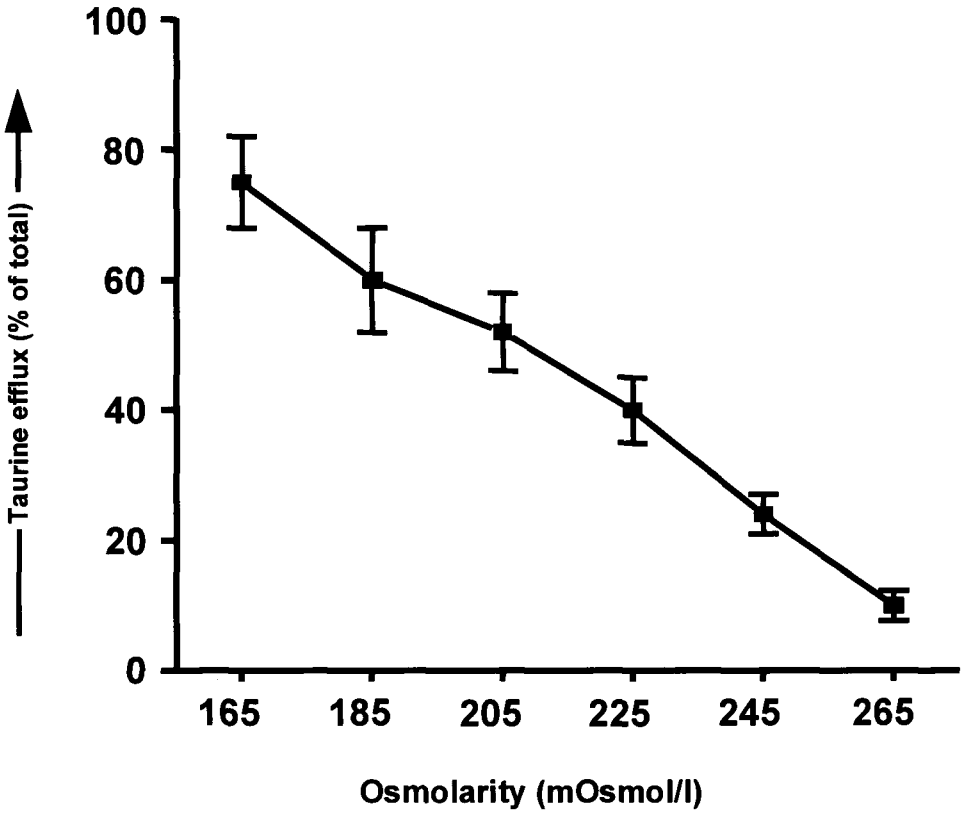


Fig: 52 Effect of different osmolarities on taurine efflux by the isolated RBC of *C. batrachus*. Values are expressed as mean ± SEM (n = 5).

DISCUSSION

Effects of anisotonicity on amino acid metabolism in the perfused liver of *C. batrachus*

The regulation of volume in response to changes in osmolarity is a fundamental property of most cells. Following cell swelling in hypotonic medium, cells tend to recover their initial volume by a process termed regulatory volume decrease (RVD). Likewise, following cell shrinkage in hypertonic medium, cells tend to recover their initial volume by a process termed regulatory volume increase (RVI). In mammalian cells, the RVD and RVI are the results of cellular release/uptake of Na^+, K^+, Cl^-, HCO_3^- and H^+ ions with the involvement of various ionic channels present in the plasma membrane (Häussinger, 1996a,b). This was also found to be true in the case of the air-breathing walking catfish (*C. batrachus*) hepatocytes (Goswami, 2002). Furthermore, it was found that some of the organic osmolytes such as Tau, also play important roles in cell volume regulation, thus indicating that the hepatocytes of the walking catfish possess various volume regulatory mechanisms to adapt under osmotic stress (Goswami, 2002). However, it is to be noted that although they possess various volume regulatory mechanisms, the hepatocytes remain partly in swollen or shrunken state as long as they are exposed to anisotonic conditions (Goswami, 2002). This minute changes of cell volume is reported to alter various metabolic processes in mammals (Häussinger et al., 1994; Häussinger, 1996a,b) and also in the walking catfish (Goswami, 2002). Hepatocyte cell swelling causes stimulation of glycogenesis, hexose monophosphate (HMP) pathway and protein synthesis, and inhibition of glycogenolysis, GSSG release into bile, proteolysis and less oxidation stress; whereas the hepatocytes cell shrinkage causes stimulation of glycogenolysis, proteolysis, more oxidative stress, inhibition of glycogenesis, HMP pathway and protein synthesis in the walking catfish (Goswami and

Saha, 1998; Goswami et al., 2004; Saha et al., 2004; Biswas and Saha, unpublished results). It has been known for sometimes that amino acids (AAs) play an important role as cellular osmolytes in many marine organisms for the maintenance of constant cell volume (Gilles, 1975; Perlman and Goldstein, 1988). Role of AAs in cell volume regulation under osmotic stress have also been reported to play very important roles in different cell types of terrestrial animals (Chamberlin and Strange, 1989; Häussinger et al., 1994). Therefore, in the present investigation the effects of anisotonicity on the changes of concentrations of different FAAs in the perfused liver of walking catfish (*C. batrachus*) was studied along with the changes of activity of certain key enzymes related to amino acid metabolism in addition to the studies on the efflux of ammonia-N, urea-N, total-N, glutamate and glutamine by the perfused liver under osmotic stress.

When the liver of walking catfish was perfused with hypotonic medium for 90 min after initial perfusion with isotonic medium for 30 min, there was significant decrease of both non-essential FAAs by 41% (mainly attributable to the decrease of Tau, Ala, Cit, Ser and Asp) and essential FAAs by 36% (mainly attributable to the decrease of Ile, Lys, Val and Met). The role of Tau in cell volume regulation has been well documented both in mammalian (Warskulat et al., 1997, Satsu et al., 1999) and fish hepatocytes (Goldstein et al., 1990, Goldstein and Brill, 1991, Fincham et al., 1987). In this walking catfish liver, the concentration of Tau, which constitutes the major AA, appeared to decrease maximally. This was possibly due to more efflux of Tau under hypotonic condition as reported recently from our laboratory, followed by release of excess water, thus maintaining cell volume homeostasis (Goswami, 2002). Our present investigation indicated that few more AAs are possibly also transported out from the perfused liver under hypotonic perfusion condition possibly by activating

volume-sensitive ion channels as suggested by Motais et al. (1997). Activation of one anion channel (Band 3 protein) due to swelling of hepatocytes under hypotonic condition has been confirmed in our fish hepatocytes (Goswami, 2002), in the trout hepatocytes (Michael et al., 1994) and also in various fish hepatocytes (Brill et al., 1992, Goldstein and Musch, 1994; Jensen, 1995), thus causing more efflux of Tau and few more AAs. Similar to our suggestion, more efflux of some AAs other than the Tau under hypotonic cell swelling has been suggested in the trout hepatocytes (Michael et al., 1994). Another possible reason of decrease of FAAs levels in the liver of walking catfish under hypotonic condition could be more oxidation of AAs as evidenced by the fact that the efflux of ammonia-N, urea-N and total-N from the perfused liver increased significantly when the liver was infused with different amino acids under hypotonic perfusion condition (Table 7; Fig. 13). King et al. (1980) reported the more oxidation of β-Ala and Sar in the liver of little skate, *Raja erinacea* during cell swelling. Ballantyne et al. (1986) have further shown the appropriate stimulation or inhibition of the mitochondrial oxidation of various AAs when varying the medium osmolarity.

When the walking catfish liver was perfused with hypotonic medium in presence of 5 mM NH_4Cl, the decreasing pattern of both non-essential and essential FAAs levels was almost similar as in the absence of NH_4Cl except for more accumulation of certain non-essential FAAs under isotonic condition (Tables 1 & 2; Figs. 3 & 5). No significant changes of any of the amino acid metabolism-related enzymes activity (both tissue and specific) was observed both in the absence or presence of NH_4Cl in the perfused liver under hypotonic condition (Tables 3 & 4; Figs. 7 & 9). This is an indication to the fact that hypotonic cell swelling does not have any effect on AA synthetic pathways except

for the possibility of decreasing production of AAs by proteolysis as cell swelling was found to cause inhibition of proteolysis (Biswas and Saha, unpublished results).

In contrast to hypotonic cell swelling, hypertonic cell shrinkage caused significant accumulation of both non-essential and essential FAAs in the perfused liver of walking catfish both in the absence and presence of NH_4Cl (Tables 1 & 2; Figs. 3 & 5). In the absence of NH_4Cl, there was about 71% increase of non-essential FAAs, which were mainly attributable to the increase of Tau, Asp, Ala, Ser, Glu, Gln and Cit, and the essential FAAs increased by 51%, which were mainly attributable to the increase of Thr, Ile, Leu, Orn and Arg. In the presence of NH_4Cl, similar increase of non-essential and essential FAAs was observed. One of the major reasons of increase of FAAs levels in the liver under hypertonic condition was again probably attributable for maintaining the cell volume homeostasis thus causing re-uptake of water as against the water loss caused due to exposure to hypertonic medium. There could be various possible mechanisms of increase of FAAs levels in the liver of walking catfish during hypertonic exposure. One of which could be by more synthesis of non-essential FAAs by the enhanced activites of various amino acid metabolism-related enzymes such as GSase, GDH and AST, both in the absence or presence of NH_4Cl, as observed in the present study.

Second, mechanism could be by stimulating proteolysis (Biswas and Saha, unpublished results), thus causing an increase of both non-essential and essential FAAs level in the perfused liver. Various works have been initiated recently with the protein turnover and proteolysis in relation to cell volume changes in the mammalian hepatic cells. It is found that the hepatic proteolysis is partly under the control of amino acids and hormones, such as insulin and glucagon (Haüssinger et al., 1994a). It has

been shown clearly that hypotonic cell swelling inhibits proteolysis in mammalian liver, whereas hypertonic cell shrinkage stimulates protein breakdown (Hallbrucker et al., 1991; Haüssinger et al., 1991; Haüssinger et al., 1990b), thereby indicating that the cellular hydration status and the proteolysis activity have a direct correlation. Proteolytic effects of various mammalian hepatocytes by various amino acids, hormones like insulin and glucagon were also suggested to be due to changes of hydration status of the cell (for review, see Haüssinger et al., 1994a).

If we compare the total of FAAs with and without NH_4Cl, it was seen that there was more increase of non-essential FAAs from 30 to 45 μmoles/g wet wt under hypertonic condition in presence of NH_4Cl. Increase of non-essential FAAs may be due to transamination, as amino group of the amino acids 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, which may be converted to glutamine by fixing one more molecule of ammonia by the induced GSase activity in addition to the conversion of some glutamate to various other non-essential FAAs by the induced transaminase enzymes.

The third possibility could be inhibition of AA oxidation, which has been generated due to stimulation of proteolysis under hypertonic stress, as evidenced by the fact that the efflux of ammonia-N, urea-N and total-N from the perfused liver while infusing with different AAs, were significantly inhibited during hypertonic stress (Table 7; Fig.13). It is difficult at this moment with the results available to explain the mechanism(s) of stimulation/inhibition of various enzymes in the walking catfish of liver observed in the present study under hypertonic stress. However, it could be due to changes of phosphorylation status of the enzymes with the involvement of certain

signaling molecules under osmotic stress. Accumulation of certain effector molecules under hypertonic stress could be another possible means of stimulation of enzymes activity. Regulation of these enzymes at the transcriptional level cannot also be ruled out under osmotic stress. However, a detailed investigation is required to be performed before drawing any definite conclusion.

When the liver of *C. batrachus* was perfused with 1 mM NH_4Cl under hypotonic perfusion condition, ammonia-N efflux significantly increased, and urea-N, Glu and Gln efflux significantly decreased (Fig. 11). The decrease in the efflux of urea-N, Gln and Glu under hypotonic condition may be due to less uptake of ammonia from the medium leading to less conversion of ammonia to urea-N, Glu and Gln, and hence less efflux from the perfused liver under hypotonic stress. In contrast, when the liver of *C. batrachus* was perfused with 1 mM NH_4Cl under hypertonic condition, ammonia-N and Glu efflux significantly decreased, and urea-N and Glu efflux increased significantly. Increase of urea-N efflux may be due to active conversion of ammonia-N to urea-N under hypertonic stress possibly by enhancing the GSase and other OUC enzymes activity.

There was significant increase in the efflux of ammonia-N, urea-N and Glu when the liver of *C. batrachus* was perfused with Gln (0.25 mM) under hypotonic perfusion condition, and decrease in the efflux of urea-N, Glu and Gln under hypertonic perfusion condition (Fig.12). Increase of efflux of ammonia-N, urea-N and Glu under hypotonic stress could be due to more oxidation of Gln, thus resulting more formation of ammonia-N, urea-N and Glu and hence more efflux. Whereas under hypertonic stress it may so happen that there was less oxidation of Glu thus forming less of ammonia-N, urea-N and Glu.

In conclusion, it appears that AAs and AA metabolism play very important roles in the liver of the walking catfish (*C. batrachus*) for osmotic balance, and thus in maintaining the cell volume homeostasis, which is done either by retaining more of AAs as osmolytes under hypertonic stress or by removing out AAs from the cell under hypotonic stress through the involvement of certain carrier mediated transporter system. Certain adjustments of amino acid metabolism as a whole under osmotic stress also appears to play an important role in the maintenance of cell volume homeostasis as a sort of physiological adaptation in this walking catfish.

Effect of hypertonicity on amino acid metabolism and ureogenesis in *C. batrachus*

In comparison to mammals, teleost fishes face more problems of osmotic stress primarily due to osmolarity changes in their external environment at different seasons of the year. Apart from the role of supply of metabolic energy, free amino acids (FAAs) are also known as important solutes in the intracellular osmolyte pool of numerous marine invertebrates and marine fishes (Gilles, 1975; Goldstein and Perlman, 1995). Several species of freshwater air-breathing teleosts including the walking catfish (*C. batrachus*) inhabit stagnant, slow flowing swampy water bodies or wetlands that are usually unhabitable to purely aquatic fishes such as carps. During summer, when these water bodies are dried up, they usually burrow inside the mud peat to avoid total dehydration. Because of the type of habitat they occupy, these fishes regularly face problems associated with osmolarity changes. Amphibians also inhabit a wide variety of osmotic environments that differ in their relative availability of free water. These include completely aquatic to fully terrestrial and arboreal habitats, and even full strength sea water. Accumulation of urea, the major nitrogenous waste, as a solute in the intra- and extra-cellular fluid compartments by some amphibians provides the opportunity to change significantly the water potential of the body fluids, and this may result in the formation of a more favorable osmotic relationship between the animal and the environment (Gordon et al., 1961; McClanahan, 1972, 1975). The walking catfish tolerated a 250 mOsmol/l mannitol solution for months without any deleterious affects. As mannitol fails to enter into the animal body, it causes dehydration of tissues by exosmosis, and adaptation to such condition could be complex. Different solutes have been known to be retained in the body such as urea, amino acids and inorganic ions

under hypertonic stress. Therefore, in the present investigation the effects of hypertonicity, caused due to exposure of the whole fish to 250 mOsmol/l mannitol for 7 days in situ on the possible alterations of FAAs levels along with the changes of certain key amino acid metabolism-related enzymes, and also the possible induction of ureogenesis via the functional OUC were elucidated in the walking catfish, *C. batrachus*.

Liver is the most important organ with relation to nitrogen metabolism and also it is the most important organ governing amino acid homeostasis in fish (Campbell, 1991). There was a significant increase in the levels of various non-essential FAAs in the liver such as Asp, Gly, Ala, Asn, Glu, Gln and Tau in the mannitol-exposed fish. There was also significant increase in the levels of various essential FAAs in the liver such as Met, Leu, Trp, and Phe in the mannitol-exposed fish. Similar observation with minor variation was also made in the kidney, muscle, brain and plasma of the walking catfish exposed to mannitol. Increase of the various FAAs, in general, though not necessarily to the same extent may be due to stimulation of proteolysis under hypertonic stress. Hypertonic stress is reported to stimulate the proteolysis in the perfused liver of the walking catfish (Biswas and Saha, unpublished results) and also in rat liver (Häussinger and Lang, 1991). Among these non-essential FAAs, Tau increased maximally. Taurine is a relatively inert molecule, which even at high concentration is not only compatible with the function and organization of macromolecules, but may in addition act as an osmoprotectant counteracting the deleterious effects of denaturing solutes as inorganic ions. The occurrence of Tau synthetic pathway, which constitutes the most predominant amine in most teleosts including the walking catfish, is not very clear in fish (Waarde, 1988). However, in the present investigation significant increase

of Tau concentration in all the tissues including the plasma was noticed under hypertonic stress. Similar increase of Tau concentration was also noticed in this fish under hyper-ammonia stress (Saha et al., 2000, 2002b). Therefore, it is necessary to investigate the occurrence of the Tau synthetic pathway with its physiological significance in this fish. However, it appears that FAAs play significant roles in this fish in cell volume regulatory processes during hypertonic stress.

The adjustment of intracellular FAA concentrations in response to osmotic stress has also been widely documented among crustaceans as well as other marine invertebrates (for reviews, see Gilles, 1975; Schoffeniels, 1976; Pierce, 1981). Rapid accumulation of Ala and Pro was observed during acclimatization to hypertonic stress in the intertidal copepod, *Tigriopus californicus* (Burton, 1986; Burton and Feldman, 1983). FAAs are reported to be major organic osmolytes for intracellular osmoregulation in marine and brakish water mollusks (Lynch and Wood, 1966; Pierce, 1971; Bedford, 1971; Matsushima et al., 1984). Similar increase in the levels of various FAAs (such as Ala, Asp, Gln, Glu, Gly and Tyr) in hypertonic media has been reported in mammalian tissues (Olson and Goldfinger, 1990; Beetsch and Olson, 1993; Hoffmann and Dunham, 1995; Beetsch and Olson, 1998; Lang et al., 1998). The basal levels of activity of some of the key enzymes related to amino acid metabolism such as the GSase, GDH (reductive amination), AST and ALT were also quite high in this walking catfish compared to many ammoniotelic teleosts including the gobiid fishes (Wilson, 1973; Waarde and Kesbeke, 1982; Chew and Ip, 1987; Iwata, 1988; Walton and Cowey, 1977, Peng et al., 1998, Anderson, 2001; Lim et al., 2001), which were further stimulated in different tissues (except for AST in liver and brain) under hypertonic stress. The increased activity of most of the enzymes in different tissues of mannitol-

exposed fish might have helped them for extra synthesis of non-essential FAAs from ammonia, generated due to protein and AA catabolism through the involvement of coupled GDH (reductive amination), which synthesizes Glu by assimilating ammonia with α-ketoglutarate, and GSase enzymes to incorporate another molecule of ammonia to Glu forming Gln, and also other AAs by transamination reactions. Stimulation of activity of the above mentioned enzymes were reported in different tissues of this walking catfish under hyper-ammonia stress and suggested that higher accumulation of ammonia could be one of the possible reasons of stimulation of activity of these enzymes (Saha et al., 2000, 2002). The cause of stimulation of these enzymes under hypertonic stress may be the same as it also caused significant accumulation of ammonia in different tissues. However, whatever may be cause of increase of FAAs levels, it appears that FAAs play important roles for osmotic balance in this walking catfish under hypertonic stress.

There was a reduction of ammonia excretion by the walking catfish immediately after being exposed to mannitol solution, whereas the urea-N excretion rate increased significantly (Table 17; Fig. 34). Seiter et al. (1978) also found the decrease in ammonia excretion in juveniles of *X. laevis* when treated with hypertonic mannitol. The initial decrease of ammonia excretion in walking catfish was accompanied by the increase in the concentration of ammonia in liver, kidney and muscle. Although there was an increase in ammonia level in the mannitol-exposed fish, but due to having its various adaptational abilities, ammonia possibly did not accumulate to a lethal concentration. This was further supplemented by the presence of a functional and inducible OUC in this fish as reported from our laboratory (Saha and Ratha, 1989; Saha et al., 1999; Saha and Das, 1999; Saha et al., 2003) resulting to a significant increase of urea level

in all the tissues. The significant accumulation of urea (as a good osmolyte) in different tissues of this fish under hypertonic stress might have also helped in maintaining the osmotic equilibrium to reduce the loss of water and prevent the dehydration.

Increased excretion and tissue urea levels were accompanied by the induction of the activity of at least three enzymes of the OUC such as CPS, ASS and ASL both in the liver and kidney of walking catfish under hypertonic stress, thus enhancing further the urea synthesis capacity from ammonia, and resulting to more excretion and accumulation of urea under hypertonic stress. Janssens and Cohen (1968) reported five fold increase of CPS activity in the liver of *X. laevis* placed in 0.9% sodium chloride solution for 14 days. McBean and Goldstein (1970) reported five fold increase in the liver of *X. laevis* treated with hypertonic saline solution. However, *X. laevis*, kept in isotonic mannitol solution, induced CPS activity immediately by two fold in the liver, but returned to normal level on the 28^{th} day (Funnkhouser and Goldstein, 1973). The decrease in ammonia excretion and accumulation, observed in walking catfish under hypertonic stress, probably the cause of increase of urea synthesis from ammonia, more accumulation and excretion of urea. Thus, it appears that urea, which is also known to be a good osmolyte, plays a critical role in this fish for cell volume regulation and to avoid desiccation under hypertonic stress. In the plasma, although there was no significant increase of ammonia concentration in the mannitol-treated fish, but there was significant increase of urea concentration. Increase of urea concentration accompanied with the increase of FAAs concentration observed in the present study could be the major causes of increase of plasma osmolarity (Table 24; Fig. 47), thus counteracting the higher external osmolarity to some extent mainly to avoid the water loss under hypertonic stress. Increase of urea level has been reported in the plasma of

carp, *Cyprinus carpio* (Gupta and Hanke, 1982a) and in anura, *Rana cancrivora* (Colley et al., 1972) treated with hyper- and hypotonic mannitol.

The mechanism(s) by which different amino acid metabolism-related enzymes and three key OUC cycle enzymes are stimulated in this fish under hypertonic stress is difficult to explain with the available data. However, the possible strategies of enzymatic induction could be through changes in the concentration of various effector molecules, and/or by changing the phosphorylation status of the pre-existing enzymes as observed in the case of glycogen phosphorylase and glycogen synthase enzymes in walking catfish (Goswami and Saha, 1998), and glycogen phosphorylase enzyme in another catfish, *Amereiurus nebulosus* (Hallgreen et al., 2003) under anisotonic conditions. Numerous protein kinases have been implicated in metabolic alterations with volume changes in mammalian hepatocytes (Weiergräber and Häussinger, 2000) and in fish chloride cells (Kültz and Avila, 2001), and several osmosensing receptors responsible for activation of various kinases have been identified (Maeda et al., 1995). Molecular crowding may also exert long-term effects, such as cAMP-mediated changes in gene transcription under osmotic stress (Burg 2000; Minton 2001). Recently it has been shown that the GSase and CPS are upregulated by increasing the enzyme protein concentrations under hyper-ammonia stress in this walking catfish (Saha et al., 2005). Therefore, the regulation at the transcriptional and translational level under osmotic stress cannot be ruled out. However, a thorough investigation needs to be performed about the regulation of enzymatic activities under osmotic stress in this fish to know the exact mechanism(s) of regulation of these metabolic pathways under various environmental stresses.

The capacity of increased urea synthesis and accumulation during osmotic stress observed in *C. batrachus*, a fresh water air-breathing teleost, was uncommon among fresh water teleosts. It resembled more with some aquatic toads and elasmobranchs. The results suggest that freshwater air-breathing teleosts have better physiological adaptation to tolerate a wide range of environmental variations unlike that of other freshwater teleosts known. The freshwater air-breathing teleosts probably have retained the ureosmotic character to synthesize and retain urea inside the body for osmotic balance, in addition to the capacity of more synthesizing and accumulating various AAs within the body for osmotic balance.

Effects of anisotonicity on the taurine (Tau) efflux/uptake by the isolated RBC of *C. batrachus*

Taurine (Tau), a sulphur-containing nitrogenous compound (NH_2-CH_2-CH_2-SO_3H) is found in all fish species. Tau also occurs in substantial amounts in marine migratory fishes, where it is the major nitrogenous substance after the histidine and imidazole dipeptides (Suyama and Yoshizawa, 1973). In all these species, as well as in invertebrates and higher vertebrates, intracellular Tau has been found to be important in the regulation of cell volume (King and Goldstein, 1983). The whole-animal balance of Tau is the result of four processes: synthesis of amino acid, its catabolism, excretion, and dietary absorption. In addition, studies of Tau metabolism have shown that fish tissues have little or no capacity for Tau metabolism (King et al., 1980a), but more recently it was suggested that probably the walking catfish (*C. batrachus*) has the capacity of synthesizing Tau within the body (Saha et al., 2000; 2002b). Erythrocytes from two euryhaline fish species, the eel (*Anguilla japonica*) and the starry flounder (*Platichthys stellatus*) were found to contain high intracellular concentrations of this amino acid. In fish red cells (Fugelli and Thoroed, 1986) and heart cells (Vislie, 1983), Tau efflux accounts for 30 to 45%, respectively, of regulatory volume decrease (RVD). The cellular accumulation of Tau occurs in cotransport with Na^+. Such Na^+-Tau symporters have been found both in the luminal and basolateral membranes of renal proximal tubules of the teleost fish species, *Carassius auratus* (Dantzler and Sibernagl, 1976; King et al., 1982). Tau helps to maintain a constant volume in a variety of cell types under hypotonic condition (Huxtable, 1992).

A reduction in extracellular osmolarity was reported to cause an increase in cell volume, and reversibly to decrease the activity of the transporter in the erythrocytes of

the euryhaline fish species, the eel and the starry flounder (Fincham et al., 1987). In contrast, low medium osmolarity was reported to stimulate the activity of a Na^+-independent nonsaturable transport route selective for Tau, GABA and small neutral amino acids, producing a net efflux of Tau from the cells. It is suggested that these functionally distinct transport routes participate in the osmotic regulation of intracellular Tau levels, and hence contribute to the homeostatic regulation of cell volume in the erythrocytes of the euryhaline fish species. When these cells are subjected to hypotonic stress, they swell and release increased amounts of Tau as part of their volume regulatory responses (Leite and Goldstein., 1987, Ziyadeh et al., 1988). The increased efflux of Tau from skate RBC is induced by cell swelling (Goldstein and Brill, 1989). However, the membrane transport system involved in the release of Tau is unknown. Therefore, in the present investigation, Tau concentration in the isolated RBC of walking catfish under iso- and anisotonic conditions, pre-incubated with/without 2 mM Tau, along with the studies on the uptake of Tau by the isolated RBC from the external medium both under iso- and anisotonic conditions, the efflux of Tau by the isolated RBC under hypo- and hypertonic conditions, and the effect of different osmolarities on the Tau efflux by the isolated RBC of walking catfish (*C. batrachus*) were studied.

Under hypotonic condition, the Tau concentration in the isolated RBC of walking catfish significantly decreased either pre-loaded with or without Tau. Whereas, under hypertonic condition no significant changes of Tau concentration was observed in the isolated RBC again either pre-loaded with or without Tau (Table 25), which was similar to the situation as observed in the little skate, *Raja erinaceae* (Goldstein and Brill, 1990). Decrease of Tau concentration from the isolated RBC of waking catfish under hypotonic condition could be due to more efflux of Tau (discussed later). More

decrease of Tau concentration under hypotonic condition from the pre-loaded RBC compared to without pre-loaded one, possibly suggests that Tau played a major role in cell volume regulation in the pre-loaded RBC, whereas in without pre-loaded RBC, various inorganic ions like Na^+, K^+, and Cl^- took major part in cell volume regulation.

Significant uptake of Tau by the isolated RBC was seen both under iso- and hypertonic conditions when the osmolarity was maintained with NaCl, which appeared to be concentration dependent with a V_{max} of 0.41 ± 0.29 and 9.41 ± 0.59 μmoles/g dry wt, respectively, under iso- and hypertonic conditions at 1 mM external Tau concentration (Table 26; Fig. 48). However, the uptake of Tau under hypertonic condition was about 2.4 fold higher compared to isotonic conditions. Similarly, the same concentration dependent uptake of Tau was also seen by the isolated RBC under both iso- and hypertonic condition when the osmolarity was maintained with mannitol with a Na^+-free medium with a V_{max} of 1.15 ± 0.08 and 2.61 ± 0.25 μmoles/g dry wt, respectively, at 1.5 mM external Tau concentration, but the uptake was at much reduced rate compared with the experiment where the osmolarity was maintained with NaCl (Table 27; Fig. 49). Thus, it appears that hypertonic stress mainly stimulated the Na^+-dependent Tau-transporters causing more uptake of Tau compared to the hypertonic condition in the absence of Na^+. The uptake of Tau, both under iso- and hypertonic conditions, although at a lower rate, in the Na^+-free medium could be by two possible means: one could be mediated by a diffusional pathway, and the second one could be mediated by Na^+-independent process as suggested in flounder erythrocytes (Thoroed and Fugelli, 1994). Goldstein and Brill (1991) also suggested that Tau uptake may occur by both Na^+-dependent and Na^+-independent processes since the Tau-transporter is known to operate in both directions across the cell membrane. Perlman

and Goldstein (1999) have further reported that the flux of Tau under anisotonic conditions could be a bi-directional process. Warskulat et al. (1997) also showed in H4IIE cells that hypertonic stress stimulates Na^+-dependent Tau uptake and leads to an increase in Tau transporter mRNA levels. As expected, hypotonic cell swelling, caused due to decrease in osmolarity in the presence or absence of Na^+, did not modulate any significant uptake of Tau from the external medium. Nonetheless, whatever may be means of uptake of Tau during hypertonic cell shrinkage either in the presence or absence of Na^+, significant increase in the concentration of Tau in the RBC might have played an important role as an osmolyte in cell volume regulation, thus causing only 10% decrease of water content in the isolated RBC during hypertonic exposure (Table 33).

Several cell types, including the kidney and brain cells have been shown to use Tau as an osmolyte (for reviews, see Chamberlin and Strange, 1989; Burg 1995; Huxtable, 1992), and in addition to its role as a compound to balance intracellular osmolarity, Tau may play a role as an antioxidant in conjugation reactions, in radioprotection, membrane utilization and in phospholipid interactions (Huxtable, 1992; Wettstein and Haüssinger, 2000). Hypertonic stress is known to cause more of oxidative stress in the hepatocyte of walking catfish under hypertonic stress (Saha and Goswami, 2004). Therefore, more uptake of Tau under hypertonic stress may have also some protective role against the oxidative stress in the erythroycyte of this fish.

In contrast to hypertonic condition, hypotonic exposure resulted to very prompt efflux of Tau from the isolated RBC of walking catfish, as evidenced by the fact that about 80% of the total Tau was released from the isolated RBC without pre-loaded with extra Tau (Figs. 50 & 51). This was found to be true both in the presence and absence

of Na^+, thus indicating that the Tau efflux from the isolated RBC of this fish is a Na^+-independent process. However, when the isolated RBC was exposed to hypertonic condition (both in the presence or absence of Na^+) after initial incubating with DIDS, an inhibitor for anion exchange transporter, the efflux of Tau was almost inhibited causing only about 20-25% efflux of the total Tau present in the RBC.

Role of Tau in cell volume regulation has been well documented in various marine teleosts and in marine elasmobranchs during exposure to dilute sea water (Thoroed and Fugelli, 1994, Goldstein and Musch, 1994; Goldstein and Brill, 1990; Fincham et al., 1987; Goldstein and Perlman, 1995). Various experimental evidences by them further suggested that the release of Tau during volume regulatory process in marine fish erythrocytes mostly takes place through a anion exchange transporter, the band 3 protein but not through the involvement of certain carrier proteins. However, no reports are available in any freshwater fish species on the role of Tau in cell volume regulation and the involvement of band 3 protein for Tau transport. The results of the present experiment certainly indicated that Tau is one of the important organic osmolytes playing a major role in cell volume regulation both under hyper- and hypotonic conditions. Further, the presence of band 3 protein in the membrane of walking catfish RBC is almost certain since the efflux of Tau was inhibited by the presence of DIDS, which is a known inhibitor for band 3 protein (Motais et al., 1997). The initial swelling of RBC, which took place during exposure to hypotonic medium, probably was taken care off by a volume regulatory release of Tau within 10 min since 80% efflux of Tau took place within this period. This observation also strongly suggests that probably quite a good number of band 3 proteins are expressed in the RBC membrane thus leading to a prompt volume regulatory release of Tau in contrast to the

situation in hagfish (*Myxine glutinosa*) and lamprey (*Petromyzon marinus*) (Brill et al., 1992). Role of Tau in cell volume regulation has also been well documented in the hepatocytes of the walking catfish (Goswami, 2002). Due to having this volume regulatory mechanism, the water content of the isolated RBC of walking catfish increased by only 13% during hypotonic exposure (-80 mOsmol/l) compared to the isotonic control (Table 33).

Further more, their exists a direct correlation with the amount of osmotic cell swelling and Tau release ($r = 0.99$, $P < 0.00002$) by the isolated RBC of walking catfish, since the percentage of efflux of Tau increased with the decreasing osmolarity of the incubation medium (Table 32; Fig. 52). However, it is not clear from this set of experiment about the mechanism of activation of Tau transporter thus leading to a more release of Tau under hypotonic condition from the isolated RBC. One of the reasons could be tyrosine phosphorylation of band 3 protein transporter by hypotonic cell swelling-induced activation of tyrosine kinase p72syk and p56lyn as suggested in skate (Hubert et al., 2000). Another mechanism could be volume-sensitive changes of Ca^{2+} influx/efflux, thus causing activation of Tau transporter as suggested in flounder erythrocytes (Thoroed and Fugelli, 1994). Hubert et al. (2000) also suggested that protein kinase C does not involve in volume-sensitive changes of Tau transport in the skate erythrocytes. However, the regulation of this transporter at the transcriptional level cannot be ruled out in the erythrocyte of walking catfish since increase of Tau transporter mRNA was reported in the HAIIE cells under hypertonic condition (Warskulat et al., 1997). Thus, it is necessary to study in detail to explain the real mechanism of stimulation of Tau uptake/efflux under hyper/hypotonic conditions in the erythrocyte of this walking catfish.

In conclusion, it appears that Tau, as an important organic osmolyte, plays a critical role in cell volume regulation in the erythrocyte of walking catfish which faces the problem of environmental osmolarity changes regularly in their natural habitat as seen in many marine fish species mainly due to the presence of a specific Tau transporter band 3 protein in the erythrocyte membrane.

CONCLUSION

Conclusion:

The Indian air-breathing walking catfish, *Clarias batrachus* possess various unique physiological and biochemical adaptations related to nitrogen metabolism. They possess various adaptations related to amino acid metabolism enabling them to survive under various environmental constraints. Further, in the present study it was seen that hypertonicity causes more accumulation of both non-essential and essential free amino acids (FAAs) in the hepatocytes of *C. batrachus*, thus suggesting that FAAs serve as very good osmolytes in cell volume regulation under osmotic stress. Under hypertonic condition, possibly there is an increase in synthesis of non-essential FAAs as evidenced by the induction of activity of some key enzymes related to amino acid synthesis, and also when supplemented with external ammonia into the perfused liver. Decrease of FAA levels in the hepatocytes under hypotonic stress is possibly caused by more efflux of amino acids and also more oxidation of amino acids. Hypotonic stress causes less synthesis of urea, glutamate and glutamine from ammonia, whereas hypertonic stress causes more synthesis of urea and glutamate and less synthesis of glutamine from ammonia in the perfused liver. Hypotonic stress causes more accumulation of ammonia, urea and glutamate from glutamine, whereas hypertonic stress causes less synthesis of ammonia, urea and glutamate from glutamine in the perfused liver. It appears that amino acids and their metabolism play very important roles in the liver of the walking catfish (*C. batrachus*) for osmotic balance, and thus maintaining the cell volume homeostasis, which is done either by retaining more of AAs as osmolytes under hypertonic stress or by removing out AAs from the cell under hypotonic stress through the involvement of certain carrier mediated transporter systems. Certain adjustments of amino acid metabolism as a whole under osmotic

stress also appears to play an important role in the maintenance of cell volume homeostasis as a sort of physiological adaptation in this walking catfish.

Hypertonicity in the external environment in situ results in more accumulation of both non-essential and essential FAAs in different tissues and in the plasma of *C. batrachus*, and possibly more synthesis of non-essential FAAs as evidenced by the induction of activity of certain related key enzymes in different tissues. Increased accumulation of FAAs, as very good osmolytes, probably helps in regulating the constant cell volume and also to avoid desiccation under hypertonic stress. Hypertonicity in the external environment in situ also causes increased synthesis, accumulation and excretion of urea by this fish by the induction of some key ornithine-urea cycle (OUC) enzymes as a sort of biochemical adaptation. Thus, urea, which is also known to be a good osmolyte, also plays a critical role in this fish for cell volume regulation and also to avoid desiccation under hypertonic stress in this walking catfish. The capacity of increased urea synthesis and accumulation during osmotic stress observed in *C. batrachus*, a fresh water air-breathing teleost, was uncommon among fresh water teleosts. It resembled more with some aquatic toads and elasmobranchs. The results suggest that freshwater air-breathing teleosts have better physiological adaptation to tolerate a wide range of environmental variations unlike that of other freshwater teleosts known. The freshwater air-breathing teleosts probably have retained the ureosmotic character to synthesize and retain urea inside the body for osmotic balance, in addition to the capacity of more synthesizing and accumulating various AAs within the body for osmotic balance.

Hypotonicity results in less or no uptake of taurine (Tau) from the external medium by the isolated RBC of *C. batrachus,* but causes more efflux of Tau by Na-

independent process through band 3 protein transporter as evidenced by the blockage of Tau efflux by DIDS. Hypertonicity causes more uptake of Tau through Na-dependent process, but no efflux of Tau by the isolated RBC of *C. batrachus.* Thus, Tau plays a very important role in cell volume regulation and also in the hydration status of RBC of this fish under osmotic stress. In conclusion, it appears that Tau, as an important organic osmolyte, plays a critical role in cell volume regulation in the erythrocyte of walking catfish, which faces the problem of environmental osmolarity changes regularly in their natural habitat in different seasons of the year, thus enabling them to survive in extreme habitat unlike that of other freshwater typical teleosts.

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1. Saha N., Kharbuli Z.Y., Bhattacharjee A., Goswami C. and Haussinger D. (2002). Effect of alkalinity (pH 10) on ureogenesis in the air-breathing walking catfish, *Clarias batrachus*. ***Comparative Biochemistry and Physiology*** **132A:** 353-364.

2. Saha N., Dutta S., and Bhattacharjee A., (2002). Role of amino acid metabolism in an air-breathing catfish, *Clarias batrachus* in response to exposure to a high concentration of exogenous ammonia. ***Comparative Biochemistry and Physiology*** **133B:** 235-250.

3. Saha, N., Datta, S., Kharbuli, Z.Y. and Bhattacharjee, A. (2005). Air-breathing walking catfish, *Clarias batrachus* upregulates the glutamine synthetase and carbamyl phosphate synthetase III to avoid ammonia toxicity during exposure to high external ammonia (Submitted).

11. Papers presented in Seminar/Symposia

1. Role of amino acid metabolism in an air-breathing catfish, *Clarias batrachus* in response to exposure to a high concentration of exogenous ammonia. Comparative Biochemistry and Physiology 133(B): 235-250. In: The National Academy of Sciences, India, Seventy Second Annual Session from October 25-27, 2002 held at North Eastern Hill University, Shillong.

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Effect of alkalinity (pH 10) on ureogenesis in the air-breathing walking catfish, *Clarias batrachus*

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Abstract

Exposure of fish to alkaline conditions inhibits the rate of ammonia excretion, leading to ammonia accumulation and toxicity. The purpose of this study was to determine the role of ureogenesis via the urea cycle, to avoid the accumulation of ammonia to a toxic level during chronic exposure to alkaline conditions, for the air-breathing walking catfish, *Clarias batrachus*, where a full complement of urea cycle enzyme activity has been documented. The walking catfish can survive in water with a pH up to 10. At a pH of 10 the ammonia excretion rate by the walking catfish decreased by approximately 75% within 6 h. Although there was a gradual improvement of ammonia excretion rate by the alkaline-exposed fish, the rate remained 50% lower, even after 7 days. This decrease of ammonia excretion was accompanied by a significant accumulation of ammonia in plasma and body tissues (except in the brain). Urea-N excretion for alkaline-exposed fish increased 2.5-fold within the first day, which was maintained until day 3 and was then followed by a slight decrease to maintain a 2-fold increase in the urea-N excretion rate, even after 7 days. There was also a higher accumulation of urea in plasma and other body tissues (liver, kidney, muscle and brain). The activity of glutamine synthetase and three enzymes operating in the urea cycle (carbamyl phosphate synthetase, argininosuccinate synthetase, argininosuccinate lyase) increased significantly in hepatic and extra-hepatic tissue, such as the kidney and muscle in *C. batrachus*, during exposure to alkaline water. A significant increase in plasma lactate concentration noticed during alkaline exposure possibly helped in the maintenance of the acid–base balance. It is apparent that the stimulation of ureogenesis via the induced urea cycle is one of the major physiological strategies adopted by the walking catfish (*C. batrachus*) during chronic exposure to alkaline water, to avoid the in vivo accumulation of ammonia to a toxic level in body tissues and for the maintenance of pH homeostasis.

Keywords: Alkaline water; Ureogenesis; Ammonia; Urea; Urea cycle; Air-breathing; Walking catfish; *Clarias batrachus*

1. Introduction

An alkaline environment (pH 8.5–10) causes severe physiological disturbances for most fish and changes in the pattern of nitrogen excretion in teleosts (for reviews, see Danulat, 1995; Wilkie and Wood, 1996). A high pH will initially inhibit diffusion of ammonia[1] across the gills, resulting in an increase in plasma ammonia concentration (Wright et al. 1993; Wilkie and Wood, 1996), as well as a disturbance in the internal acid–base and electrolyte balance (Heming and Blumhagen, 1988; Lin and Randall, 1990; Wilkie and Wood,

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[1] Throughout the remainder of the paper, 'ammonia' will be used to refer to total ammonia ($NH_3+NH_4^+$). When referring to a specific forms, the chemical formula will be used, NH_3 or NH_4^+ for un-ionized or ionized ammonia, respectively.

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1991, 1995; Yesaki and Iwama, 1992). The causes for a decrease in ammonia excretion rates in a high pH medium are well explained in a recent review by Wilkie and Wood (1996). Fishes living in highly alkaline waters have generally evolved adaptations to facilitate nitrogenous waste excretion. To date, three teleostean species that thrive in their natural alkaline habitats have been investigated, the Lake Magadi tilapia (*Oreochromis alcalicus grahami*) from Kenya, the Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*) from Pyramid Lake Nevada and the anadromous tarek (*Chalcalburnus tarichi*) endemic to Lake Van, Turkey. All three species have evolved strategies that allow them to circumvent problems associated with ammonia excretion in alkaline waters (for reviews, see Danulat, 1995; Wilkie and Wood, 1996). Perhaps the most significant 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). Lahontan cutthroat trout excrete approximately 25–35% of total nitrogenous wastes as urea-N, a slightly higher percentage than reported for 'typical' freshwater teleosts (Wright et al., 1993; McGeer et al., 1994); however, the rate of urea-N excretion doubled during acute exposure to alkaline water (pH 10) (Wilkie et al., 1993). It was suggested that in lahontan cutthroat trout, the urea production takes place mainly via the typical teleost pathway of uricolysis, due to the presence of significant level of uricolytic enzyme activity, but not via the urea cycle, since the activity of certain key enzymes in the urea cycle, such as carbamyl phosphate synthetase III (CPS III), are reported to be very low (Wilkie et al., 1993; Wilkie and Wood, 1994). Tarek (*C. tarichi*) excretes approximately 37% of its nitrogenous wastes as urea-N, but it too has no functional urea cycle (Danulat and Kempe, 1992). Thus, urea production in this fish is also probably via uricolysis (Wilkie and Wood, 1996).

Fish in aquaculture may also be accidentally exposed to high pH. For instance, calcium carbonate, added to pond water for improved fish production (Bandt, 1935) or high rates of photosynthesis caused by unusual phytoplankton blooms due to high nutrient input in fish ponds (Schreckenbach et al., 1975), can also elevate pH levels. There are reports of diurnal increases in water pH in eutrophic lakes that result from high rates of algal and macrophytic photosynthesis (Barica, 1974, 1990; Ayles et al., 1976; Wetzel, 1983). The air-breathing, walking catfish (*Clarias batrachus*) that are predominantly found in the Indian subcontinent usually inhabit stagnant, slow-flowing swampy water bodies or wet lands, which are often covered with macrovegetation like water hyacinth and are characterized by low dissolved oxygen levels, and higher bicarbonate and ammonia levels (for review, see Saha and Ratha, 1998). During summer, when the ponds dry up, they usually burrow inside the mud to avoid total dehydration. They usually live in water with a pH of 7–8.5, but also face the problem of high alkalinity (up to pH 8.5–9.5) in some seasons of the year due to the reasons mentioned above. It is now well-documented that the functional urea cycle, present in *C. batrachus*, plays an important role in facing the problem of ammonia toxicity under hyper-ammonia stress (Saha and Ratha, 1989, 1998; Saha and Das, 1999; Saha et al., 1999). The present investigation demonstrates the role of ureogenesis, via the functional urea cycle, in this air-breathing, walking catfish in avoiding the in situ ammonia build-up when living in alkaline water (pH 10) and in helping maintain the acid–base balance.

2. Materials and methods

2.1. Animals

Walking catfish, *Clarias batrachus*, weighing 85 ± 15 g, were purchased from commercial sources and acclimatized in the laboratory for approximately 1 month at a constant room temperature (28 ± 2 °C), with a 12:12 h light and dark photoperiod. Minced pork liver and rice bran (5% of body wt.) was given as food and the water was changed on alternate days. Sexes were unknown. Food was withdrawn 24 h prior to the experiment.

2.2. Research protocol

Initially, to test the tolerance limit of *C. batrachus* to alkaline water, three different sets of fish with a group of 10 were exposed separately in 10 l of bacteria-free filtered stream water, whose pH was adjusted to 9, 10 and 11, respectively, with the addition of NaOH. The water also contained 20 mM Na_2HPO_4 to maintain the pH of the water. On alternate days, the water media were replaced with a fresh medium. All 10 fishes exposed to

water at a pH 11 died within 12 h. However, at pH 9 and 10 no mortality of fish was observed during the period of 1 month. Further studies were conducted by exposing another set of fish for 7 days in alkaline water, with the pH adjusted to 10. Thirty-five fishes were weighed and placed individually in a plastic bucket containing 1 l of bacteria-free filtered stream water, whose pH was adjusted to 10 with the addition of NaOH. The water also contained 20 mM Na_2HPO_4 to maintain the pH of the water at 10 throughout the experiment. Another 35 *C. batrachus* were kept one fish per plastic bucket in 1 l of bacteria-free filtered stream water containing 20 mM Na_2HPO_4, with the pH adjusted to 7 with HCl. The medium was changed initially after every 3 h for the first 12 h, followed by a complete water change every 12 h, after collecting a water sample for determination of ammonia and urea-N excreted by individual fish. The pH of the water was continuously monitored during the experiment. Water samples collected at different time intervals were acidified with 1 M perchloric acid and stored at −20 °C. Every 24 h, five fish from each treatment were removed and killed by decapitation, after collecting blood from the caudal vein with a heparinized syringe. Liver, kidney, muscle and brain were dissected out, and tissue samples plunged into liquid nitrogen and stored at −60 °C. All enzyme assays and estimations were completed within 1 week of collecting the tissue. Blood collected from each fish was centrifuged at 10 000×*g* for 10 min and plasma was processed for the estimation of ammonia and urea level.

2.3. Assays

2.3.1. Ammonia and urea-N

Levels of ammonia and urea-N excreted by the fish, kept in alkaline water (pH 10) and in the water with a pH 7, were measured enzymatically (Kun and Kearney, 1974) after neutralizing the sample with 1 M NaOH, the details of which were described by Saha et al. (1995). Ammonia and urea levels in organ tissue and blood plasma were also measured by the same enzymatic methods described in Saha and Ratha (1989).

2.3.2. Lactate

In the deproteinized and neutralized plasma samples (Saha and Ratha, 1989), the lactate concentration was determined enzymatically using the method described in Bergmeyer (1974).

2.3.3. Plasma pH

The plasma pH was determined with an Orion pH meter fitted with a pH microelectrode.

2.4. Enzyme assay

A 10% homogenate (w/v) of different tissues were prepared in a homogenizing buffer containing 100 mM Tris–HCl buffer (pH 7.5), 50 mM KCl, 1 mM ethylenedinitro tetra acetic acid (EDTA) and 1 mM dithiothreitol (DTT), using a motor-driven Potter–Elvehjem glass homogenizer, with a Teflon pestle. The homogenate was treated with 0.5% Triton X-100 in 1:1 ratio for 30 min. The homogenate was then subjected to mild sonication for proper breakage of mitochondria and centrifuged at 10 000×*g* for 10 min. The supernatant was used for assaying the enzymes. All steps were carried out at 4 °C. The five enzymes of the urea cycle, carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) were assayed following the method described in Saha et al. (1995). However, for the assay of urea cycle-related CPS activity, 1 mM of uridine-5′-triphosphate (UTP) was also added to the reaction mixture, to inhibit the pyrimidine synthesis-related CPS II activity (Saha et al., 1997). 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). Hence, a part of ammonia (as NH_4Cl) taken as a nitrogen-donating substrate in the reaction mixture might be converted to glutamine in the presence of GS enzyme, thus serving as a nitrogen-donating substrate for CPS III. The reaction for all the enzymes was stopped by adding 0.5 ml of 10% perchloric acid per 1 ml of reaction mixture after a specific time of reaction, followed by centrifugation 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 spectrophotometrically (Beckman, DU 640) 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 is defined as the amount that catalyzes 1 μmol of product formed or substrate used per h at 30 °C. Glutamine synthetase (GS) was assayed by the γ-glutamyl transferase reaction as described by Webb and Brown (1976). The reaction mixture contained 60 mM glutamine, 15 mM hydroxylamine-HCl, 20 mM Na-arsenate, 0.4 mM ADP, 3 mM $MnCl_2$, 100 mM imidazole-HCl buffer (pH 6.8) and 200 μl of tissue extract in a final volume of 1 ml. After 30 min of incubation at 30 °C, the reaction was stopped by the addition of 1 ml of a solution containing 0.2 M trichloroacetic acid, 0.67 M HCl and 0.37 M of $FeCl_3$. The solution was centrifuged at $14\,000 \times g$ for 10 min and the γ-glutamyl hydroxamate formed during the reaction was measured in the supernatant at 500 nm. One unit of enzyme activity for GS is expressed as that amount which catalyzes the formation of 1 μmol of γ-glutamyl hydroxamate per hour at 30 °C.

2.5. Chemicals

Enzymes, co-enzymes, substrates, glycylglycine, Na-glycinate, DTT and UTP were purchased either from Sigma Chemical Co. (St. Louis, USA) or Boehringer Mannheim (Mannheim, Germany). Other chemicals used were of analytical grade obtained from local sources. Deionised double-glass-distilled water was used in all preparations.

2.6. Statistical analysis

Data collected from different replicates were statistically analyzed and presented as mean ± S.E.M. (n) where n equals the number of animals in the sample. Comparisons of the paired mean values between the experimental and the respective controls were made using Student's t-test, and one-way ANOVA followed by Student–Neuman–Keul's multiple range test were used to compare the initial control with the respective controls in the case of ammonia and urea-N excretion (Croxton et al., 1982). Differences with $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Survival in alkaline water

The walking catfish exposed to alkaline water of pH 9 showed minor irritational symptoms, followed by gradual calming down within a few hours. However, fish exposed to pH 10 and 11 showed increased irritational symptoms. Any physical movement of water in the experimental bucket resulted in violent escape attempts. The hyper-excitability calmed down within 12 h at pH 10, but at pH 11 all the fishes died within 12 h of exposure. At pH 10, only one fish died out of 35 after 24 h of exposure; a mortality rate of 3%.

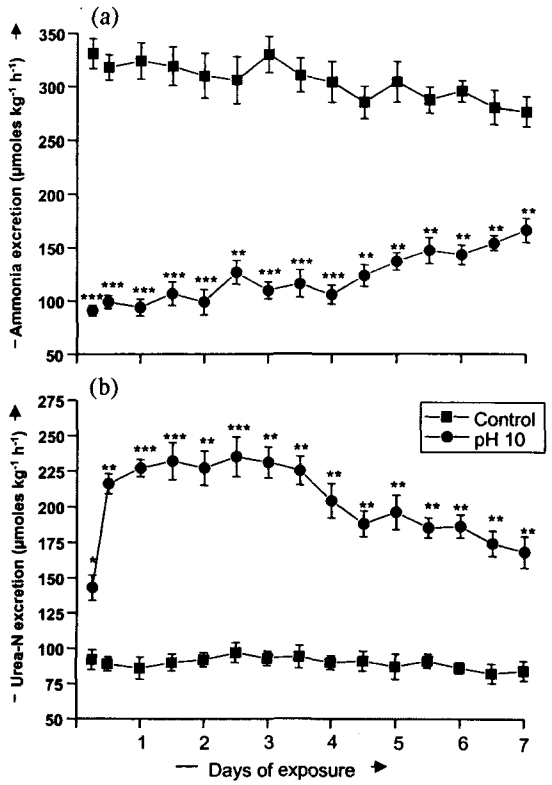

Fig. 1. Effect of alkalinity (pH 10) on excretion rate (μmol kg^{-1} h^{-1}) of ammonia (a) and urea-N (b) by *C. batrachus*. Values are plotted as mean ± S.E.M. ($n = 5$). *, **, ***: P values significant at < 0.05, < 0.01 and < 0.001, respectively. The experimental values were compared with respective controls (Student's t-test).

3.2. Excretion of ammonia and urea-N

The pattern of excretion of ammonia and urea-N by the walking catfish during exposure to alkaline water (pH 10) are shown in Fig. 1. The ammonia excretion at control pH 7 averaged at 305 μmol kg^{-1} h^{-1} during the period of 7 days. A slight decrease of ammonia excretion was

observed in the control fish over the period of 7 days, which was non-significant (one-way ANOVA). High pH (pH 10) exposure led to a 73% decrease in ammonia excretion rate (from 330±14 to 90±5 μmol kg^{-1} h^{-1}) after 6 h of alkaline exposure (Fig. 1a). Although there was a gradual recovery of ammonia excretion towards the control level, the rate remained almost reduced to half as against the control value even on day 7. Urea-N excretion rates at a control pH of 7 after 6 h averaged at 89.5 μmol kg^{1} h^{-1}, which increased approximately 2.4-fold (from 90±8 to 214±9 μmol kg^{-1} h^{-1}) after the first day of high pH exposure and this high rate of excretion was maintained until day 3, followed by a gradual decrease at later stages of exposure (Fig. 1b). However, the urea-N excretion rate was maintained approximately 2-fold higher (164±11 μmol kg^{-1} h^{-1}) as against the control level (83±7 μmol kg^{-1} h^{-1}) even after the seventh day of exposure. The ratio of ammonia/urea-N excretion decreased initially from 3.5 to approximately 0.54 between 1 and 3 days of exposure, and increased to approximately 0.97 by day 7.

3.3. Changes in tissue and plasma levels of ammonia and urea in the alkaline-exposed fish

As shown in Fig. 2, a significant increase in the level of ammonia was observed in liver, kidney, muscle and plasma, but not the brain, of walking catfish within the first day of exposure to alkaline water. In the liver, the ammonia level increased from 8.6±0.57 to 13.2±1.4 μmol g^{-1} wet wt. (53%) by day 3, in the kidney from 8.1±0.46 to 12.1±1.2 μmol g^{-1} wet wt. (49%) by day 3, in the muscle from 3.4±0.21 to 4.7±0.41 μmol g^{-1} wet wt. (38%) by day 2 and in the plasma, it increased maximally from 0.40±0.05 to 2.02±0.34 μmol ml^{-1} (405%) by day 3 of exposure to alkaline water. Although the ammonia level in liver, kidney, muscle and plasma decreased gradually after reaching a peak between 2 and 3 days, it remained significantly higher than for control fish, even after seventh day of alkaline exposure.

There was a significant difference in the concentration of urea in the liver, kidney, muscle and plasma, but not in the brain, compared with the controls for fishes exposed to a pH of 10 (Fig. 3). In the liver, the urea level increased from 5.6±0.36 to 8.4±0.38 μmol g^{-1} wet wt. (50%) by day 2,

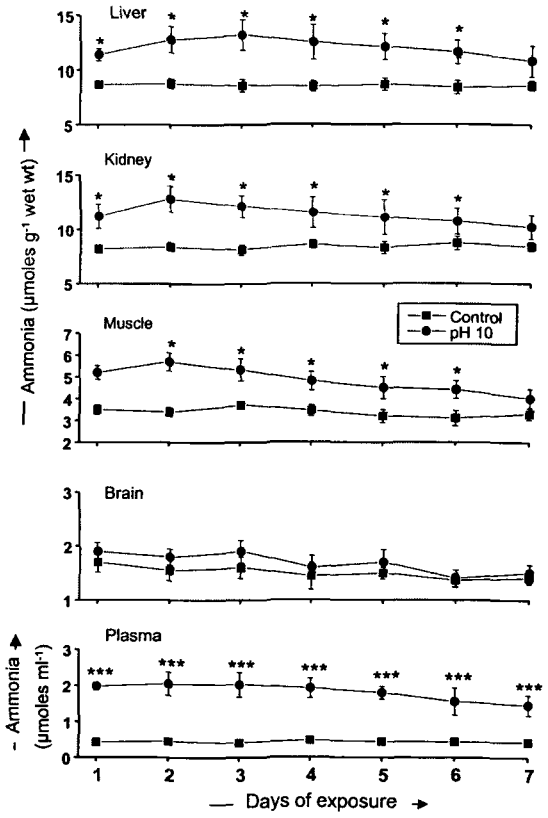

Fig. 2. Effect of alkalinity (pH 10) on ammonia levels in different tissues (μmol g^{-1} wet wt.) and in the plasma (μmoles ml^{-1}) of *C. batrachus*. Values are plotted as mean±S.E.M. (n=5).*, **: P values significant at <0.05 and <0.001, respectively. The experimental values were compared with respective controls (Student's t-test).

in the kidney from 4.6±0.38 to 7.1±0.81 μmol g^{-1} wet wt. (54%) by day 3, in the muscle from 1.5±0.18 to 2.5±0.21 (66%) by day 3 and in the plasma, it increased maximally from 0.35±0.04 to 0.91±0.05 μmol ml^{-1} (160%) by day 3 of exposure to alkaline water. Higher level of urea in the body tissues (except brain) of walking catfish was maintained until seventh day of exposure to alkaline water.

3.4. Enzyme activities in the alkaline-exposed fish

Levels of GS and all the urea cycle enzymes are compared for fish in a pH of 10 and 7 after 1, 3 and 7 in Table 1. The GS activity increased significantly in the liver, kidney and muscle of alkaline-exposed fish after the first day of exposure

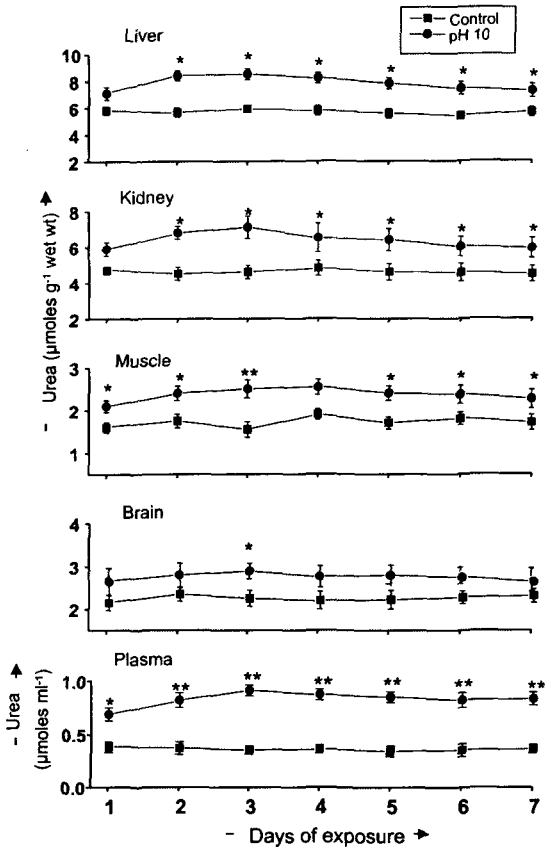

Fig. 3. Effect of alkalinity (pH 10) on urea levels in different tissues (μmol g^{-1} wet wt.) and in the plasma (μmol ml^{-1}) of *C. batrachus*. Values are plotted as mean ± S.E.M. ($n=5$).*, **: *P* values significant at <0.05 and <0.01, respectively. The experimental values were compared with respective controls (Student's *t*-test).

to high alkaline water. Out of the five urea cycle enzymes, the activity of CPS, ASS and ASL increased significantly after 1 or 3 days of exposure in the liver, kidney and muscle. The CPS activity increased maximally in the kidney (58%) on day 7, followed by the liver (52%) and muscle (37%) on day 3 of exposure. The ASS activity increased maximally in the kidney (35%) by day 3, followed by the liver (29%) after 1 day and in the muscle (28%) after 7 days of exposure. The ASL activity increased maximally in the kidney (48%) and then in the liver (33%) after 3 days of exposure. The ASL activity in the muscle could not be detected, both in the control and alkaline-exposed fish, by the assay method used in the present study. The activities of OTC and ARG did not show any significant change in either of the tissues of the alkaline-exposed fish studied.

3.5. Total ammonia-N production by the alkaline-exposed fish

Total ammonia-N production by the alkaline-exposed walking catfish was calculated by adding the total amount of ammonia and urea-N excreted by the fish in 24 h, with the extra accumulation of ammonia and urea-N, which had taken place mainly in the muscle (which constitutes approximately 65% of the total body mass), and in the liver (2%), kidney (1%) and plasma (constituting approximately 2.5% of the total body mass) (Fig. 4). There was only 23% decrease in ammonia-N production on day 1 by the alkaline-exposed fish, which restricted to only 10–15% decrease at the later part of experiment.

3.6. Changes of plasma lactate level

The mean plasma lactate concentration in the control fish exposed to water with a pH of 7 was found to be 1.2 ± 0.03 ($n=35$) mmol l^{-1} (Fig. 5a). The plasma lactate concentration increased by 110% in fish exposed to pH 10 water after day 1, a value that persisted until the third day of exposure. Plasma lactate concentration decreased slightly thereafter but was still significantly higher (60%) compared to the control fish after 7 days (Fig. 5a).

3.7. Changes of plasma pH

The plasma pH in the control fish exposed to a water of pH 7 was unchanged over 7 days, with a pH of 7.5 (Fig. 5b). The plasma pH of fish exposed to water with a pH of 10 did not change significantly compared to the control fish (Fig. 5b).

4. Discussion

The walking catfish (*C. batrachus*) survives in alkaline water up to pH 10 for several days, although the experimental fish made violent attempts to escape on the first day. Fish kept at alkaline water of pH 11 died within 12 h. Possible causes of death in alkaline water in non-specialized teleosts have been proposed by several workers, such as (i) increase in plasma ammonia level

Table 1
Levels of GS and the urea cycle enzymes (units g^{-1} wet wt.) in various tissues of *C. batrachus* while exposed to alkaline water (pH 10)

Enzymes		Tissues								
		Liver			Kidney			Muscle		
		Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
GS	Control	85.4±4.8	87.6±5.9	83.8±6.2	41.7±5.1	39.6±4.8	42.4±5.6	8.25±0.74	8.76±0.67	8.4±0.89
	pH 10	127.5±9.1[a]	138.2±11.4[a]	125.7±12.6[a]	60.2±6.2[a]	62.4±7.8	57.7±7.1[a]	11.61±0.51[a]	12.62±0.62[a]	10.8±0.48[a]
CPS	Control	5.36±0.74	5.47±0.58	5.18±0.37	3.68±0.45	3.76±0.38	3.50±0.48	0.72±0.12	0.82±0.15	0.77±0.16
	pH 10	7.66±0.62[a]	8.22±0.51[a]	7.89±0.55[a]	5.07±0.34[a]	5.71±0.62[a]	5.54±0.45[a]	0.88±0.11	1.12±0.25[a]	0.98±0.18[a]
OTC	Control	151.3±8.9	157.5±6.7	154.3±7.2	61.7±3.8	62.3±2.6	63.5±5.4	10.2±0.51	11.6±0.62	10.8±0.78
	pH 10	157.5±9.9	161.2±8.7	157.2±9.5	59.2±4.2	66.4±6.1	61.2±5.2	9.6±0.48	10.7±0.58	12.1±0.71
ASS	Control	47.9±4.6	49.1±4.2	48.2±5.7	24.7±2.6	26.4±3.5	22.3±3.7	8.92±0.67	8.65±0.88	9.12±0.78
	pH 10	61.7±6.9[a]	62.4±5.8[a]	58.3±5.6	30.6±2.8	33.8±3.6[a]	30.2±3.9[a]	10.6±0.41	10.89±0.45	11.7±0.51[a]
ASL	Control	38.7±2.8	40.4±5.3	39.5±4.8	32.4±3.8	33.3±3.5	35.2±4.4	BLD	BLD	BLD
	pH 10	49.2±4.1	53.6±4.4[a]	47.7±4.5	46.5±4.2[a]	49.3±5.6[a]	45.5±4.7[a]	BLD	BLD	BLD
ARG	Control	1038±88	1066±97	968±49	995±34	1059±68	1034±81	130±12	135±16	128±17
	pH 10	1089±87	1162±91	1021±78	1005±61	987±44	1088±58	137±15	132±16	118±14

[a] *P* value significant at <0.05 level. Experimental values were compared with respective controls (Student's *t*-test).

Values are expressed as mean±S.E.M. ($n=5$). *Abbreviations*: BLD, below the level of detection; GS, glutamine synthetase; CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase. One unit of enzyme activity is defined as the amount of enzyme that catalyzes 1 μmol of product formed or substrate used h^{-1} at 30 °C.

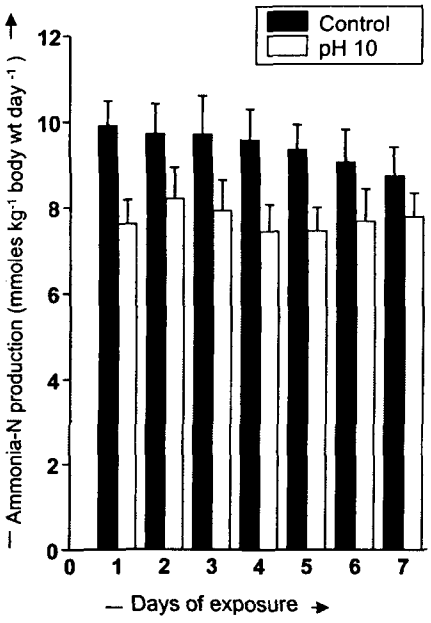

Fig. 4. Effect of alkalinity on ammonia-N production rate (mmol kg^{-1} day^{-1}) by *C. batrachus*. Values are plotted as mean ± S.E.M. ($n=5$). The production rate of ammonia-N was calculated from the total excretion of ammonia and urea-N, and also from the extra accumulation of ammonia and urea in different major tissues and plasma.

resulting from sudden decrease in ammonia excretion rate, (ii) acute disturbances in acid–base balance and ionoregulation, and/or (iii) acute increase of plasma pH (for review, see Danulat, 1995). Plasma ammonia concentration and pH was measured in the walking catfish exposed to pH 11 water just before death. The plasma ammonia level increased to 3.2 ± 0.24 ($n=8$) μmol ml^{-1} (approximately 8-fold higher than the control) and the plasma pH increased to 9.3 ± 0.25 ($n=8$) (approximately 1.8 pH units higher than the control). Increase in ammonia concentration may not be major cause of death of this fish at pH 11, because the walking catfish is reported to accumulate up to approximately 4 μmoles ml^{-1} of plasma ammonia under hyper-ammonia stress without having any apparent deleterious affects (Saha and Ratha, 1998; Saha and Das, unpublished data). Restriction of oxygen uptake due to pathological changes at the gill lamellae was suggested as a cause of death in eight freshwater teleosts exposed to varying alkaline pH water by Bandt (1935, 1936). However, this is unlikely to be the cause of death for the walking catfish, since they are predominantly air-breathers, with a gas exchange occurring through their accessory respiratory organs (for review, see Saha and Ratha, 1998). The acute increase in plasma pH, possibly due to disturbances of acid–base balance, could be the primary cause of death of these fish at pH 11 water, suggesting that alkalosis could be more critical factor than the hyper-ammonia stress in fish.

The cause of immediate decrease in ammonia excretion rate by this fish at a high pH water was possibly due to decrease in blood-gill water NH_3 gradient and/or inhibiting Na^+/NH_4^+ exchange processes, as suggested in other teleosts adapted in alkaline water (Wilkie and Wood, 1991; Wilkie et al., 1993, 1996; Wilson et al., 1998). Decrease in ammonia excretion was accompanied with 400% (5-fold) increase of plasma ammonia concentration along with a 30–50% increase in the liver, kidney and muscle, similar to that reported for rainbow trout (*Oncorhynchus mykiss*) adapted to alkaline water (Wilkie and Wood, 1991; Wilkie et al., 1996). Extra loading of ammonia, primarily in the muscle and also in other tissues at high pH exposure, probably occurred as a result of elevated NH_4^+ electrochemical and NH_3 partial pressure

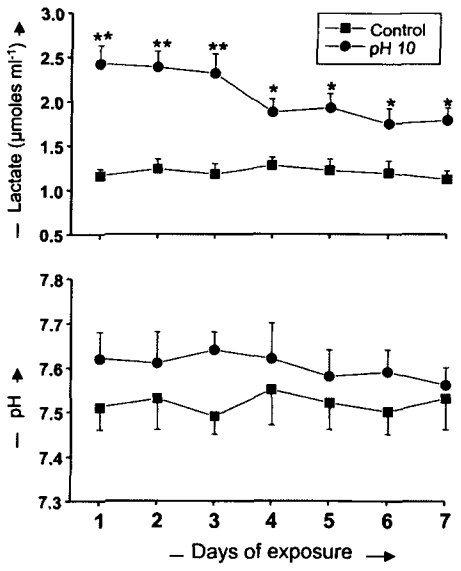

Fig. 5. Effect of alkalinity (pH 10) on plasma lactate concentration (μmol ml^{-1}) and plasma pH levels of *C. batrachus*. Values are plotted as mean ± S.E.M. ($n=5$).*, **: P values significant at <0.05 and <0.01, respectively. The experimental values were compared with respective controls (Student's t-test).

gradients from the plasma to different tissues, as suggested in rainbow trout during high pH exposure (Wilkie and Wood, 1995). In rainbow trout, the ammonia excretion rate recovered almost to pre-exposure level within 3 days of high pH exposure and was suggested to be due to an increase in the ammonia concentration of blood, leading to the re-establishment of favorable blood-gill water NH_3 gradients (Wilkie and Wood, 1991, 1994; Wilkie et al., 1996; Yesaki and Iwama, 1992), which was not true in the case of walking catfish, except for a partial recovery of ammonia excretion rate (Fig. 1). There was an approximately 2.5-fold increase in urea-N excretion rate by the walking catfish within the first day of chronic high pH exposure and this high rate of urea-N excretion (approx. 2-fold) was maintained until the seventh day of exposure. Thus, approximately 70% of the total nitrogenous waste (in the form of ammonia and urea-N) was initially excreted as urea-N, which later decreased to 50% on the seventh day of alkaline exposure, thus maintaining the ratio of ammonia/urea-N excretion as 1:1. This is dissimilar to the excretion of nitrogen in salmonids, where a transient increase in urea-N excretion rate was observed in response to chronic high pH exposure (Wilkie et al., 1993, 1996). This air-breathing walking catfish is a potential ureogenic species, due to having relatively high levels of activity for all the five urea cycle enzymes in hepatic and also in extra-hepatic tissues (Saha and Ratha, 1989; Saha et al., 1999). Furthermore, the partial change for nitrogenous waste excretion from ammonia to urea-N was accompanied with an increase in activity of GS, the critical enzyme related to urea synthesis via the urea cycle in fish (Campbell and Anderson, 1991), and other key enzymes of the urea cycle such as CPS, ASS and ASL both in hepatic and extra-hepatic tissues in the high pH exposed fish (Table 1). Therefore, this catfish might have adapted by chronically elevating the rate of urea-N excretion synthesized via the induced urea cycle, for the long-term maintenance of nitrogenous waste excretion during high pH exposure. Furthermore, the induction of ureogenesis via the induced urea cycle during alkaline exposure might have helped a better maintenance of acid–base balance.

Induction of ureogenesis under hyper-ammonia stress accompanied by an increase in urea-N excretion and higher accumulation of urea in vivo have already been reported in this walking catfish (Saha and Das, 1999; Saha and Das, unpublished data), and in another air-breathing singhi catfish (Saha and Ratha, 1990, 1994; Saha et al., 1995), suggesting that the higher accumulation of ammonia in vivo under hyper-ammonia stress probably served as a potential modulator in this induction process. In addition to the increase in urea-N excretion, a significant increase in the concentration of urea in plasma and other tissues was also observed in the high pH exposed fish (Fig. 3). Therefore, a hyper-ammonia stress-like situation was established in the fish exposed to high pH water, due to the higher accumulation of ammonia-stimulating ureogenesis. The transient increase of urea-N excretion of approximately 2-fold, demonstrated in lahontan cutthroat trout (Wilkie et al., 1993) and in the rainbow trout (Wilkie and Wood, 1991) due to chronic exposure to high pH water, was presumably through enhanced rates of uricolysis, but did not persist beyond 2 or 3 days, possibly because of lack of a functional urea cycle in both the cases. Wright et al. (1995) reported that embryonic rainbow trout larvae increased urea-N excretion by 6-fold, following acute (4-h) exposure to pH 9.5, which was possible probably because of the expression of a functional urea cycle at the larval stages of rainbow trout (Wright et al., 1995; Korte et al., 1997). In the Lake Magadi tilapia (*O. alcalicus grahami*) also, exclusive excretion of urea into its alkaline (pH 10) surroundings was possible due to the presence of a functional urea cycle, both in the hepatic and extra-hepatic tissues (Randall et al., 1989; Wood et al., 1989; Lindley et al., 1999). The possible mechanism(s) of stimulation of ureogenesis during alkaline exposure could be by changes in cellular events, such as enzyme phosphorylation, allosteric effects or changes in the concentration of low molecular weight effectors like *N*-acetyl glutamate (NAG) (a positive allosteric modulator of CPS III and I). Increases in the concentration of NAG are known to enhance flux through the CPS III enzyme in the gulf toadfish during confinement stress (Julsrud et al., 1998). Release of certain stress-related hormones, such as cortisol, could be another means of stimulation of ureogenesis under alkalinity stress in this fish as shown by Hopkins et al. (1995) in the gulf toadfish (*Opsanus beta*). As such, high accumulation of ammonia (substrate) in vivo, observed in different tissues, including the plasma in the alkaline-exposed fish, could be another means of stimulation of ureoge-

nesis, suggested in this fish species and in singhi catfish (Saha and Ratha, 1990, 1994; Saha et al., 1995; Saha and Das, 1999). A detailed investigation on these aspects would possibly clarify the mechanism(s) of stimulation of ureogenesis in this walking catfish under various environmental constraints faced regularly by them.

Another possible strategy to avoid the accumulation of ammonia to toxic levels in salmonids, while living in alkaline environments, was by decreasing the rate of ammonia production (Wilkie et al., 1996; Wilson et al., 1998). However, in the case of walking catfish, there was only 23% decrease in ammonia production rate (calculated from the total efflux of ammonia and urea-N, and extra accumulation of ammonia and urea in different tissues) after the first day of high pH exposure, which was restricted to only 10–15% after 7 days. Furthermore, some of the accumulated ammonia might have also been converted to some non-essential free amino acids (FAAs) in alkaline-exposed fish, which was not measured in our present study. The possible conversion of accumulated ammonia to non-essential FAAs in the perfused liver of walking catfish under hyperammonia stress have been reported (Saha et al., 2000). Therefore, the decrease in ammonia production rate to avoid its toxicity is possibly not adopted by *C. batrachus* during high pH exposure.

Metabolic acidosis associated with the production and accumulation of lactate has been reported in salmonids as one of the major processes for acid–base balance during exposure to alkaline water (Wilkie and Wood, 1991, 1995; Wilkie et al., 1993, 1996). In the walking catfish, the plasma lactate concentration also increased to approximately 130% compared to the control within first day of alkaline exposure, which was maintained at least for 3 days, followed by a partial decrease at later stages of exposure (Fig. 5a). The role of the urea cycle in pH homeostasis has been well-documented in mammalian systems, since equimolar amounts of HCO_3^- and NH_4^+ are disposed of via urea synthesis (Häussinger et al., 1984; Häussinger and Gerok, 1985). Atkinson (1992) also emphasized the importance of the presence of the urea cycle in the alkaline Lake Magadi tilapia for acid–base regulation. Therefore, the induction of ureogenesis via the induced urea cycle, in addition to the increased production and accumulation of lactate, might have played some role in maintaining the acid–base balance during chronic exposure to alkaline water (Fig. 5b). However, this would need to be confirmed by further investigation of the urea cycle in pH homeostasis in this fish.

In conclusion, it appears that in the air-breathing walking catfish, unlike that of salmonids, ureogenesis is stimulated by the induced urea cycle, as one of the major physiological strategies during chronic exposure to alkaline water, to avoid the in vivo accumulation of ammonia to a toxic level. These adaptive physiological strategies probably do not require a decrease in the production of amino acid catabolism as a major energy source.

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Role of amino acid metabolism in an air-breathing catfish, *Clarias batrachus* in response to exposure to a high concentration of exogenous ammonia

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Abstract

The air-breathing ureogenic walking catfish (*Clarias batrachus*) faces various environmental constraints throughout the year leading to the problem of accumulation of toxic ammonia. In the present study, the possible role of conversion of accumulated ammonia to various non-essential free amino acids (FAAs) was tested in this fish under hyper-ammonia stress caused by exposing the fish at 25 mM NH_4Cl for 7 days. Significant accumulation of ammonia of approximately two- to threefold was observed in different tissues (except in the brain), which was accompanied with the significant accumulation of non-essential FAAs in the NH_4Cl-exposed fish. There was approximately two- to threefold increase of non-essential FAAs in different tissues and in the plasma of the NH_4Cl-exposed fish compared to the control fish after 7 days of exposure, which was mainly attributable to the increase of Asp, Ala, Gly, Glu, Gln and taurine (Tau) concentrations in general, with certain tissue-specific variations. This was also accompanied with significant increase of activity of certain amino acid metabolism-related enzymes such as the glutamine synthetase (approx. two- to threefold), glutamate dehydrogenase (ammonia utilizing direction) (approx. twofold), aspartate and alanine aminotransaminases (approx. twofold) mainly in the liver, kidney and muscle of the NH_4Cl-exposed fish. Thus, it appears that the walking catfish has the capacity of active conversion of accumulated ammonia to non-essential FAAs under condition of high concentrations of external ammonia. However, the increase of urea excretion rate due to active conversion of ammonia to urea via the induced urea cycle appears to be quantitatively much more important pathway than the increase of tissue levels of FAAs in dealing with a severe ammonia load.

Keywords: Ammonia; Urea; Amino acid metabolism; Free amino acids; Hyper-ammonia stress; Ureogenesis; Air-breathing catfish; *Clarias batrachus*

1. Introduction

The vast majority of teleost fishes are ammoniotelic excreting ammonia as the major nitrogenous end product in response to their aquatic habitat (for reviews, see Randall and Wright, 1987; Campbell, 1991; Wood, 1993; Saha and Ratha, 1998). However, under certain circumstances such as high ambient ammonia or aerial exposure, ammonia excretion is inhibited, and toxic ammonia becomes concentrated in blood and body tissues. Fishes are generally known to tolerate relatively higher accumulation of ammonia than mammals (for reviews, see Saha and Ratha, 1998; Wood, 1993). It is, therefore, interesting to study the different mech-

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anism(s) of how fish manage accumulated ammonia especially those teleosts that are regularly faced with ammonia loading situations as part of their life cycle.

More recently, the expression of high urea cycle enzymes with an accompanying active urea cycle has been reported in several teleost species as an adaptation to unique environmental circumstances. Examples include the marine toadfishes, *Opsanus beta* and *Opsanus tau* (Read, 1971; Mommsen and Walsh, 1989), the alkaline lake-adapted tilapia, *Alcolapia grahami* (Randall et al., 1989), some Indian freshwater air-breathing fishes (Saha and Ratha, 1987, 1989; Saha et al., 1999), and gobiid fish, *Mugilogobius abei* (Iwata et al., 2000). These fishes may excrete significant amounts of urea in response to adverse environmental conditions such as confinement (stress), severely alkaline water, and ammonia loading during exposure to semidry conditions and higher ambient ammonia (Randall et al., 1989; Saha and Ratha, 1990, 1994, 1998; Walsh et al., 1990, 1994; Ratha et al., 1995; Walsh and Milligan, 1995; Saha and Das, 1999; Saha et al., 2001, 2002).

The freshwater amphibious air-breathing walking catfish (*Clarias batrachus*) that are found predominantly in the Indian subcontinent, spend a substantial part of their lives on mudflats in response to habitat drying and are observed to migrate terrestrially during wetter periods (Liem, 1987). This facultative air-breather usually inhabits stagnant, slow-flowing swampy water bodies or wet lands, which are often covered with macrovegetation such as water hyacinth, and these waters are also characterised by low dissolved oxygen, and high bicarbonate and ammonia levels (for review, see Saha and Ratha, 1998). During summer, when the swamps dry up, they usually burrow into the mud to avoid total dehydration. In addition to the presence of a functional urea cycle (Saha and Ratha, 1989, 1998; Saha et al., 1999), this air-breathing catfish has the potential to switch from ammoniotelism to ureotelism under hyper-ammonia stress (Saha and Das, 1999), and also while exposed to air or living inside the mud (Saha and Das, unpublished data). Another unique characteristic reported in this catfish is extreme tolerance to a high concentration of ambient ammonia, surviving exposure to 75 mM NH_4Cl for several weeks. This was suggested mainly due to the presence of a functional ureogenesis via the urea cycle (for review, see Saha and Ratha, 1998). Furthermore, there could be other possible mechanism(s) present in this walking catfish to tackle the problem of ammonia toxicity such as the conversion of accumulated ammonia to various non-essential free amino acids (FAAs) under hyper-ammonia stress as previously shown in the non-ureogenic ammoniotelic mudskippers, *Periophthalmus cantonensis* (Iwata et al., 1981; Iwata, 1988), *Periophthalmus schlosseri* (Peng et al., 1998), and in the carp, *Cyprinus carpio* (Dabrowska and Wlasow, 1986). Ip et al. (2001) in a recent review, however, emphasised the importance of a decrease or suppression of proteolysis and/or amino acid catabolism by some fishes during aerial exposure and ammonia loading.

In the present study, we report the effects of higher ambient ammonia on FAA synthesis and accumulation in various tissues along with the changes of activities of some key enzymes related to amino acid metabolism such as glutamine synthetase (GS), glutamate dehydrogenase (GDH, both reductive amination and oxidative deamination), aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) in the walking catfish (*C. batrachus*) while exposed to 25 mM NH_4Cl solution for 7 days. Although the walking catfish can survive at 75 mM NH_4Cl, we decided to expose the fish to 25 mM NH_4Cl, the concentration they may face in their natural habitat in some seasons of the year (Saha and Ratha, 1998).

2. Materials and methods

2.1. Animals

Walking catfish, *Clarias batrachus*, weighing 85 ± 15 g body wt., were purchased from commercial sources and acclimatised in the laboratory approximately for 1 month at a constant room temperature (28 ± 2 °C) with a 12:12-h light and dark photoperiod before using for experiments. No sex differentiation of the fish was done while performing these studies. Minced pork liver and rice bran (5% of the body wt.) was given as food, and the water, collected from a nearby natural stream, was changed on alternate days. Food was withdrawn 24 h prior to the experiment.

2.2. Experimental protocol

Nine fishes of similar sizes were weighed and placed individually in plastic buckets containing 2

l of 25 mM NH_4Cl solution prepared in bacteria-free filtered stream water (pH 6.95 ± 0.11). Fishes were treated in NH_4Cl solution for 7 days. Another nine *C. batrachus* were kept individually in plastic buckets containing 2 l of bacteria-free filtered stream water (pH 7.04 ± 0.10), which served as controls. Both NH_4Cl solution and the water from each bucket was replaced with a fresh medium everyday at a fixed time after collection of few media from each bucket for the measurement of ammonia and urea-N concentrations. On day 1, 3 and 7, three fishes from each treatment were removed, killed immediately by decapitation after collecting blood from the caudal vein with a heparinised syringe. Liver, kidney, muscle and brain were dissected out and tissue samples were plunged into liquid nitrogen before storing at −60 °C for analysis of free amino acids (FAAs), ammonia and urea, and also for assaying the enzymic activities. All enzyme assays and analysis were completed within 2 weeks of collecting the tissue. Blood collected from each fish was centrifuged at $10\,000 \times g$ for 10 min, and the plasma was processed for estimation of ammonia and urea, and for analysis of FAAs as described by Saha and Ratha (1989).

2.3. Extraction procedure

For the assay of enzymes, a 10% homogenate (w v^{-1}) of different tissues were prepared in a homogenizing buffer containing 50 mM Tris–HCl (pH 7.4), 0.3 M sucrose, 1 mM EDTA and phenyl methyl sulfonyl fluride (10 mg ml^{-1}). The homogenate was treated with 0.5% Triton X-100 for 30 min, followed by mild sonication to facilitate proper breakage of mitochondria. The homogenate was then centrifuged at $10\,000 \times g$ for 10 min and the resultant supernatant was used for enzyme assay. All steps for the preparation of tissue extracts were carried out at 4 °C.

For the analysis of FAAs, a 10% homogenate (w v^{-1}) of different tissues was prepared in HPLC grade water (Saha et al., 2000). Protein was immediately precipitated from the homogenate by adding 2 M perchloric acid (PCA) in 1:1 ratio, followed by centrifugation at $10\,000 \times g$ for 10 min. The plasma was also treated with 2 M PCA in a 1:1 ratio to precipitate out the protein, and further processed as above. All these steps were performed at 0 °C. The pH of the resultant supernatant was adjusted to 2.2 by adding a known volume of 0.4 N Li-hydroxide. The supernatant was passed through a Millipore micro filter (0.45 μM pore size) before analysis.

2.4. Enzyme assays

Glutamate dehydrogenase (GDH, both reductive amination and oxidative deamination) activity was assayed following the method of Olson and Anfinsen (1952) with modifications of substrate (optimal) concentrations (Saha et al., 2000). The alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) activities were assayed following the method of Foster and Moon (1986) with modifications in the substrate (optimal) concentrations (Saha et al., 2000). All these enzymes were assayed at 30 °C with a Beckman UV-visible spectrophotometer (Model DU 640) at 340 nm (Em $M_{340} = 6.22$). Enzyme activities were expressed as units g^{-1} wet wt. of tissue and corrected for any non-specific activity in the absence of substrate. One unit of enzyme activity was expressed as that amount which oxidised 1 μmol of NADH or reduced 1 μmol of NAD per hour at 30 °C.

Glutamine synthetase (GS) was assayed by the γ-glutamyl transferase reaction as described by Webb and Brown (1976) the details of which were mentioned earlier by Saha et al. (2000). One unit of GS activity was expressed as that amount which catalyzed the formation of 1 μmol of γ-glutamyl hydroxamate per hour at 30 °C.

2.5. Analysis of ammonia, urea-N and free amino acids (FAAs)

Levels of ammonia and urea-N excreted or taken up by the fish, kept either in water or in 25 mM NH_4Cl, were measured enzymatically (Kun and Kearney, 1974). Ammonia and urea levels in organ tissue and blood plasma were also measured by the same enzymatic methods described in Saha and Ratha (1989).

The total FAA concentration in different tissues and in the plasma was analyzed with a Shimadzu HPLC (Model LC 4A) with a post-column derivatisation method using *o*-phthaldehyde (OPA) reagent as a fluorescent dye, following the method of Fujiwara et al. (1987) with certain modifications by Saha et al. (2000).

Table 1
Changes in the concentration (μmol g^{-1} wet wt.) of different FAAs and related compounds in the liver of *C. batrachus* exposed to 25 mM NH_4Cl

	Days of exposure					
	1		3		7	
	Control	Treated	Control	Treated	Control	Treated
Non-essential						
Asp	1.47±0.11	2.59±0.18**	1.36±0.14	4.91±0.31***	2.05±0.15	5.84±0.61***
Gly	1.64±0.17	3.37±0.24**	1.56±0.11	4.34±0.24***	1.42±0.11	4.11±0.42***
Ala	2.95±0.23	4.54±0.27***	2.77±0.17	6.17±0.89**	2.49±0.23	7.37±0.81***
Ser	1.83±0.21	2.53±0.18*	1.28±0.07	3.13±0.36**	1.82±0.18	3.36±0.34**
Asn	0.48±0.08	1.32±0.14***	0.41±0.05	2.82±0.18***	0.45±0.05	2.68±0.18***
Glu	3.28±0.31	6.26±0.89**	2.76±0.21	9.12±1.10***	2.51±0.19	9.98±0.65***
Gln	1.59±0.12	4.64±0.62***	1.45±0.11	7.64±0.89***	1.41±0.08	8.85±0.71***
Cit	0.28±0.07	0.46±0.10*	0.35±0.04	0.39±0.05	0.25±0.04	0.18±0.01
Tyr	1.08±0.09	1.22±0.05**	1.00±0.07	1.10±0.08	0.91±0.07	1.05±0.08
β-Ala	0.75±0.05	0.81±0.04	0.69±0.08	2.12±0.12***	0.51±0.04	1.28±0.06**
Pro	0.84±0.09	1.41±0.12*	0.85±0.10	1.51±0.11*	1.18±0.08	1.55±0.12*
Tau	15.61±1.71	19.12±1.27	14.74±1.88	20.64±1.82*	13.68±1.31	20.29±1.65*
Total	31.80±2.24	48.32±3.12*	29.22±1.89	63.85±4.56**	28.73±2.17	66.56±4.15**
Essential						
Thr	2.46±0.21	2.92±0.36	2.42±0.36	3.26±0.21*	2.31±0.18	3.07±0.38
Val	1.95±0.16	1.97±0.21	1.87±0.12	1.91±0.11	1.66±0.12	1.72±0.18
Met	0.77±0.10	0.91±0.12	0.71±0.05	1.15±0.04*	0.67±0.07	0.91±0.08*
Ile	1.22±0.07	1.45±0.06	1.1±0.07	1.51±0.05*	1.02±0.11	1.48±0.09*
Leu	1.68±0.12	1.97±0.09	1.78±0.11	2.62±0.11*	1.61±0.08	2.32±0.12*
Trp	0.22±0.02	0.23±0.005	0.18±0.01	0.21±0.03	0.17±0.04	0.22±0.04
Phe	0.60±0.04	0.65±0.05	0.69±0.05	0.64±0.05	0.62±0.05	0.60±0.03
His	0.81±0.10	0.77±0.04	0.74±0.06	0.82±0.05	0.68±0.07	0.60±0.03
Orn	1.81±0.09	1.91±0.12	1.82±0.17	1.94±0.12	1.87±0.12	1.82±0.12
Arg	0.67±0.07	0.71±0.07	0.64±0.05	0.72±0.08	0.74±0.09	0.78±0.05
Total	12.29±1.07	13.43±1.18	12.00±0.95	14.81±1.62	11.39±0.88	13.55±1.44
Ammonia	8.81±1.34	14.4±1.09*	8.96±0.62	19.7±1.88**	9.19±0.5	22.4±2.02***
Urea	4.17±0.22	7.12±0.57*	4.29±0.37	10.7±0.87***	4.37±0.27	14.2±0.59***

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.
Values are expressed as mean±S.E.M. ($n=3$).

2.6. Calculation for nitrogenous balance sheet

The calculation for nitrogenous balance sheet was made assuming the total fish body wt. as 100 g. The total excretion of ammonia and urea-N by the fish was calculated by the average rate of excretion of ammonia and urea-N for a period of 7 days, and then finally converted for 100 g fish body wt. The uptake of ammonia by the fish exposed to 25 mM NH_4Cl was calculated by subtracting the concentration of ammonia present in the medium after exposing the fish for 24 h from the concentration of ammonia in the control bucket containing only 25 mM NH_4Cl. The extra accumulation of ammonia, urea-N, and non-essential FAAs (taking 2 N in the case of Gln and Asn) by day 7 in major fish tissues such as the muscle, liver, kidney, brain and blood plasma were calculated taking the average weight of each tissue as 50, 2, 1, 0.5 and 4 g, respectively, for 100 g fish (Saha and Das, unpublished observations).

2.7. Chemicals

Enzymes, co-enzymes, substrates, *o*-phthaldehyde and standard physiological free amino acid mixture were purchased from Sigma Chemicals (St. Louis, MO). The other chemicals used were

Table 2
Changes in the concentration (μmol g^{-1} wet wt.) of different FAAs and related compounds in the kidney of *C. batrachus* exposed to 25 mM NH_4Cl

	Days of exposure					
	1		3		7	
	Control	Treated	Control	Treated	Control	Treated
Non-essential						
Asp	3.34±0.24	5.59±0.42*	4.01±0.38	8.64±1.10**	3.17±0.22	8.23±0.85***
Gly	3.50±0.28	5.53±0.38*	3.19±0.42	6.10±0.81**	2.85±0.18	6.81±0.54**
Ala	7.94±0.88	11.56±0.98*	7.01±0.62	13.28±1.32**	6.45±0.37	14.32±1.41**
Ser	6.06±0.95	7.51±0.61	5.55±0.38	8.19±0.45*	5.42±0.42	10.05±0.81**
Asn	0.81±0.12	1.41±0.17**	0.75±0.15	1.94±0.17***	0.78±0.10	1.81±0.15**
Glu	6.34±0.85	8.99±0.66*	5.46±0.32	12.29±1.21**	5.32±0.38	13.16±0.65***
Gln	2.79±0.21	5.08±0.41**	2.92±0.18	6.42±0.56**	2.52±0.18	7.74±0.42***
Cit	0.69±0.07	1.10±0.10*	0.54±0.12	0.82±0.11*	0.65±0.08	0.75±0.15
Tyr	2.02±0.12	2.53±0.21	2.16±0.14	3.46±0.17*	1.92±0.12	4.39±0.21**
β-Ala	4.16±0.61	5.09±0.31	2.84±0.14	5.53±0.31**	3.55±0.24	6.17±0.32**
Pro	1.82±0.14	2.38±0.27*	1.61±0.17	2.45±0.17*	2.07±0.24	2.81±0.16*
Tau	10.71±0.81	12.57±1.67	9.81±1.12	14.47±1.81*	9.27±0.91	15.11±1.61*
Total	50.17±3.57	69.34±4.25*	45.75±3.85	83.55±5.68**	43.95±2.89	91.38±5.12**
Essential						
Thr	3.58±0.32	2.92±0.32	3.16±0.17	3.57±0.18	2.81±0.31	3.50±0.24
Val	3.93±0.24	3.88±0.21	3.55±0.21	3.18±0.24	3.12±0.12	3.41±0.18
Met	1.08±0.12	1.41±0.10*	0.95±0.08	1.57±0.07*	1.22±0.10	1.77±0.05*
Ile	2.02±0.11	2.21±0.14	2.18±0.31	2.52±0.12	1.68±0.12	2.08±0.11
Leu	4.04±0.38	4.48±0.24	4.16±0.21	4.81±0.24	3.87±0.21	4.56±0.28
Trp	0.62±0.05	0.71±0.06	0.69±0.04	0.75±0.05	0.56±0.05	0.81±0.05
Phe	1.05±0.05	1.14±0.10	0.95±0.07	1.11±0.10	1.14±0.07	1.27±0.11
His	1.28±0.11	1.42±0.14	1.24±0.10	1.08±0.07	1.27±0.11	1.38±0.14
Orn	1.58±0.12	1.69±0.11	1.81±0.12	1.44±0.11	1.41±0.12	1.21±0.12
Arg	1.02±0.05	1.21±0.07	1.36±0.07	1.49±0.10	0.97±0.05	1.21±0.08
Total	20.23±1.05	22.15±1.42	20.03±1.38	21.55±1.85	18.28±1.66	21.24±2.07
Ammonia	10.61±1.12	16.94±1.33*	9.69±0.74	23.43±1.54**	10.18±0.57	24.62±1.34***
Urea	4.61±0.38	6.17±0.42*	3.91±0.24	19.12±0.72**	4.27±0.31	10.68±0.62***

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.
Values are expressed as mean±S.E.M. ($n=3$).

of the analytical grade and obtained from local sources. Deionised and double-glass-distilled water were used in all preparations.

2.8. Statistical analysis

Data collected from three replicates were statistically analyzed and presented as mean±S.E.M. Comparisons of the unpaired mean values between the experimental and respective controls were made using unpaired Student's *t*-test and differences with $P<0.05$ were regarded as statistically significant.

3. Results

3.1. Changes of tissue levels of ammonia, urea and free amino acids

As shown in Tables 1–5, the concentration of ammonia increased significantly in all the tissues studied (except in the brain), and in the plasma of the walking catfish within first day of exposure to 25 mM NH_4Cl, followed by further increase of approximately two- to threefold compared to controls after 7 days of exposure. The concentration of urea also increased significantly to approximately two- to fivefold in all the tissues studied

Table 3
Changes in the concentration (μmol g^{-1} wet wt.) of different FAAs and related compounds in the muscle of *C. batrachus* exposed to 25 mM NH_4Cl

	Days of exposure					
	1		3		7	
	Control	Treated	Control	Treated	Control	Treated
Non-essential						
Asp	0.56±0.05	0.71±0.08*	0.44±0.03	1.21±0.16***	0.61±0.05	1.68±0.19***
Gly	15.28±1.47	18.81±2.13	14.49±1.32	18.12±1.28	13.18±1.61	16.81±2.15*
Ala	7.65±0.68	13.17±1.21**	6.82±0.87	15.26±1.51**	8.12±1.16	15.79±1.62**
Ser	0.64±0.10	0.61±0.07	0.52±0.07	0.71±0.06*	0.44±0.04	0.81±0.07**
Asn	0.23±0.04	0.78±0.10***	0.18±0.03	1.11±0.10***	0.21±0.006	1.21±0.06***
Glu	0.53±0.03	1.91±0.12***	0.47±0.05	2.01±0.12***	0.42±0.02	2.41±0.31***
Gln	0.36±0.04	2.67±0.11***	0.39±0.02	3.98±0.38***	0.32±0.01	4.31±0.25***
Cit	0.11±0.01	0.15±0.02*	0.10±0.01	0.26±0.02***	0.12±0.003	0.31±0.02***
Tyr	0.55±0.07	0.58±0.05	0.48±0.05	0.72±0.05*	0.43±0.05	0.81±0.03**
β-Ala	0.37±0.02	0.39±0.03	0.41±0.06	1.38±0.09***	0.36±0.007	1.46±0.07***
Pro	0.25±0.12	0.35±0.04*	0.32±0.02	0.45±0.05*	0.39±0.01	0.84±0.02**
Tau	13.75±1.18	13.81±1.13	12.91±0.87	13.68±1.61	10.17±0.68	12.81±1.36
Total	40.28±2.35	53.92±3.17*	36.98±2.54	58.07±4.12*	34.00±2.36	58.25±0.68**
Essential						
Thr	0.67±0.05	0.79±0.07	0.75±0.04	0.99±0.06*	0.88±0.04	1.15±0.06*
Val	0.15±0.02	0.17±0.03	0.19±0.02	0.20±0.02	0.31±0.02	0.41±0.006*
Met	0.26±0.02	0.35±0.02*	0.31±0.03	0.39±0.006*	0.35±0.02	0.51±0.007*
Ile	0.15±0.01	0.24±0.01*	0.13±0.006	0.17±0.005*	0.17±0.005	0.25±0.004*
Leu	0.86±0.12	1.12±0.04*	0.71±0.05	1.02±0.03*	0.60±0.04	0.81±0.01
Trp	0.11±0.01	0.14±0.005	0.08±0.002	0.11±0.002	0.11±0.002	0.13±0.005
Phe	0.14±0.005	0.17±0.006	0.17±0.005	0.21±0.01	0.15±0.003	0.19±0.01
His	0.12±0.004	0.10±0.01	0.10±0.003	0.12±0.003	0.13±0.004	0.10±0.002
Orn	0.22±0.03	0.18±0.03	0.27±0.03	0.34±0.02	0.22±0.015	0.22±0.004
Arg	0.31±0.04	0.35±0.02	0.25±0.04	0.21±0.006	0.27±0.02	0.31±0.015
Total	3.02±0.7	3.61±0.44	2.96±0.51	3.62±0.88	3.20±0.63	4.04±0.41
Ammonia	5.0±0.21	8.5±0.54**	5.1±0.62	11.8±0.68***	5.9±0.38	13.2±0.56**
Urea	1.4±0.12	2.5±0.23**	1.8±0.14	4.4±0.34***	1.9±0.17	4.6±0.34**

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.
Values are expressed as mean±S.E.M. ($n=3$).

including the plasma of the walking catfish after 7 days of exposure.

There was a significant increase in the concentration of different non-essential FAAs in the liver of NH_4Cl-exposed fish within the first day of exposure, followed by a further increase of approximately 2.5-fold after 7 days (Table 1, Fig. 1). This was mainly attributable to the increase of Asp (10% of the total increase), Ala (13%), Glu (20%), Gln (20%), and taurine (Tau, 17.5%) concentrations. Significant increases of a few essential FAAs such as Thr, Met, Ile and Leu were also observed after 3 days of exposure, but resulted in no significant changes in the concentration of total essential FAAs even after 7 days of exposure. In the kidney of the NH_4Cl-exposed fish, the concentration of total non-essential FAAs increased maximally to approximately twofold within 3 days of exposure, and was maintained after 7 days of exposure (Table 2, Fig. 1). It was mainly due to an increase of Asp (11% of the total increase), Ala (17%), Glu (17%), Gln (11%), and Tau (12.5%) concentrations. In the muscle of NH_4Cl-exposed fish, the concentration of some non-essential FAAs increased within 3 days of exposure, followed by a further increase of approx-

Table 4
Changes in the concentration (μmol/g^{-1} wet wt.) of different FAAs and related compounds in the brain of *C. batrachus* exposed to 25 mM NH_4Cl

	Days of exposure					
	1		3		7	
	Control	Treated	Control	Treated	Control	Treated
Non-essential						
Asp	0.55±0.04	1.51±0.11***	0.51±0.03	2.15±0.23***	0.55±0.02	2.06±0.22***
Gly	1.18±0.08	1.48±0.12	1.28±0.06	1.59±0.14	0.92±0.04	1.24±0.15
Ala	0.91±0.04	1.72±0.08**	0.76±0.05	1.61±0.13**	0.74±0.05	1.52±0.17**
Ser	0.48±0.05	0.81±0.05*	0.42±0.03	0.81±0.08**	0.45±0.03	0.75±0.10*
Asn	0.12±0.005	0.56±0.04***	0.14±0.004	0.50±0.04***	0.18±0.006	0.41±0.05**
Glu	3.77±0.17	6.81±0.32**	3.61±0.17	7.72±0.54**	3.12±0.21	7.91±0.62**
Gln	2.51±0.21	9.57±1.03***	2.75±0.21	14.81±1.33***	2.38±0.15	17.21±1.51***
Cit	0.14±0.006	0.21±0.002*	0.18±0.015	0.38±0.05**	0.15±0.006	0.33±0.02**
Tyr	0.15±0.004	0.19±0.002	0.17±0.018	0.23±0.05	0.11±0.004	0.21±0.01
β-Ala	0.27±0.007	0.51±0.03**	0.22±0.02	0.68±0.03***	0.24±0.005	0.71±0.04***
Pro	0.17±0.01	0.19±0.03	0.15±0.01	0.21±0.04	0.16±0.01	0.21±0.02
Tau	12.21±1.23	12.61±1.41	11.65±1.11	12.21±1.07	12.33±0.68	12.89±0.88
Total	22.44±1.15	36.12±1.86*	21.97±1.53	42.85±2.18**	21.36±1.35	45.77±2.68**
Essential						
Thr	0.24±0.02	0.27±0.005	0.27±0.02	0.29±0.02	0.24±0.03	0.22±0.005
Val	0.21±0.005	0.24±0.01	0.24±0.01	0.21±0.005	0.21±0.01	0.27±0.01
Met	0.12±0.002	0.12±0.002	0.11±0.002	0.13±0.003	0.14±0.005	0.17±0.006
Ile	0.17±0.005	0.15±0.003	0.19±0.003	0.17±0.004	0.16±0.004	0.19±0.005
Leu	0.87±0.04	0.89±0.03	0.71±0.03	0.76±0.02	0.68±0.02	0.64±0.02
Trp	0.12±0.002	0.16±0.003	0.17±0.005	0.21±0.005	0.14±0.004	0.17±0.006
Phe	0.15±0.003	0.13±0.004	0.12±0.003	0.14±0.003	0.18±0.005	0.26±0.002
His	0.29±0.004	0.36±0.004	0.15±0.005	0.17±0.006	0.17±0.002	0.13±0.005
Orn	0.34±0.01	0.24±0.01	0.24±0.01	0.21±0.01	0.23±0.007	0.21±0.007
Arg	0.29±0.004	0.37±0.006	0.30±0.02	0.33±0.03	0.24±0.008	0.27±0.004
Total	2.80±0.35	2.93±0.45	2.50±0.25	2.62±0.37	2.40±0.38	2.55±0.42
Ammonia	1.81±0.24	2.00±0.32	1.61±0.22	1.95±0.17	1.71±0.22	2.12±0.15
Urea	1.90±0.14	2.40±0.15	2.40±0.15	2.90±0.18*	2.32±0.14	3.01±0.32*

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.
Values are expressed as mean±S.E.M. ($n=3$).

imately 1.7-fold after 7 days (Table 3, Fig. 1). This was mainly attributable to an increase of Gly (16% of the total increase), Ala (33%), Glu (9%), and Gln (17.5%) concentrations. In the brain, the maximum increase of the total non-essential FAAs was approximately 2.2-fold after 7 days of exposure to NH_4Cl, and was mainly due to an increase of Gln (60%) and Glu (19.5%) concentrations (Table 4, Fig. 1). In the plasma, significant increases of some non-essential FAAs were also observed within first day of exposure to NH_4Cl. The total non-essential FAAs had increased by twofold after 7 days, and was mainly due to an increase of Asp (10.5% of the total increase), Ala (18.5%), and Gln (13%) concentrations (Table 5, Fig. 1).

3.2. Excretion/uptake of ammonia and urea-N

The pattern of excretion/uptake of ammonia and urea-N by the walking catfish exposed to 25 mM NH_4Cl and in the water are shown in Fig. 2. The ammonia excretion rate by the control fish averaged to 215 μmol kg^{-1} h^{-1} for a period of 7 days. High NH_4Cl exposure led to a total inhibition of ammonia excretion, accompanied with a significant ($P<0.001$) uptake of ammonia from the

Table 5
Changes in the concentration (μmol ml^{-1}) of different FAAs and related compounds in the plasma of *C. batrachus* exposed to 25 mM NH_4Cl

	Days of exposure					
	1		3		7	
	Control	Treated	Control	Treated	Control	Treated
Non-essential						
Asp	0.155±0.013	0.234±0.015*	0.154±0.016	0.303±0.018**	0.151±0.003	0.314±0.023**
Gly	0.165±0.021	0.25±0.031	0.18±0.009	0.20±0.021	0.161±0.023	0.171±0.035
Ala	0.215±0.024	0.431±0.032**	0.221±0.045	0.547±0.042**	0.232±0.027	0.521±0.045**
Ser	0.067±0.003	0.12±0.003**	0.061±0.005	0.108±0.004*	0.058±0.006	0.092±0.012*
Asn	0.023±0.005	0.051±0.005**	0.033±0.004	0.057±0.005**	0.023±0.004	0.068±0.004***
Glu	0.055±0.007	0.091±0.007*	0.057±0.005	0.105±0.009**	0.044±0.003	0.117±0.006***
Gln	0.139±0.014	0.227±0.026*	0.132±0.004	0.252±0.015**	0.149±0.016	0.354±0.025***
Cit	0.005±0.001	0.009±0.001**	0.007±0.001	0.020±0.003***	0.005±0.001	0.025±0.005***
Tyr	0.055±0.006	0.059±0.005	0.057±0.006	0.061±0.004	0.051±0.004	0.059±0.004
β-Ala	0.039±0.003	0.045±0.007	0.041±0.005	0.054±0.008	0.032±0.001	0.036±0.003
Pro	0.015±0.004	0.012±0.003	0.017±0.002	0.014±0.005	0.019±0.003	0.024±0.005
Tau	0.75±0.056	0.91±0.062	0.74±0.75	1.25±0.18*	0.77±0.09	1.38±0.59**
Total	1.68±0.21	2.40±0.27*	1.70±0.17	2.84±0.21*	1.67±0.15	2.90±0.22**
Essential						
Thr	0.072±0.003	0.075±0.006	0.080±0.009	0.089±0.007	0.087±0.007	0.089±0.004
Val	0.065±0.005	0.060±0.005	0.077±0.005	0.079±0.005	0.081±0.005	0.075±0.005
Met	0.013±0.002	0.017±0.003	0.017±0.003	0.022±0.004	0.019±0.002	0.023±0.003
Ile	0.055±0.006	0.051±0.004	0.050±0.006	0.054±0.003	0.059±0.004	0.064±0.007
Leu	0.114±0.04	0.104±0.025	0.091±0.007	0.085±0.007	0.085±0.006	0.091±0.008
Trp	0.024±0.006	0.022±0.007	0.027±0.003	0.029±0.002	0.029±0.005	0.025±0.004
Phe	0.284±0.057	0.278±0.051	0.251±0.06	0.243±0.038	0.224±0.041	0.217±0.022
His	0.027±0.005	0.029±0.004	0.022±0.005	0.025±0.003	0.020±0.003	0.025±0.002
Orn	0.105±0.02	0.117±0.035	0.115±0.032	0.117±0.019	0.105±0.012	0.114±0.019
Arg	0.032±0.005	0.037±0.008	0.037±0.002	0.039±0.005	0.035±0.002	0.039±0.002
Total	0.80±0.03	0.79±0.04	0.76±0.03	0.79±0.05	0.74±0.04	0.77±0.03
Ammonia	0.66±0.05	1.01±0.14*	0.69±0.05	2.21±0.18***	0.71±0.05	2.47±0.25***
Urea	0.38±0.05	0.55±0.03*	0.40±0.05	1.02±0.15***	0.42±0.05	1.32±0.17***

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.
Values are expressed as mean±S.E.M. ($n=3$).

medium over the period of 7 days. The urea-N excretion by the control fish averaged to 71 μmol kg^{-1} h^{-1} for a period of 7 days. There was an immediate significant ($P<0.001$) increase of urea-N excretion rate by the NH_4Cl-exposed fish of approximately 3.4-fold, followed by a maximum increase of approximately 5.7-fold after 3 days.

3.3. Changes in the activity of some key amino acid metabolism-related enzymes

Table 6 shows the changes of activity of certain key amino acid metabolism-related enzymes such as the GS, GDH (both reductive amination and oxidative deamination), AST and ALT in the NH_4Cl-exposed fish along with the normal physiological levels of activity of these enzymes in different tissues. Relatively high levels of activity of the mentioned enzymes could be detected in all the tissues studied including the liver, kidney, muscle and brain, with tissue specific variations. The activity of GS and GDH (reductive amination) were maximum in the brain, followed by the liver, kidney and muscle. Whereas, the activity of other enzymes such as GDH (oxidative deamination), AST and ALT were maximum in the liver. In the NH_4Cl-exposed fish, the activities of GS, GDH (only in the reductive amination direction), AST and ALT increased significantly in the liver, kidney

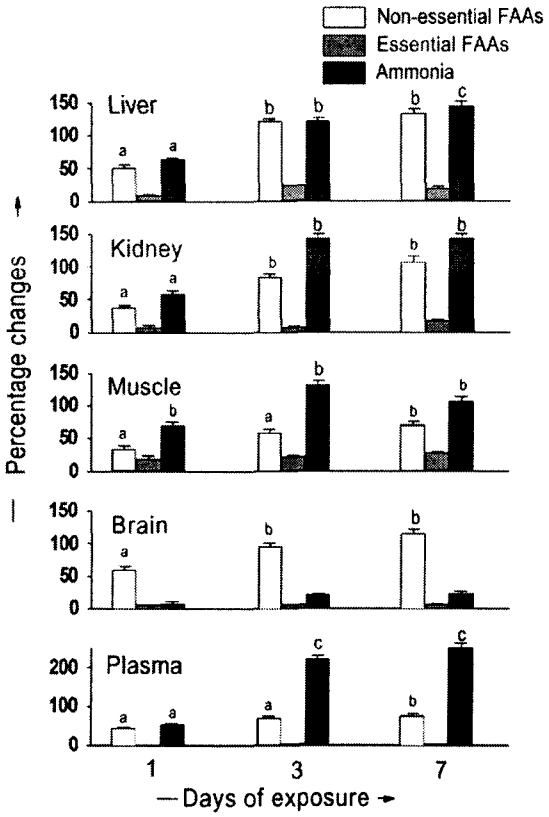

Fig. 1. Percentage changes in the concentration of total non-essential and essential FAAs, and ammonia in different tissues and in the plasma of *C. batrachus* exposed to 25 mM NH_4Cl. Values are plotted as mean ± S.E.M. ($n=3$). a, b, c: P values significant at <0.05, <0.01, <0.001, respectively.

and muscle. In the brain, no significant changes in the activity of any of these enzymes were observed. Overall, in most the tissues the maximum increases of enzyme activity were observed after 3 days of exposure to NH_4Cl.

4. Discussion

In the present study, the possible conversion of some of the accumulated ammonia to different non-essential FAAs by the walking catfish was looked upon as another potential mechanism of survival at a high concentration of ambient ammonia. There was a significant accumulation of various non-essential FAAs in different tissues and in the plasma of walking catfish while exposed to 25 mM NH_4Cl for 7 days.

The significant accumulation of ammonia observed in various tissues of walking catfish, exposed to higher ambient ammonia (25 mM NH_4Cl), was tissue specific with a maximum concentration in the kidney, followed by the liver, muscle and plasma. This was possibly due to total inhibition of ammonia excretion generated endogenously, accompanied with the uptake of ammonia by the fish from the external medium containing high concentration of ammonia (Fig. 2a). The capacity for accumulation of ammonia in this catfish was found to be much higher than many teleosts and gobiid fishes (Dabrowska and Wlasow, 1986; Iwata, 1988; Jow et al., 1999; Iwata et al., 2000), but similar to the situation observed in another air-breathing catfish, *Heteropneustes fossilis* (Saha and Ratha, 1994) and in loach, *Misgurnus anguillicaudatus* (Tsui et al., 2002). This could be one of the reasons of tolerating such high ambient ammonia. Furthermore, the in vivo accumulation of ammonia to a lethal concentration might have been prevented in this catfish by converting a part of the accumulated ammonia to urea, since this fish has a functional urea cycle with a full complement of the urea cycle enzymes in hepatic as well as in some extra-hepatic tissues (Saha and Ratha, 1989; Saha et al., 1999), thus causing higher accumulation of urea in different tissues and in the plasma of the NH_4Cl-exposed fish as reported in the present study. In addition, this catfish has the potential of transition to ureotelism from ammoniotelism by stimulating the activity of certain key enzymes of the urea cycle during confrontation with an ammonia load (Saha and Das, 1999). Approximately four- to fivefold increase of urea-N excretion rate during exposure to high concentration of external ammonia was also observed in the present study (Fig. 2b). This was similar to the situation observed in the singhi catfish (*Heteropneustes fossilis*) under higher ammonia conditions (Saha and Ratha, 1990, 1994; Saha et al., 1995, 2001). Several recent studies have suggested, however, that ureotelism (i.e. increased urea cycle pathway activity) is not the universal response to fish to these kinds of environmental circumstances (for review, see Ip et al., 2001).

The basal levels of activity of some of the key enzymes related to amino acid metabolism such as the GS, GDH (reductive amination), AST and ALT were also quite high in this walking catfish compared to many ammoniotelic teleosts including gobiid fishes (Wilson, 1973; Waarde and Kesbeke,

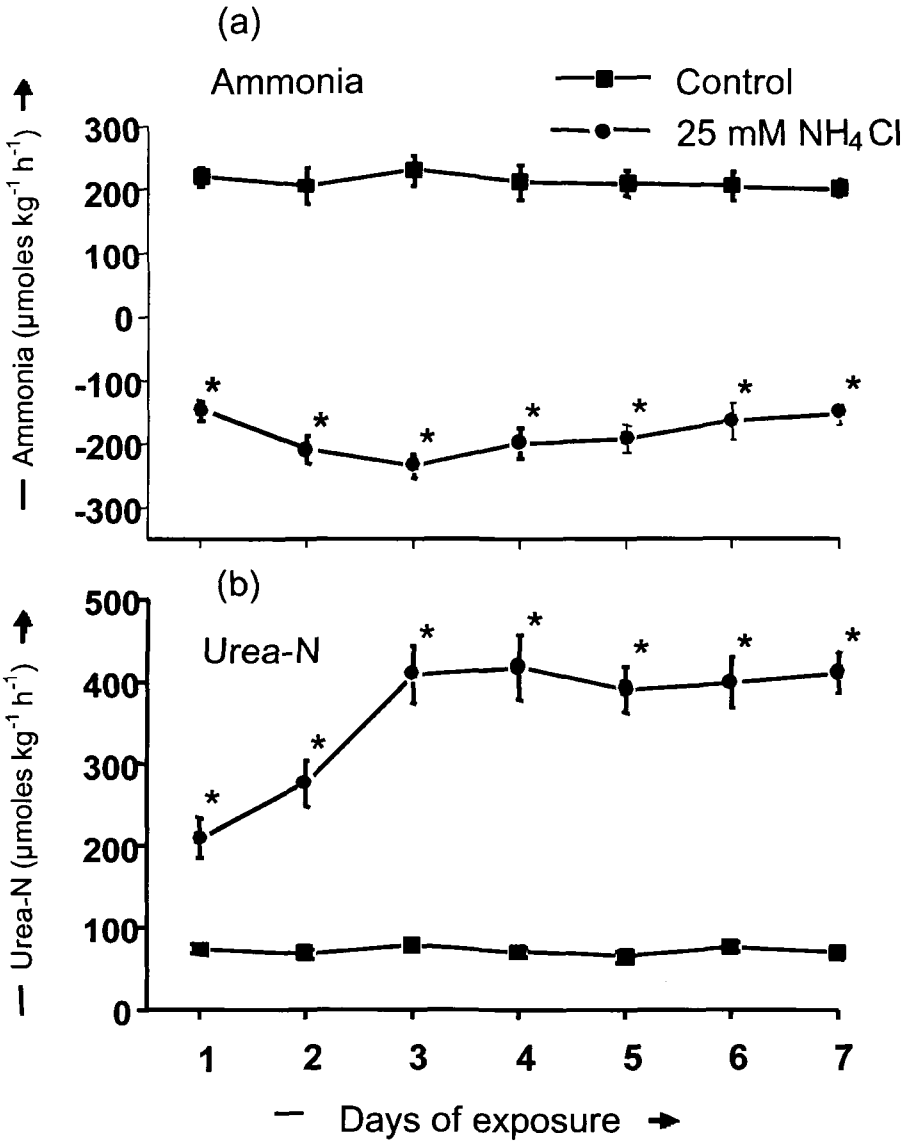

Fig. 2. Changes in the excretion rate (μmol kg^{-1} h^{-1}) of ammonia (a) and urea-N (b) by *C. batrachus* exposed to 25 mM NH_4Cl. Values are plotted as mean ± S.E.M. ($n = 4–9$). * Significantly different from respective control values ($P < 0.001$).

1982; Chew and Ip, 1987; Iwata, 1988; Walton and Cowey, 1977; Peng et al., 1998; Anderson, 2001; Lim et al., 2001), which were further stimulated in different tissues of NH_4Cl-exposed fish (Table 6).

Significant increase in the concentration of many of the non-essential FAAs was observed in the NH_4Cl-exposed fish liver (approx. 2.3-fold), 80% of which were attributable to the increase of Asp, Ala, Glu, Gln and Tau concentrations. Similar observations were also made in the kidney of the walking catfish exposed to NH_4Cl. In the kidney, the concentration of total non-essential FAAs increased to approximately twofold, 90% of which were attributable to the increase of Asp, Ala, Glu, Gln and Tau concentrations. It is known that the synthesis of glutamate by assimilating ammonia with α-ketoglutarate by GDH (reductive amina-

Table 6
Changes of activity (units g^{-1} wet wt.) of different enzymes related to amino acid metabolism in different tissues of *C. batrachus* exposed to 25 mM NH_4Cl

Enzymes		Tissues											
		Liver			Kidney			Muscle			Brain		
		Days of exposure											
		1	3	7	1	3	7	1	3	7	1	3	7
GS	Control	111±12.2	91±10.7	118±5.2	45±3.3	36±2.2	46.4±4.6	10.6±1.6	8.5±0.9	8.2±0.8	226±11.1	234±12.7	233±14.5
	25 mM NH_4Cl	184±9.4*	194±8.1**	324±14.6***	60±1.8*	87±5.3**	114±7.2***	18.6±1.9**	21.2±2.1***	18.8±1.23**	244±18.8	260±22.1	227±28.9
		(+66)	(+113)	(+175)	(+33)	(+142)	(+148)	(+75)	(+146)	(+129)	(+8)	(+11)	(−2)
GDH (R.A)	Control	922±73	858±36	854±54	204±10.5	197±26.9	178±8.4	152±8.5	163±9.0	162±8.1	2496±64	2586±69	2715±71
	25 mM NH_4Cl	1137±56	1683±76**	1954±75**	250±14.1	372±22.0**	363±28.1**	222±14.7*	285±17.3**	356±15.8**	3034±77	3085±82	2935±89
		(+23)	(+96)	(+129)	(+23)	(+89)	(+104)	(+46)	(+75)	(+120)	(+21)	(+19)	(+8)
GDH (O.D)	Control	395±11.1	392±15.9	413±22.4	66±5.7	68±5.9	59±2.3	72±1.9	83±9.9	84±6.7	95±5.5	89±3.2	113±8.3
	25 mM NH_4Cl	424±10.5	411±27. 8	402±23.9	71±4.8	60±5.7	55±4.7	76±2.1	81±4.1	89±7.5	100±6.5	91±7.8	119±10.3
		(+7)	(+5)	(−3)	(+8)	(−12)	(−7)	(+6)	(−2)	(+6)	(+5)	(+2)	(+5)
AST	Control	442±17.1	454±26.9	471±19.7	148±5.7	127±7.2	157±6.2	165±6.8	187±19.5	189±17.6	54±3.1	63±4.3	68±3.8
	25 mM NH_4Cl	878±36.5**	985±53.8**	848±42.3**	287±21**	375±19.7***	595±30.4***	216±10.9*	446±29.4***	558±21.9***	89±4.1*	129±5.9**	156±10.2**
		(+99)	(+117)	(+80)	(+94)	(+195)	(+279)	(+31)	(+139)	(+195)	(+65)	(+105)	(+129)
ALT	Control	180±9.1	183±11.9	174±8.8	64±5.8	66±3.3	65±4.4	297±8.9	273±11.1	262±17.3	57±7.5	60±6.4	73±4.5
	25 mM NH_4Cl	396±15.9**	302±15.7*	299±17.7**	84±7.6*	107±6.7*	112±8.7**	356±17.9	458±25.1*	479±19.4**	62±3.4	71±5.8	87±7.5
		(+120)	(+65)	(+72)	(+31)	(+62)	(+72)	(+20)	(+68)	(+83)	(+9)	(+18)	(+19)

* P values significant at <0.05.
** P values significant at <0.01.
*** P values significant at <0.001.

Values are expressed as mean±S.E.M. ($n=3$). Percentage increase (+) or decrease (−) of activity compared to control are given in parentheses. GS, glutamine synthetase; GDH (R.A/O.D), glutamate dehydrogenase (reductive amination/oxidative deamination); AST, aspartate aminotransaminase; ALT, alanine aminotransaminase.

tion) plays a key role in the detoxification of ammonia created endogenously from the catabolism of amino acids and proteins in various tissues including the brain (Cooper and Plum, 1987; Jürss and Bastrop, 1995). The activity of GDH in the ammonia utilizing direction was found to be approximately two- to threefold higher than in the ammonia forming direction both in the liver and kidney, and were comparatively higher than in many ammoniotelic teleosts (Wilson, 1973; Chew and Ip, 1987; Iwata, 1988; Walton and Cowey, 1977; Peng et al., 1998), but comparable to ureogenic teleosts (Walsh et al., 1993, 1994). The activity of GS, which helps to form glutamine from ammonia, was also relatively high in the liver and kidney of walking catfish and was comparable to other ureogenic teleosts (Anderson, 2001). The activities of AST and ALT were also high. The increased activity of most of these enzymes in different tissues of the NH_4Cl-exposed fish might have fixed accumulated ammonia to Glu, Gln and other non-essential FAAs under hyper-ammonia stress.

Non-essential FAAs were also reported to significantly increase in different non-ureogenic ammoniotelic teleosts such as the carp (Dabrowska and Wlasow, 1986), mudskippers (Iwata et al., 1981; Iwata, 1988; Iwata and Deguchi, 1995; Peng et al., 1998), and goldfish (Levi et al., 1974), along with the induction of certain related enzymes under hyper-ammonia stress. Iwata et al. (1981) reported a significant increase of GDH activity (assayed in the glutamate forming direction) in the mudskipper, *Periophthalmus cantonensis* under increased ammonia content. Kong et al. (1998) reported a significant increase of GDH activity (reductive amination) in the largemouth bass, *Micropterus salmoides* after exposing the fish to 0.25 mM NH_4Cl for 12 days, but without affecting the activity of GS. Peng et al. (1998) also reported significant increase of GDH (reductive amination) and ALT activities in the liver and brain of *Periophthalmus schlosseri* under hyper-ammonia stress. Significantly elevated GS activity was also observed in different tissues of sleeper *Bostrichthys sinensis* during high ammonia exposure (Anderson et al., 2002). Induced activity of GS both in the liver and kidney (potential ureogenic tissues) might have also played a significant role in the conversion of toxic ammonia to urea via the urea cycle, since GS is also known to play a central role in urea synthesis in ureogenic teleosts by supplying Gln as one of the substrates for the urea synthesis via carbamyl phosphate synthetase III (CPS III) (Campbell and Anderson, 1991; Anderson, 1995), thereby playing a pivotal role in the process of ammonia detoxification.

Several studies have reported that the muscle has a large storage capacity for ammonia when ammonia excretion is blocked or inhibited at the gills (Iwata, 1988; Wilkie and Wood, 1995; Peng et al., 1998). In the walking catfish also muscle served as the major site of ammonia accumulation, since it constitutes approximately 50% of the total body mass (Saha and Das, unpublished observation), and its large storing capacity for ammonia seems to contribute largely to lowering of the circulating blood ammonia level while a fish is suffering from ammonia loading. The increase of ammonia concentration (approx. 2.5-fold) in the walking catfish muscle exposed to 25 mM NH_4Cl was accompanied by a 1.7-fold increase of total non-essential FAAs, 75% of which were mainly due to the increase of Gly, Ala, Glu and Gln concentrations. Although the activity of different amino acid metabolism-related enzymes were low in the muscle compared to other tissues, the total activity in the whole muscle should be much higher, since it constitutes a major part of the total body mass. The activity of GS, GDH (reductive amination), AST and ALT was also significantly stimulated in the muscle under hyper-ammonia stress, possibly to convert accumulated ammonia to various non-essential FAAs.

The brain is the organ most sensitive to ammonia toxicity (Cooper and Plum, 1987). Virtually all fish brain have high GS activity, which is thought to detoxify ammonia (Webb and Brown, 1976; Webb, 1980; Chew and Ip, 1987; Iwata, 1988; Chakravorty et al., 1989; Peng et al., 1998). This is also true in walking catfish. The mode of detoxification of ammonia in vertebrate brain was suggested to be through Glu and subsequent Gln synthesis through the coupled steps of GDH and GS (Cooper and Plum, 1987; Mommsen and Walsh, 1991; Wright and Wright, 1996). The physiological level of activity of GDH (reductive amination) was also very high in the brain of walking catfish with a very high ratio (approx. 25–30) of reductive amination vs. oxidative deamination reactions taking place (Table 6). The high levels of activity of these two enzymes probably helped converting accumulated ammonia to Glu and Gln and other non-essential FAAs, thus caus-

ing a significant increase of Gln (60% of the total increase), Glu and other non-essential FAAs, without affecting the concentration of ammonia in the brain under hyper-ammonia stress. This arrangement would therefore enable the catfish to tolerate high ambient ammonia (up to 75 mM NH_4Cl). Significant increases of various non-essential FAAs in the plasma of NH_4Cl-exposed fish, mainly due to increase in the concentrations of Gln (60%), Glu (20%), Asp (6%) and Ala (5%) could possibly be for inter-organ transportation of amino acids under hyper-ammonia stress.

The occurrence of a Tau synthetic pathway, which constitutes the most predominant amine in most teleosts including the walking catfish, is not very clear in fish (Waarde, 1988). However, in the present study significant increase of Tau concentration in the liver, kidney and also in the plasma was noticed under hyper-ammonia stress. Significant increase in the efflux of Tau was observed from the perfused liver infused with 5 and 10 mM NH_4Cl (Saha et al., 2000). It is, therefore, necessary to investigate the occurrence of the Tau synthetic pathway with its physiological significance in this fish.

The mechanism(s) by which different amino acid metabolism-related enzymes are stimulated in this fish under hyper-ammonia stress is difficult to explain with the available data. However, the possible strategies of enzymatic induction could

Table 7
A balance sheet of nitrogenous excretion/uptake and accumulation (all as mmol N) for 100 g body wt. of *C. batrachus* exposed to water as control and 25 mM NH_4Cl

	Nitrogen excretion/uptake/accumulation (mmol)		
	Control	25 mM NH_4Cl	Difference
Excretion/uptake from 100 g fish[a]			
Ammonia	+25.3	−21.9	−47.2
Urea-N	+8.15	+43.8	+35.3
Reduction in nitrogenous excretion			−11.9
Retention in different tissues[b]			
Liver (2 g)			
Ammonia	0.018	0.045	+0.027
Urea-N	0.018	0.056	+0.038
Non-essential FAAs[c]	0.061	0.158	+0.095
Muscle (50 g)			
Ammonia	0.295	0.66	+0.365
Urea-N	0.190	0.46	+0.270
Non-essential FAAs	1.726	3.189	+1.463
Kidney (1 g)			
Ammonia	0.01	0.025	+0.015
Urea-N	0.008	0.022	+0.014
Non-essential FAAs	0.047	0.100	+0.053
Brain (0.5 g)			
Ammonia	0.0009	0.001	+0.00001
Urea-N	0.002	0.003	+0.001
Non-essential FAAs	0.007	0.013	+0.006
Plasma (4 g)			
Ammonia	0.003	0.01	+0.007
Urea-N	0.004	0.01	+0.006
Non-essential FAAs	0.007	0.013	+0.053
Increase in nitrogenous accumulation			+2.359

[a] Calculated from the rate of excretion/uptake (Fig. 2) (see Section 2).
[b] Amount present in the tissues on day 7 of the experiment.
[c] For glutamine and asparagine the nitrogen accumulation was taken as 2 N.

be through changes in the concentration of various effector molecules, and/or by changing the phosphorylation status of the pre-existing enzymes (for review, see Anderson, 2001). Kong et al. (2000) reported that increases of mRNA levels of GS in the gulf toadfish during 48 h of confinement stress, along with the increased GS enzyme concentration. Increases of GS protein and GS mRNA in different tissues of *Bostrichthys sinensis* were also observed at high ammonia exposure (Anderson et al., 2002). Therefore, the regulation of these enzymes at the transcriptional level cannot be ruled out in the walking catfish under hyper-ammonia stress. Recently, a significant increase of GDH (reductive amination), GS and AST activities accompanied significant accumulation and efflux of non-essential FAAs in the perfused liver of this fish following infusion with NH_4Cl (Saha et al., 2000). This suggests that the accumulation of ammonia in various tissues under hyper-ammonia stress could be another potential means to stimulate the activity of these enzymes.

It is evident from the results of this experiment that active synthesis and accumulation of non-essential FAAs in this walking catfish play significant roles for the detoxification of accumulated ammonia under hyper-ammonia stress. However, to assess the importance of amino acid synthesis vs. urea synthesis in the process of ammonia detoxification, the nitrogenous balance was calculated from the total accumulation/uptake of ammonia, urea and amino acids, and also from the total excretion of ammonia and urea-N (Table 7). In the NH_4Cl-exposed fish, there was total inhibition of ammonia excretion. This was accompanied by more uptake of ammonia from the external medium, thus causing a total uptake of 47.2 mmol of ammonia assuming that the production rate of ammonia remained same as in the control fish. Out of this total uptake of ammonia, 35.3 mmol were excreted as urea-N, and 0.41, 0.33 and 1.67 mmol were accumulated in the form of ammonia, urea and non-essential FAAs, respectively, by day 7, leaving a balance of 9.36 mmol of ammonia. Thus, suggesting that the enhanced rate of urea synthesis due to accumulation of ammonia during exposure to high ambient ammonia possibly play a more significant role in the process of ammonia detoxification in this walking catfish. From the nitrogenous balance sheet, it is also evident that there was overall decrease in the nitrogen excretion by the NH_4Cl-exposed fish. There could be two possible reasons of decreasing the nitrogenous waste excretion by the NH_4Cl-exposed fish: (i) decrease in amino acid catabolism rate as suggested in mudskippers (Lim et al., 2001) and loach (Chew et al., 2001) during aerial exposure mainly to avoid the ammonia toxicity, and (ii) excretion of nitrogenous wastes in some other forms other than the ammonia and urea such as the amino acids. A detailed investigation on these aspects would possibly clarify these points. However, due to the presence of these physiological mechanisms related to amino acid metabolism, along with the capacity to stimulate ureogenesis via the induced urea cycle, this unique group of fish is able to tolerate high ambient ammonia concentrations which they face at some seasons of the year.

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