UREOGENESIS AND ITS REGULATION IN A FRESHWATER AIR-BREATHING TELEOST, *Heteropneustes fossilis*

ABSTRACT

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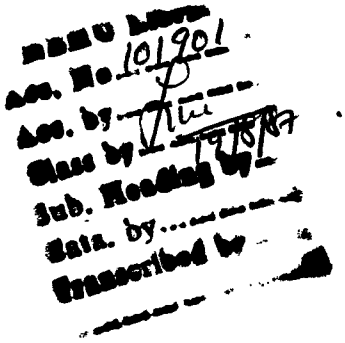


ABSTRACT

Nature of the excretory product in animals has been strictly dependent upon the availability of water. Freshwater teleosts are ammoniotelic excreting primarily ammonia mostly by diffusion through gills. Ammonia being highly toxic has to be excreted out immediately after its production from the catabolism of proteins and amino acids **in vivo**. It cannot be stored in the body due to its toxicity and needs large amount of water for its excretion. This has been possible in the aquatic animals such as fishes making them ammoniotelic. Fishes excrete ammonia by diffusion through gills to the abundantly available water in their natural habitat. Presence of small amount of urea in the excretory products and also in the tissues has been reported in some freshwater teleosts. However, the source of this urea and its physiological significance are not yet clear. In terrestrial animals such as amphibians and mammals ammonia diffusion was not possible. The accumulated ammonia was converted to urea through ornithine-urea (o-u) cycle. Urea is less toxic and being highly soluble needs lesser amount of water for excretion. It can also be retained in the body upto a considerable concentration. There have been reports of amphibians being ammoniotelic in water and ureotelic on land. This shift to ureotelism has been attributed to water restriction. Some fishes such as lungfish and mudskipper fish are capable of spending sometime outside

water and some of them also undergo aestivation. Ammonia accumulates due to lack of its excretion during these conditions. The presence of functional o-u cycle and the conversion of greater part of accumulated ammonia to urea through o-u cycle as physiological adaptation during water restriction have been reported in these fish.

Urea is also known to play a significant role for osmoregulation in addition to detoxification of ammonia in some groups of animals. In all the marine amphibians, elasmobranchs and also in many primitive fishes urea has been reported to be synthesised actively via o-u cycle and retained inside the body mainly for maintaining osmotic balance.

Urea can be formed through uricolytic pathway or by the catabolism of dietary arginine besides o-u cycle. In all ureotelic animals urea is formed mostly through o-u cycle. Presence of a functional o-u cycle in freshwater teleosts was questioned by various workers. Traces of urea found in the excretory products and also in some tissues of freshwater teleosts were suggested to come either from uricolytic pathway or by the catabolism of dietary arginine or both. Ammonia excretion being easy and osmotic problem reversed during the evolution of freshwater teleosts, the presence of the energy linked complicated o-u cycle was not expected in freshwater fishes. Some attempts have been made to find out a

functional o-u cycle in freshwater teleosts since 1960. Brown and Cohen (1960) could not detect some of the enzymes of o-u cycle in several freshwater teleosts studied and suggested that the genes responsible for these enzymes might have got deleted in freshwater teleosts in the process of evolution. This has been known as the 'deletion' hypothesis. Wilson (1973) also supported this view as he could not detect some of the enzymes of o-u cycle in the channel catfish, **Ictalurus punctatus**. However, Huggins **et al** (1969) could detect the full complement of o-u cycle in some freshwater teleosts with of course very low enzyme activity. They suggested that the expression of the genes responsible for the synthesis of o-u cycle enzymes in freshwater teleosts might have altered due to an adaptational change instead of being deleted. Most of the freshwater teleosts studied so far by various groups did not show any promise for adaptation to environmental changes at the level of ureogenesis particularly through o-u cycle.

However, there are some freshwater teleosts which are primarily aquatic but breath predominantly air through their secondary respiratory organs. Sometimes, they are exposed to air and tolerate short periods of dehydration. Some of them are capable of living successfully in sewage fed water bodies and in paddy fields with high ammonia concentration. Such freshwater teleosts might have some special adaptive

physiological regulatory mechanism(s) in their nitrogen metabolism and excretion like amphibians and lungfishes to manage ammonia toxicity during the periods of water deprivation or higher ambient ammonia in aquatic medium. There has not been any systematic study on this aspect which can also throw some light on the evolutionary modifications in the nitrogen metabolism and excretion in freshwater fishes.

Several species of freshwater air-breathing teleosts are available in this sub-continent including **Heteropneustes fossilis, Clarias batrachus, Anabas testudineus, Channa punctatus** and **Amphipnous cuchia** which were used in the present study. They are primarily aquatic but breath predominantly air. They inhabit usually in stagnant and slow flowing shallow water bodies. During drought conditions, they live inside mud and tolerate temporary dehydration when kept outside water. **A. cuchia** has an eel or snake like body and has almost completely lost the power of aquatic respiration with rudimentary gills.It spends most of its time on the bank of ponds and rivers and also can burrow inside mud during drought conditions.

Therefore, the present study has been aimed to find out the following in the above mentioned five species of freshwater air-breathing teleosts.

1. Pattern of excretion and tissue levels of ammonia and urea.

2. Probable sources of urea by studying o-u cycle and uricolytic pathway enzymes.
3. The regulation in the excretion of ammonia and urea, and ureogenesis under different environmental conditions such as higher ambient ammonia, dehydration and higher-osmolar ambient medium using one of the five species (**H. fossilis**) as a model. The results have been presented in five different chapters in the thesis as follows.

CHAPTER I: (Normal excretion pattern and tissue level of ammonia and urea) This chapter contains the studies on diurnal pattern of excretion and level of ammonia and urea in different tissues such as liver, kidney, muscle, brain, gill, skin and blood plasma of the five species of freshwater air-breathing teleosts in the aquatic medium. This chapter also includes the studies on diurnal pattern of renal and extra-renal excretion of ammonia and urea in **H. fossilis** while in water. The survival period outside water for the five species was also reported.

CHAPTER II: (Normal ureogenesis) This chapter includes the findings on the activity of five ornithine-urea cycle enzymes both in liver and kidney of above mentioned five species of freshwater air-breathing teleosts while in water. It also includes the results of the assay of the three uricolytic pathway enzymes in different tissues of **H. fossilis** in aquatic medium.

CHAPTER III: (Hyper-ammonia stress) This chapter presents the results on the tolerance limit for ambient ammonia and changes in excretion pattern and concentration of ammonia and urea in different tissues. Alterations in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with higher concentration of NH_4Cl in the medium for 28 days also have been included.

CHAPTER IV: (Dehydration stress) This chapter deals with the changes in excretion pattern and concentration of ammonia and urea in different tissues, and the alterations in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** kept ouside water for 48 hrs.

CHAPTER V: (Hyper-osmotic stress) This chapter presents the results on the osmotolerance limit and changes in excretion and concentration of ammonia and urea in different tissues. It also presents the changes in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with 250 mOsm mannitol for 28 days.

Physiological level of ammonia was found maximum in various tissues of **C. punctatus** and minimum in **A. cuchia.** However, urea level was found maximum in **A. cuchia** and minimum in **C. punctatus.** This has been correlated with their capacity to survive outside water. **A. cuchia** was found to survive

maximum (90-100 hrs) while **C. punctatus** survived for minimum time (8-12 hrs) outside water. Both **H. fossilis** and **C. batrachus** survived for 60-70 hrs and **A. testudineus** for 24-36 hrs. In all the air-breathing fishes studied the urea level was found higher and ammonia level lower than other purely freshwater teleosts reported earlier.

Ammonia was found to be the major excretory product in the five species like other freshwater teleosts in aquatic medium. However, the rate of excretion of urea was found higher than reported earlier in other freshwater teleosts. Both ammonia and urea were excreted mostly through extra-renal sources in **H. fossilis**. Higher tissue level and higher rate of excretion of urea in four out of five species of freshwater air-breathing teleosts studied were suggestive of their capability for converting ammonia to urea **in vivo** through an active o-u cycle.

The above suggestion found support in the presence of functional o-u cycle in at least four (except **C. punctatus**) out of the five species studied. The activity of all the enzymes was found very high compared to purely freshwater teleosts, aquatic lungfish (**Neoceratodus forsteri**) and freshwater sting rays. The enzyme activities were similar or nearer to those of aestivating lungfish (**Protopterus**) and aquatic amphibia (**Xenopus laevis**) where urea synthesis through o-u

cycle have been confirmed. The uricolytic pathway enzymes were also found to be present in **H. fossilis.** The activity of the enzymes of this pathway were found, in general lower than other freshwater teleosts. Synthesis of urea calculated from the relative activity of the enzymes seemed to be more through o-u cycle than uricolytic pathway in **H. fossilis.** The results obtained indicate that 'deletion' hypothesis is not applicable at least to freshwater air-breathing teleosts. The ability to synthesise urea by two distinct pathways by any freshwater teleost is a unique finding resembling more with their marine ancestors and aestivating dipnoan lungfish, **Protopterus.** Some of the freshwater teleosts such as the air-breathing species studied have still retained the functional o-u cycle besides uricolytic pathway for active ureogenesis. It was also found that the o-u cycle could be regulated by various environmental factors.

H. fossilis treated with different concentrations of NH_4Cl, tolerated upto 75 mM NH_4Cl for at least 28 days without any apparent deleterious effect. This high tolerance to ambient ammonia was several times higher than those reported for other freshwater teleosts and even aquatic amphibia, **Xenopus laevis. H. fossilis** was treated with 25, 50 and 75mM NH_4Cl solution for 28 days and the pattern of excretion, levels of ammonia and urea in different tissues and in blood plasma, and the alterations in the activity of o-u cycle

enzymes in the liver and kidney were studied at different time intervals. Absorption of ammonia dominated over excretion during the exposure of the fish to all the concentrations of NH_4Cl. In 25 mM, ammonia excretion became normal during later periods of the experiment. Urea excretion was suppressed immediately after treatment in different concentrations of NH_4Cl. After 6-8 days, urea excretion rate increased by 2-3 fold and continued at that higher level till the end of the experiment. Absorption of ammonia from the medium was accompanied by the increase in the tissue ammonia level which reached its maximum on the 7th day of treatment. There was no further increase during the later periods of treatment. Accumulation of urea was also noticed in all tissues of treated fish probably for osmotic balance. All the enzymes of o-u cycle except arginase (ARG) were induced both in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl. There were correlations between the accumulation of ammonia and urea with alterations in the rate of their excretion and the induction of o-u cycle enzymes. Increase in ammonia level in different tissues might be the primary factor for induction of o-u cycle enzymes for mainly detoxification of accumulated ammonia and retention of urea for osmoregulation. The urea excretion also enhanced after the induction of o-u cycle enzymes.

H. fossilis was subjected to dehydration stress by keeping outside water in glass jar for 48 hrs. There was

an immediate suppression of both ammonia and urea excretion rate under aerial exposure. Ammonia excretion reduced by 75% at the initial stage which continued further to 85% at later period of emersion. However, the urea excretion rate which was reduced by 40% at the initial stage returned almost to normal level during the later period of the experiment. Decrease in ammonia and urea excretion was accompanied by linear increase in their levels in almost all tissues. All the enzymes of o-u cycle except ARG were also induced significantly both in liver and kidney of **H. fossilis** during dehydration. Accumulation of ammonia to the toxic level in different tissues was perhaps the main cause again for induction of o-u cycle enzymes reesulting in a shift towards ureotelism under dehydration stress in **H. fossilis**. This physiological adaptation might have helped the animal to avoid accumulation of toxic ammonia **in vivo.**

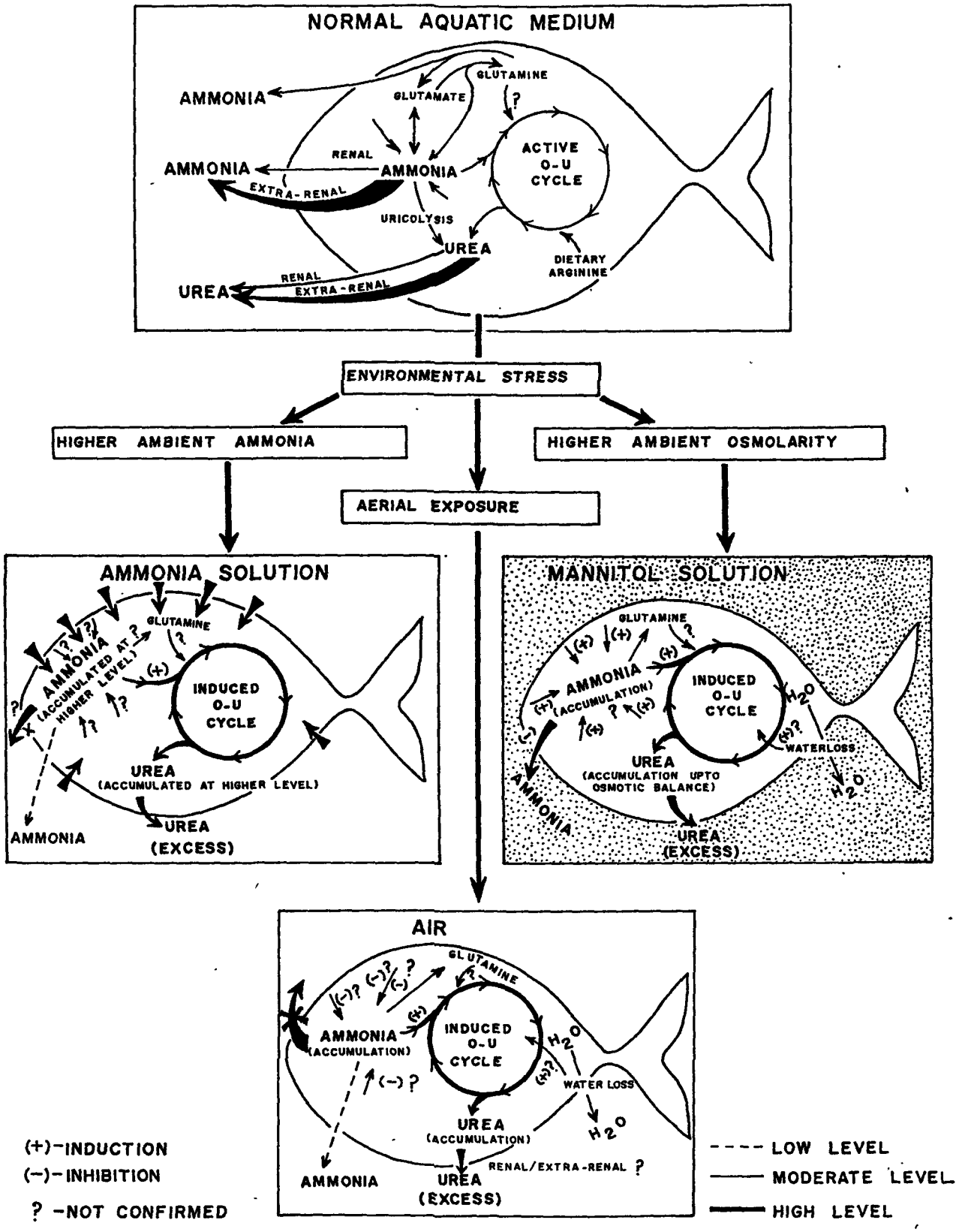
Treatment with different concentrations of mannitol indicated that **H. fossilis** could tolerate easily upto 300 mOsm of mannitol solution without any visible deleterious effect. However, the long term experiments were conducted in **H. fossilis** treated with 250 mOsm mannitol for 28 days. Immediately after treatment with 250 mOsm mannitol there was a suppression in the rate of excretion of both ammonia and urea by **H. fossilis**. The excretion of ammonia by treated fish remained at lower level than the control all through

the experiment. However, the urea excretion rate returned to normal after 8-10 days and then increased significantly during the later period of the treatment. There was no increase in ammonia level in any tissue except in the kidney estimated on 7, 14, 21 and 28 days of treatment. However, accumulation of urea was noticed in all the tissues studied. Maximum accumulation was observed by 14th day and it was maintained at that level till the end of the experiment. More excretion and accumulation of urea were accompanied by the induction of all the enzymes of o-u cycle except ARG both in the liver and kidney of **H. fossilis.** It might be possible that increase in tissue ammonia level might have occurred due to the suppression of its excretion immediately (within a day or two) after exposure of **H. fossilis** to hyper-osmotic medium. That enhanced ammonia level possibly led to the induction of o-u cycle resulting in the decrease in ammonia level (by 7th day) and increase in urea level in various tissues for ammonia detoxification and osmoregulation. Thus, induction of o-u cycle for urea synthesis and its accumulation under hyper-osmolar stress were found to play a significant role in maintaining osmotic balance in **H. fossilis.**

Above findings have made it clear that the o-u cycle is not only functional in freshwater air-breathing teleosts but also can be physiologically regulated to provide better adaptation to the freshwater air-breathing teleosts under

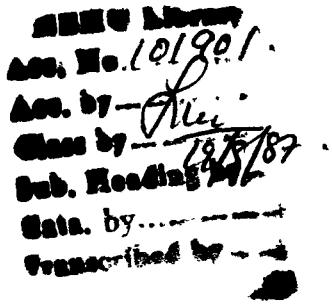
various environmental conditions such as higher ambient ammonia, dehydration and hyper-osmotic stress. Increase of ammonia level in different tissues under the above mentioned three environmental conditions was suggested to be the major factor for induction of o-u cycle enzymes in freshwater air-breathing teleosts. Water loss from the tissues might have also played some part in the process. A model has been proposed to explain the regulatory mechanism of o-u cycle in freshwater air-breathing teleosts under the three environmental conditions (see page 13).

Freshwater air-breathing teleosts have shown unique physiological adaptive mechanisms for ammonia metabolism which has given them the capacity to tolerate higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Thus, freshwater air-breathing teleosts show all the three urea producing characters such as ureogenic - having the full complement of o-u cycle enzymes, ureotelic - having the capacity to synthesise through o-u cycle and to excrete sufficient urea and ureosmotic - having the capacity to synthesise and accumulate urea for osmoregulation. It is further suggested that the freshwater air-breathing teleosts might be either relatively primitive to the present day freshwater teleosts and the genes for o-u cycle enzymes might have been repressed at a later stage of evolution. Alternately it could be possible that these genes might have been derepressed as a secondary modification in these fish for their ability



A diagramatic model for regulation of ureogenesis under environmental stress in freshwater air-breathing teleosts.

to adapt to higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Therefore, freshwater air-breathing teleosts have a separate physiological status and should be reclassified as an independent group among the freshwater teleosts. Studies on these fish might connect many missing links in the evolutionary story of regulation of nitrogen metabolism during teleostean evolution.



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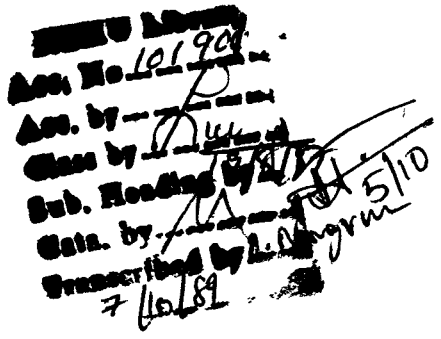

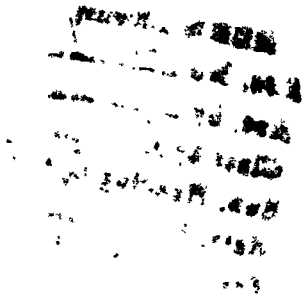

DEDICATED

TO

MY BELOVED FATHER

NORTH-EASTERN HILL UNIVERSITY

Biochemical Adaptation Laboratory : Department of Zoology,
Shillong-793014, INDIA

Dr. B. K. RATHA, Ph. D.

Certified that the thesis entitled "UREOGENESIS AND ITS REGULATION IN A FRESHWATER AIR-BREATHING TELEOST, **HETEROPNEUSTES FOSSILIS**", submitted by Mr. Nirmalendu Saha for the degree of DOCTOR OF PHILOSOPHY in Zoology of the North-Eastern Hill University, Shillong embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph. D. Degree. This work has not been submitted for any degree of any University.

SHILLONG,
Dated 29/X/1986

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Nirmalendu Saha

SHILLONG

Date: 28 October 1986

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CONTENTS

GENERAL INTRODUCTION

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Nitrogen metabolism is one of the major pathways of energy supply in all animals. Normal dietary intake of protein by animals provides amino acids excess of the amount required for the synthesis of new proteins to sustain protein turnover. Excess amino acids cannot be stored (as stored proteins) as can be carbohydrates (as glycogen) or lipids (as fat), except for the special proteins such as vitellogenin in egg. Therefore, excess amino acids are deaminated and the carbon residues either oxidized via the TCA cycle for energy production or used in glycogenesis or lipogenesis. Ammonia which is produced from deamination of amino acids is highly toxic and cannot be retained inside the animal for longer time even in very low concentrations. It is either excreted out immeditely or converted to some less toxic substance such as urea, uric acid, some amino acids for temporary storage inside the animal and excretion.

Aquatic animals usually excrete ammonia by diffusion to external aquatic medium immediately after its formation due to its high toxicity (Forster & Goldstein, 1969; Campbell, 1973; Watts & Watts, 1974). In terrestrial animals where water availability became limited ammonia excretion became difficult. Many terrestrial animals converted ammonia to either urea or some other compounds for temporary storage

and excretion with less amount of water in the form of urine. Insoluble uric acid became the excretory product in those animals where conservation of metabolic water became highly essential due to their arid environment. The animals have been classified depending on their major nitrogenous waste product into the three following groups.

(i) Ammoniotelic: Animals which excrete ammonia as the major excretory product as in most of the aquatic animals where ammonia diffuses out into the plentily available water in the environment.

(ii) Ureotelic: Animals which excrete urea as the major excretory product as in mammals and amphibians where water availability is enough to remove soluble urea in concentrated form in urine.

(iii) Uricotelic: Animals which excrete insoluble uric acid as the major excretory product as in some insects, reptiles and birds where water availability being restricted conservation of metabolic water became essential.

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

during later stages of development. It has been suggested that nitrogen excretory pattern is one of the most sensitive physiological processes to respond effectively to environmental variations (Gordon, 1970).

Ammonia is excreted primarily by diffusion through the gills of fish and a small fraction of the total nitrogen excreted by fish appears in the urine. Earlier studies have shown that urine of freshwater and marine fish both fasted and fed, had very low nitrogen values (Denis, 1913-1914; Marshall & Graffin, 1928; Edwards & Condorelli, 1928; Grollman, 1929). The classical experiment of Smith (1929) with goldfish and carp showed that about 90% of the ammonia was excreted through gills and the rest through urine and may be some amount through skin. Later this has been supported by various workers (Fromm, 1963; Fromm & Gillette, 1968; Vellas & Serfaty, 1974; McLean & Fraser, 1974; Payan & Matty, 1975). Most of the ammonia were found in the liver and some in the kidney of fish (Pequin & Serfaty, 1963; Vellas & Serfaty, 1974).

Ammonia has many advantages as an end product of nitrogen metabolism (Campbell, 1973).There is no expenditure of energy for the conversion of protein nitrogen to ammonia. Instead, some of the reactions involved in the production of ammonia such as deamination of glutamate through glutamate dehydrogenase (GDH) activity, ultimately produce energy (Bessman & Pal, 1976). Due to small molecular size, high solubility in water as free base and higher partition coefficient, ammonia

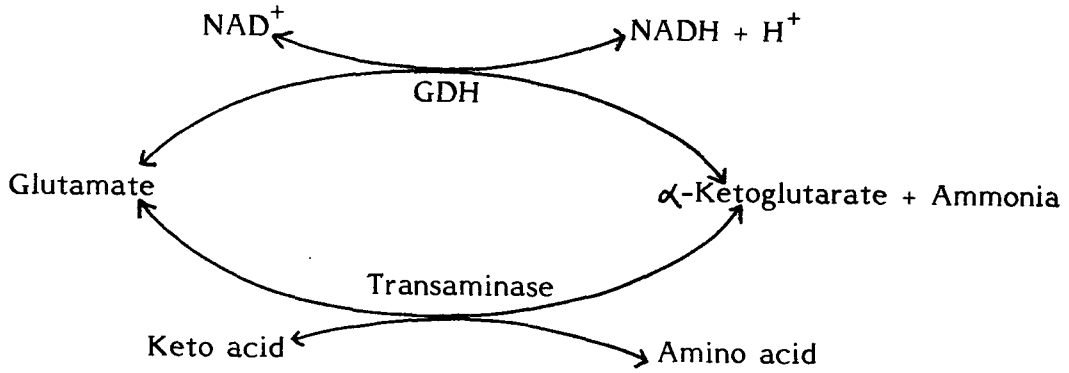
is easily eliminated by diffusion (Forster & Goldstein, 1969). Maetz and Garcia (1964) and Evans and Cameron (1986) have demonstrated the ability of NH_4^+ to exchange with Na^+ absorption by the gills of freshwater fish. Absorption of Na^+ has been critically important in maintaining salt and water balance in freshwater teleosts. Hence, in freshwater fishes the exchange of NH_4^+ for Na^+ serves the dual purpose of elimination of the nitrogenous waste product (NH_4^+) and absorption of Na^+ from the external freshwater environment.

Formation of ammonia:

Ammonia production in mammals has been shown by deamination of amino acids, amides, nucleosides and nucleotides (Cohen & Brown, 1960). However, in fish, protein and amino acids are the major sources of excreted nitrogen (Walton & Cowey, 1977, 1982). Amides, nucleosides and nucleotides have also been identified as the precursors of ammonia in many species (Forster & Goldstein, 1969; Watts & Watts, 1974). The precise mechanisms of ammonia formation are still not completely known in fish. Available reports suggest that the major pathway of ammoniogenesis is via the transdeamination of amino acids (Walton & Cowey, 1982; Campbell **et al**, 1983) besides deamination (Watts & Watts, 1974).

Transdeamination: The chief source for about 90% of the total nitrogen liberated is the -amino-nitrogen of surplus amino acids (Baldwin, 1970). In serine and probably in histidine

and cysteine the amino group is directly deaminated to form ammonia (Watts & Watts, 1974). However, in case of most of the amino acid the amino group is transferred to another keto acids forming a new amino acid. The dissociated amino group tends to be channeled directly or indirectly through the formation of glutamic acid. Glutamate undergoes an oxidative deamination catalyzed by the enzyme glutamate dehydrogenase (GDH) yielding ammonia. The over all reaction of liberation of ammonia from amino acids via glutamate formation is known as transdeamination (Braunstein, 1939) which may be summarized in the following reactions:



The reaction catalyzed by GDH is reversible and it functions in the reductive or oxidative direction depending upon the substrate being utilized (Chamalaun & Tager, 1970). Therefore, GDH has been considered to hold the regulatory link between energy production and nitrogen metabolism (Hochachka & Somero, 1973). The enzyme from mammalian sources has been shown to utilize NAD^+ or NADH equally well **in vitro**, although it has been claimed that the enzyme **in vivo** favours the glutamate (specific for NADH) formation (McGiven & Chappel,

1975). In some invertebrates GDH has been shown to favour glutamate oxidation (Goldin & Frieden, 1971; Smith **et al**, 1975, Storey **et al**, 1978).

The activity of GDH has been detected in a variety of fish species (Forster & Goldstein, 1969; Watts & Watts 1974) with liver showing the highest level of activity. GDH has been considered as the N-storage enzyme playing an important role for ammonia formation in fish (Forster & Goldstein, 1969; Walton & Cowey, 1977).

Deamination: The experiments of Van Slyke **et al** (1943) showed that glutamine, an amide, acts for temporary storage and transport of ammonia. Glutamine is deaminated through hydrolytic removal of secondary amino group by the enzyme glutaminase.

$$\text{Glutamine} + H_2O \xrightarrow[\text{(PDG/PIG)}]{\text{Glutaminase}} \text{Glutamate} + NH_3$$

The activity of glutaminase exists in the form of two isoenzymes in various organs (Curthoys & Lowry, 1973; Kalra & Bronsan, 1973, 1974). One of these isoenzymes is phosphate dependent glutaminase (PDG or glutaminase-I) which requires phosphate for its activity. The other isoenzyme is phosphate independent which is activated by maleate (PIG or glutaminase-II). In most of the fish, so far studied for glutaminase activity, only PDG activity was detected. Walton and Cowey (1977) could not detect PIG activity in different tissues of rainbow trout.

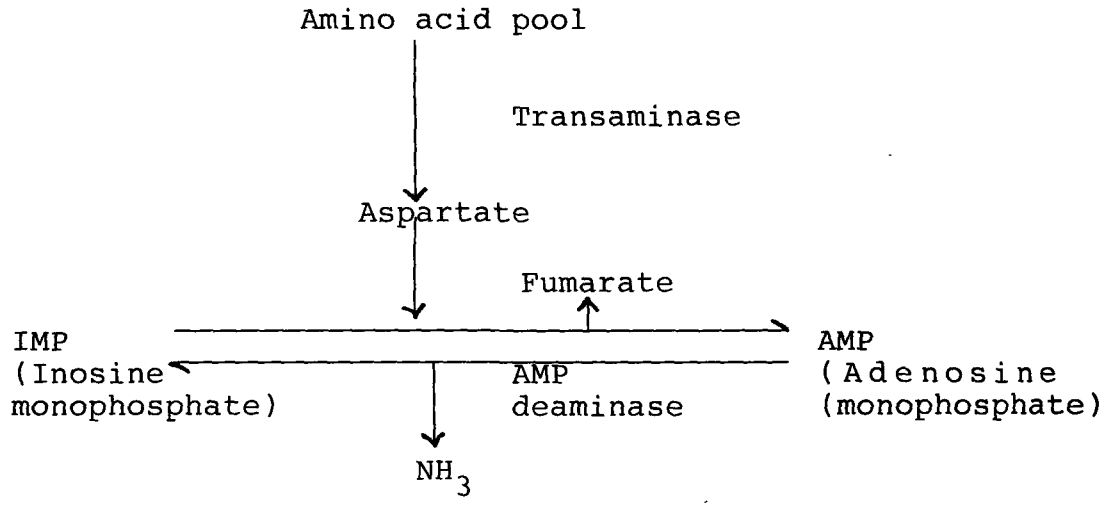
Direct deamination of aspartate, cysteine and histidine may also contribute to the production of ammonia. These deamination reactions are catalyzed by respective amino acid deaminases (Salvatore **et al**, 1965; Janicki & Lingis, 1970).

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

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

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

Hydrolysis of, particularly, adenosine monophosphate (AMP) could be ultimately utilized for deamination of amino acid as follows:

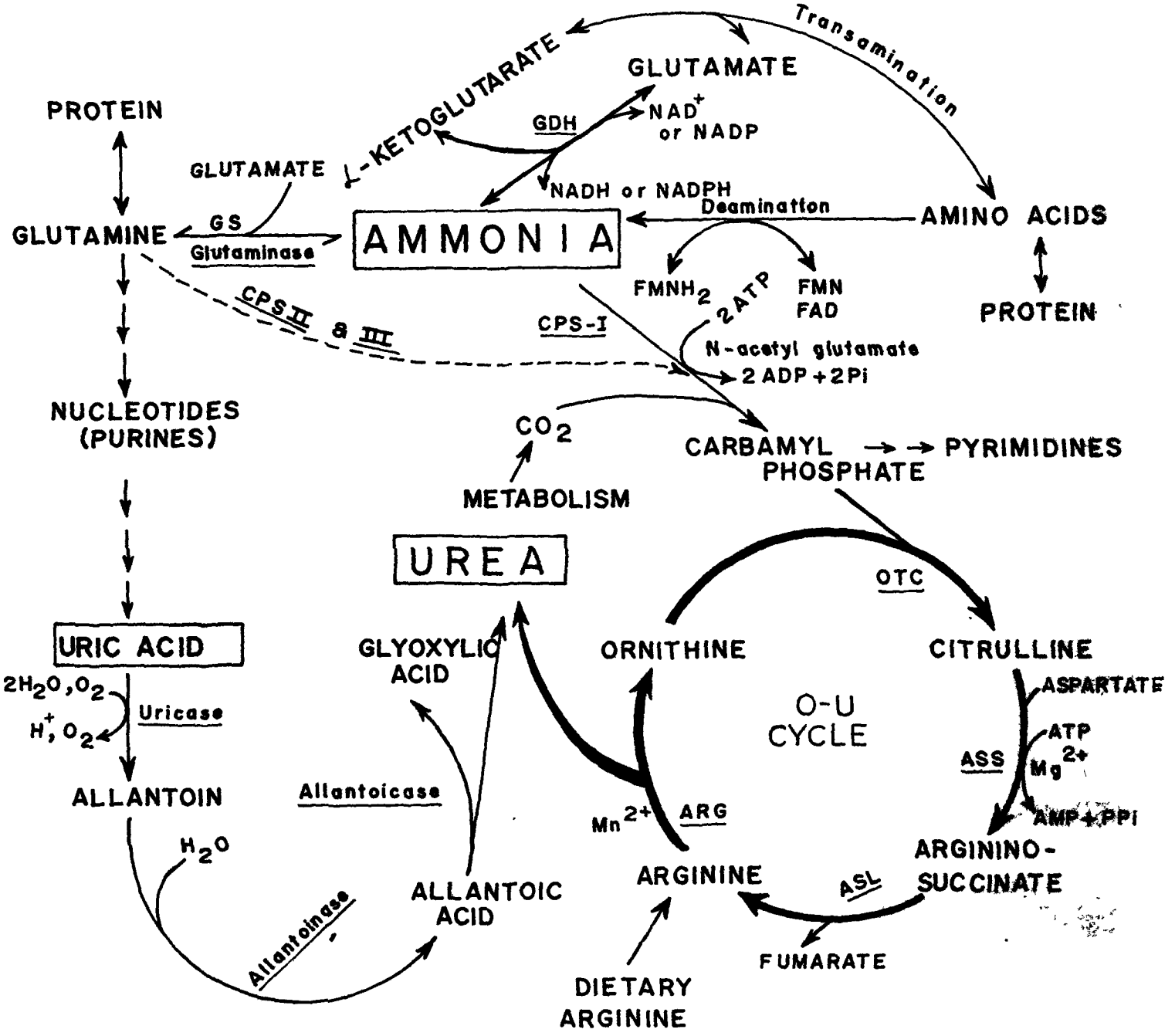


The role of AMP deaminase has been shown to be more important in ammonia production in some fishes (Makarewicz & Zydowo, 1962; Makarewicz, 1963) and glutaminase in some other (Walton & Cowey, 1977).

Formation of urea:

Excretion of ammonia being easy in aquatic animals, its conversion to urea for excretory purposes was not necessary in fishes as the process was energy dependent. However, some urea has been reported both in the excretory products and in tissues of several fishes besides the marine fishes where urea production and accumulation has been for osmoregulation. The formation of urea in fish has been suggested to be through either one or more of these three pathways such as (i) ornithine-urea (o-u) cycle, (ii) uricolytic pathway, and (iii) catabolism of dietary arginine (Fig. 1).

Ornithine-urea (o-u) cycle: In most of the terrestrial animals, ammonia is converted to a relatively less toxic form, urea via o-u cycle (Krebs & Henseleit, 1932). The o-u cycle involves five enzymatic steps, each step being catalyzed by a specific enzyme (Krebs & Henseleit, 1932; Brown & Cohen, 1959). The five enzymes involved in the o-u cycle are carbamyl phosphate synthetase (CPS), Ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) (Fig. 1).



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

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

The first enzyme of o-u cycle is CPS. Three different types of CPS have been identified depending on the utilization of nitrogen donating substrate and the cofactor used in the reaction. They have also functional variations. CPS-I is located specifically in the liver mitochondria of ureogenic animals requiring N-acetyl-L-glutamate (NAG) as cofactor and utilizing only ammonia as the nitrogen donating substrate. It is related specifically to urea synthesis (Ratner, 1973; Marshall, 1976). CPS-II does not require NAG as a cofactor and utilizes glutamine as the physiologically significant nitrogen source (ammonia can be used only at very high concentration). It has been related specifically to pyrimidine synthesis and located in the cytosol (Ratner, 1973). The presence of CPS-II activity in any species of fish has not been demonstrated. Tramell and Campbell (1970) reported a third type of CPS (CPS-III) in several species of invertebrates. It uses glutamine as nitrogen donating substrate and like CPS-I requires NAG as cofactor. CPS-III has been reported in freshwater teleost, **Micropterus salmoides** (Anderson, 1976) and in elasmobranchs (Anderson, 1980, 1981). It was located exclusively in the mitochondria (Casey & Anderson, 1982, 1985) and has been suggested to be related to urea synthesis. The second enzyme of o-u cycle, OTC has been located within the mitochondrial matrix in all ureotelic and ureosmotic vertebrates (Ratner, 1973; Gamble & Lehninger, 1973; Vorhaben & Campbell, 1977; Casey & Anderson, 1985). The third (ASS), fourth (ASL) and fifth (ARG) enzyme of o-u cycle have been

shown to be cytosolic in several ureotelic species (Ratner, 1973). However, Casey and Anderson (1985) reported the presence of arginase in the liver mitochondria of dogfish, **Squalus acanthias.**

The presence of functional o-u cycle in elasmobranchs and lungfishes (Brown & Cohen, 1959; Forster & Goldstein, 1966; Schooler **et al**, 1966; Janssens & Cohen, 1966; Huggins **et al**, 1969) and in marine teleosts (Huggins **et al**, 1969; Read, 1971) have been reported. However, a physiologically functional o-u cycle has not yet been reported in freshwater teleosts. Brown and Cohen (1960) could not detect CPS and OTC activity in several species of freshwater teleosts studied by them. However, Huggins **et al** (1969) could detect all the enzymes of o-u cycle in some freshwater teleosts. The activity of almost all the enzymes were so low that no physiologically significant function could be attributed to them. They divided the urea producing fishes into three categories on the basis of their finding "without unduly disturbing established usage". The three groups were ureogenic, ureotelic and ureosmotic as explained below.

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

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

Ureosmotic: These animals produce urea to help maintain its osmotic equilibrium with the environment.

Uricolytic pathway: In teleosts, elasmobranchs and lungfishes degradation of uric acid (uricolysis) has been proposed to be one of the sources for urea production (Brunel, 1937; Florkin & Duchateau, 1943; Goldstein & Forster, 1965; Brown **et al**, 1966; Cvancara, 1969a; Vellas & Serfaty, 1974). Brunel (1937) proposed uricolysis as a three step enzymatic process (Fig. 1) involving three enzymes such as uricase, allantoinase and allantoicase. He further suggested that in some teleosts where allantoicase was lacking the end product of purine catabolism was allantoic acid, instead of urea. This proposal was contradicted by Golstein and Forster (1965) who detected the activity of allantoicase in various groups of fishes by improved assay technique. Cvancara (1969a) studied the individual enzymes of uricolytic pathway in several freshwater teleosts and suggested that uricolysis could be one of the major sources of urea in freshwater teleosts. Brown **et al** (1966) could find more urea formation via the uricolytic pathway than o-u cycle in **Protopterus**. However, Forster and Goldstein (1966) reported that urea formation was 100 times more via o-u cycle than uricolytic pathway in lungfishes.

Dietary arginine: Arginase the last enzyme of o-u cycle which converts arginine to urea and ornithine has been reported to be present in various tissues of freshwater teleosts with high activity (Brown & Cohen, 1960; Huggins **et al**, 1969; Cvancara, 1969b; Wilson, 1973a). Cvancara (1969b), therefore suggested that dietary arginine could be one of the major sources of urea in freshwater teleosts.

Adaptation of nitrogen metabolism in fishes: The importance of adaptation in nitrogen metabolism for better survibility of animals has been already discussed. The alterations in the pattern of nitrogen metabolism have been mainly dependent upon the availability of water to the animal. Fishes are mostly ammoniotelic in their aquatic habitat where they excrete the excess ammonia from the body by diffusion through gills. Some groups of fishes such as lungfishes and mudskipper fishes are known to tolerate periods of water restriction like amphibians. They have been shown to produce urea as the nitrogenous end product for excretion during this period and the bulk of this has been suggested to be synthesized via the o-u cycle. This conclusion has not found much favour for freshwater teleosts due to the obvious reason of either absence or very low activity of o-u cycle enzymes. A highly specialized teleost, the mudskipper fish (**Periophthalmus sobrinus**) which could live in intertidal mangrove swamps, excreted both urea and ammonia (Gordon **et al**, 1969). On transfer to normal seawater this fish excreted 59.5% of the nitrogen as urea and 40.5%

as ammonia. It could also tolerate a wide range of salinities. Acclimated to 40% seawater, it excreted only 13.5% of the total nitrogen as urea and rest (86.5%) as ammonia. Forster and Goldstein (1969) explained the alteration in ammonia and urea excretion rate under various salinity on the basis of water availability **in vivo**. At low salinity more water entered the fish increasing the blood volume and also blood flow through the gills, thus facilitating the rapid clearance of excess ammonia at low salinity.

African lungfish **Protopterus aethiopicus** excreted about 50% of its excreted nitrogen as ammonia and rest as urea even when in water (Smith, 1930). During aestivation, which occured when the pools in which they lived got dried up, the animals survived in the remaining mud wrapped in a cocoon like covering in a state of near inactivity. Ammonia could neither be excreted in this condition nor retained in the body. The total waste nitrogen production was reduced so that, without significant increase in the activity of o-u cycle enzymes, all ammonia produced could be converted to urea (Forster & Goldstein, 1966). The urea accumulated inside the body upto about 1% of the body weight after one year of experimental aestivation (Smith, 1930; Janssens, 1964). The accumulated urea was rapidly excreted when the animal returned to an aquatic environment. However, the purely aquatic lungfish **Neoceratodus forsteri**, had negligible urea excretion and excreted nearly twice as much ammonia per kg body weight per hr (Goldstein **et al**, 1967).

Urea as an excretory product may be critically important during embryonic development which takes place within a limited environment inside the egg membrane. The o-u cycle enzymes have been demonstrated in the embryo of **Squalus sucklevi** and **Raja binoculata** (Read, 1968). Forster and Goldstein (1969) suggested that the adaptive use of urea production must be of very ancient origin and its selective advantage might have played a key role in the evolutionary origin of urea excretion.

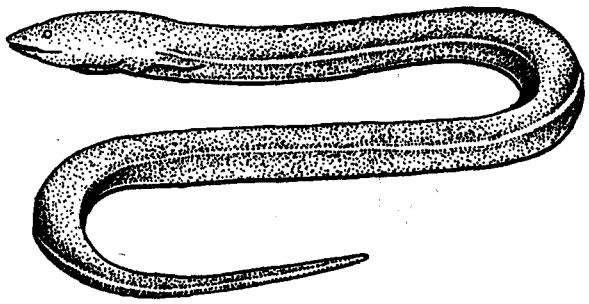
Most of the freshwater teleosts studied so far by various groups did not show any promise for adaptation to environmental changes at the level of ureogenesis particularly through o-u cycle. However, nature has also created a small group of freshwater teleosts which are primarily aquatic but have the ability to breath air through their secondary respiratory organs (Dutta & Datta Munshi, 1985). Therefore, these fish sometimes dare coming out of water and tolerate short periods of dehydration. Some of them are capable of living inside mud and some can live successfully in sewage fed water bodies with high ammonia levels. Such freshwater teleosts might possess some special adaptive physiological regulatory mechanism(s) for their nitrogen metabolism and excretion like the amphibians or lungfishes to manage their problem of nitrogen excretion during the periods of water deprivation or higher ambient ammonia in aquatic medium.

As early as 1969, Huggins **et al** had made a suggestion

while studying o-u cycle enzymes in freshwater teleosts that Indian tree fish (**Anabas**) might be having a functional o-u cycle to manage its problem of nitrogen excretion during temporary emergence. However, no systematic approach has been made in this regard to study the nitrogen metabolism in the air-breathing fishes available in India. Therefore, a beginning was made with the study of the mechanism of ureogenesis in five available species of freshwater air-breathing teleosts such as **Amphipnous cuchia, Heteropneustes fossilis, Clarias batrachus, Anabas testudineus** and **Channa punctatus** and its regulation in one of them (**H. fossilis**) under various environmental conditions which have been known to influence ureogenesis in other fishes and amphibians.

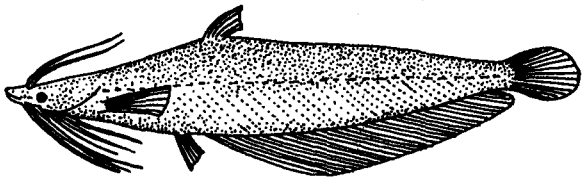
Several species of freshwater air-breathing teleosts are available in this sub-continent including **Heteropneustes fossilis, Clarias batrachus, Channa punctatus, Anabas testudineus** and **Amphipnous cuchia** (Fig-2) which were used in the present study. **H. fossilis, C. batrachus, Channa** spp. and **Anabas** spp. are widely distributed and used mainly as food fish. They are primarily aquatic but breath predominantly air. They inhabit usually in stagnant and slow flowing shallow water bodies. During drought conditions, they live inside mud and can also tolerate temporary dehydration when kept outside water (Jhingran, 1983). **A. cuchia** is found mainly in the northern part of India. It has an eel or snake like body and has almost completely lost the power of aquatic respiration with rudimentary gills. It spends most of its

Phylum - Chordata
Group - Vertebrata
Subphylum - Gnathostomata
Series - Pisces
Class - Teleostomi
Subclass - Actinopterygii
Order - Symbranchiformes
Genus - Amphipnous
Species - cuchia



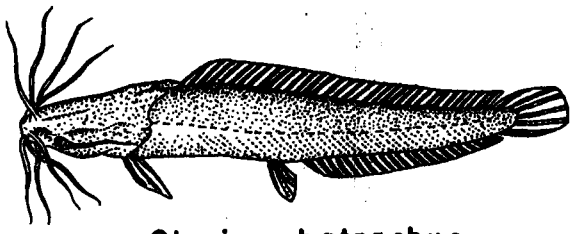
Amphipnous cuchia

Class - *Teleostomi*
Subclass - Actinopterygii
Order - Cypriniformes
Division - Siluri
Genus - Heteropneustes
Species - fossilis



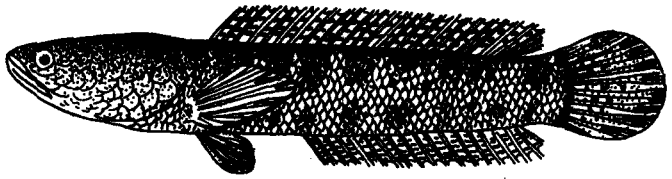
Heteropneustes fossilis

Class - Teleostomi
Subclass - Actinopterygii
Order - Cypriniformes
Division - Siluri
Genus - Clarias
Species - batrachus



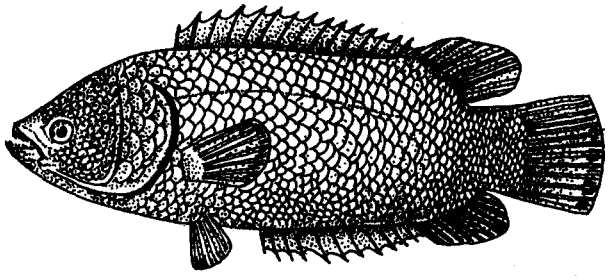
Clarias batrachus

Class - Teleostomi
Subclass - Actinopterygii
Order - Channiformes
Genus - Channa
Species - *punctatus*



Channa punctatus

Class - Teleostomi
Subclass - Actinopterygii
Order - Perciformes
Genus - Anabas
Species - testudineus



Anabas testudineus

Fig. 2. Five different types of freshwater air-breathing teleosts.

time on the bank of ponds and rivers, and can burrow inside mud during drought conditions (Beavan, 1982).

The work done has been presented in five separate chapters in this thesis besides a general discussion at the end for conclusion. A brief description of each chapter is given below.

CHAPTER I: (Normal excretion pattern and tissue level of ammonia and urea) This chapter contains the results on diurnal pattern of excretion and level of ammonia and urea in different tissues such as liver, kidney, muscle, brain, gill, skin and blood plasma of the five species of freshwater air-breathing teleosts in the aquatic medium. It also includes the studies on diurnal pattern of renal and extra-renal excretion of ammonia and urea in **H. fossilis** while in water. The survival period outside water for the five species has also been presented.

CHAPTER II: (Normal ureogenesis) This chapter includes the findings on the activities of five ornithine-urea cycle enzymes both in liver and kidney of above mentioned five species of freshwater air-breathing teleosts while in water. It also includes the results of the assay of the three uricolytic pathway enzymes in different tissues of **H. fossilis** in aquatic medium.

CHAPTER III: (Hyper ammonia stress) This chapter presents

the results on the tolerance limit for increased ambient ammonia and changes in excretion and concentration of ammonia and urea in different tissues. Alterations in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with higher concentration of NH_4Cl in the medium for 28 days also have been included.

CHAPTER IV: (Dehydration stress) This chapter deals with the changes in excretion and concentration of ammonia and urea in different tissues and the changes in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** kept outside water for 48 hrs.

CHAPTER V: (Hyper-osmotic stress) This chapter presents the results on the osmotolerance limit and changes in excretion and concentration of ammonia and urea in different tissues. It also presents the changes in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with 250 mOsm mannitol for 28 days.

The observations of each chapter has been discussed individually and all of them have been discussed together as a general discussion to find out the nature of regulation of ureogenesis in **H. fossilis** under various enviornmental stress.

CHAPTER I

NORMAL EXCRETION PATTERN AND THE TISSUE LEVEL OF AMMONIA AND UREA

INTRODUCTION

Most of the fish being carnivorous use protein and amino acids as their major source for energy production. Ammonia is produced in large quantities as the primary end product of protein or amino acid metabolism. Being highly toxic, very little amount of ammonia could be retained in the body. Either it is promptly excreted out as ammonia or converted to some less toxic molecules for temporary storage **in vivo.** Variation in the nitrogenous excretory product and the pattern of their excretion is dependent on the environment it lives. Studies on a number of representative species of fish, both freshwater and marine, have shown that the metabolic waste nitrogen in teleost fish is excreted through the gills predominantly as ammonia. Some other minor nitrogen containing molecules such as urea, uric acid, trimethylamine oxide, amino acids, creatine and creatinine have been reported as excretory products of fish. Ammonia accounted for about three quarters of the total waste nitrogen produced by metabolism in freshwater teleosts such as **Carassius auratus** (gold fish), **Cyprinus carpio** (carp) and coho salmon (Smith, 1929; McLean & Fraser, 1974). About 90% of ammonia was excreted by diffusion through the gills in freshwater fish (Smith, 1929; Vellas & Serfaty, 1974) and at least 50% of the waste nitrogen through gills in marine fish such as **Leptocottus armatus** (sculpin), **Platichthys stellatus** (starry flounder) and **Taeniotoca lateralis**

(blue sea perch) (Wood, 1958). Most of the other excretory products are also excreted through the gills and the urine contains little amount of excretory nitrogen.

Gills extract preformed ammonia from the blood for excretion rather than having a major active role in its production (Payan & Pic, 1977). However, in the marine teleost **Myoxocephalus scopius** it has been reported that 60% of the ammonia excreted was from the blood and 40% from amino acid metabolism in the gills (Goldstein **et al**, 1964). Besides gills, smooth skin also has been suggested as a possible site for ammonia excretion in freshwater fish. Analysing blood from various sites in the carp, Pequin and Serfaty (1963) reported that out of the total ammonia excreted, 66% derived from the liver and 33% from the kidney. The liver has been shown as the main organ for ammoniogenesis in fish (Goldstein **et al**, 1964; Vellas & Serfaty, 1974). Ammonia can be formed by several pathways such as deamination of amino acids, amides, purines, pyrimidines and hexosamine (Forster & Goldstein, 1969; Campbell, 1973; Watts & Watts, 1974; Walton & Cowey, 1982). In fish, all the pathways for ammonia formation have not been completely elucidated. However, it has been shown that the major pathway for ammonia formation in fish could be transdeamination (Schmidt, 1957; Makarewicz & Zydowo, 1962; Salvator **et al**, 1965; Walton & Cowey, 1977; Waarde & Kesbeke, 1981; Campbell **et al**, 1983; Casey **et al**, 1983). Cowey and Sargent (1972) suggested that most of the fish being carnivorous, dietary protein was catabolised to amino acids, most of which were deaminated with the release of ammonia and the carbon skeleton of amino acids

used as energy source. Thus, ammonia is produced in large quantities mainly in liver and kidney, and excreted primarily through the gills.

Next to ammonia, urea often contributed to the total nitrogen excretion (Smith, 1929; Burrows, 1964; Elliott, 1976; Brett & Zala, 1975; Vellas, 1981) and small amounts were also found in tissues and blood plasma of freshwater teleosts (Holmes & Donaldson, 1969; Wood, 1958). Synthesis of urea and its retention in different tissues have been reported in marine fishes for osmoregulation (Alexander **et al**, 1968; Goldstein & Forster, 1971, Campbell, 1973; Pang **et al**, 1977; Read, 1971). However, in freshwater teleosts the sources of urea is still not clear. Brown and Cohen (1960) could not detect the activity of some of the enzymes of ornithine-urea (o-u) cycle in the liver of a few freshwater teleosts studied. They proposed a genetic deletion of o-u cycle and thereby, the loss of capability to synthesize urea from ammonia in freshwater teleosts. Huggins **et al** (1969) could detect very low activity of o-u cycle enzymes only in a few species of freshwater teleosts. Wilson (1973a) also failed to detect some of the enzymes of o-u cycle in channel catfish, **Ictaluras punctatus.** It was, therefore, thought the o-u cycle was either absent or non-functional for converting ammonia to urea in freshwater teleosts where diffusion of excess of ammonia to the aquatic medium was very easy and economical.

Purine catabolism and dietary arginine were suggested

to be alternate sources of urea in freshwater teleosts (Goldstein & Forster, 1965; Cvancara, 1969a,b; Vellas & Serfaty, 1974).

The air-breathing amphibious fishes, some of which can undergo aestivation during the dry season and most others being capable of temporary terrestrial life, face the problem of ammonia excretion during their life outside water. Ammonia needs plenty of water to be diluted for excretion during terrestrial life and also cannot be stored as such inside the body for longer period due to its toxicity. It is usually converted to a less toxic substance such as urea for temporary retention **in vivo**. Urea also needs less amount of water for its excretion. Presence of such regulated physiological system to convert ammonia to urea and excretion of urea have been clearly shown in most amphibians (Janssens, 1964; McBean & Goldstein, 1970; Balinsky, 1981), African lungish, **Protopterus** (Janssens, 1964; Goldstein **et al**, 1967) and mudskipper fishes (Gordon **et al**, 1969, 1978) during their stay outside water for varying period of time.

The situation in freshwater teleosts, in general, is still not clear. They have been accepted as ammoniotelic excreting primarily ammonia through exctra-renal sources and have apparently lost the capacity of ureogenesis from ammonia through o-u cycle. However, the air-breathing species are special among freshwater teleosts due to their capacity to survive outside water for considerable period. They might be having different excretory pattern and special adaptive

physiological mechanism like amphibians and lungfishes to manage the problem of ammonia excretion during the period of water deprivation. It was suggested that studies on such fishes might explain the adaptive changes in nitrogen metabolism and excretion during the evolution of freshwater teleosts (Huggins **et al**, 1969). This chapter deals with the study of physiological level in different tissues and the diurnal pattern of excretion of ammonia and urea in five species of freshwater air-breathing teleosts in their aquatic habitat. In addition to this, diurnal pattern of renal and extra-renal excretion of ammonia and urea by one of these species i.e. in **H. fossilis** in water was also studied.

Plan of Work:

The following observations were made in five species of freshwater teleosts such as **Amphipnous cuchia, Heteropneustes fossilis, Clarias batrachus, Anabas testudineus** and **Channa punctatus.**

(1) The concentrations of ammonia and urea in different tissues such as liver, kidney, muscle, brain, gill, skin and blood plasma of five species of freshwater air-breathing teleosts acclimatised to their primary aquatic habitat were estimated.

(2) The diurnal pattern of total excretion of ammonia and urea in the aquatic habitat was determined for the five species.

(3) The diurnal pattern of renal and extra-renal excretion of ammonia and urea was determined in **H. fossilis** in

aquatic habitat.

(4) Maximum period of survival of the five species of fresh-water air-breathing teleosts kept outside water was determined.

MATERIALS AND METHODS

Animals: **Heteropneustes fossilis, Clarias batrachus, Anabas testudineus, Channa punctatus** and **Amphipnous cuchia** were purchased from commercial sources. They were maintained separately in the laboratory at 20 ± 2°C in plastic aquaria containing bacteria free filtered tap water with 12 hr : 12 hr light and dark period. **A. cuchia** was kept in mud. Minced pork liver was supplied as food on every alternate day at a fixed time. Water was changed regularly on the day after feeding. The fishes were used after their acclimatization to the laboratory condition for at least one month when death rate became zero and food consumption normal. The fishes were used for experiments 24 hrs after last food was given. The experiments were conducted under same environmental conditions as they were acclimatised unless otherwise mentioned.

Experimental:

(a) Total excretion of ammonia and urea: Six fishes of similar weight from each species were kept separately in glass jars. Each fish was kept in 500 ml of bacteria free filtered tap water containing 10 mg of streptopenicillin to block microbial growth in the medium. The jars were covered with bilayers of cheese cloth. The fishes were kept at 6 A.M. and the medium

was replaced at 12 hrs interval upto 24 hrs. The water collected at 6 P.M. and next day at 6 A.M. were used for estimation of ammonia and urea per 24 hrs.

(b) Renal and extra-renal excretion: Renal and extra-renal excretion was studied only in male **H. fossilis** as catheterisation could not be successful in other fishes. The catheters, approximately 6 cm in length were prepared from polyethylene tubings (0.8 mm diameter). A catheter was introduced into the urinary bladder through the ureter and tied along the copulatory bursa. A baloon was tied at the distal end of the catheter. Each catheterised fish was released in a separate glass jar containing 500 ml of bacteria free filtered tap water with 10 mg of streptopenicillin at 6 A.M.. The urine was collected after 12 hrs day and 12 hrs night at 6 P.M. and 6 A.M. next day respectively by puncturing the distal end of the baloon. The volume of urine collected was recorded. The concentration of ammonia and urea in the urine samples were estimated. These were expressed as renal excretion. The amount of ammonia and urea estimated in the medium, collected simultaneously at the time of urine collection, were expressed as extra-renal excretion (excretion through gills and body surface).

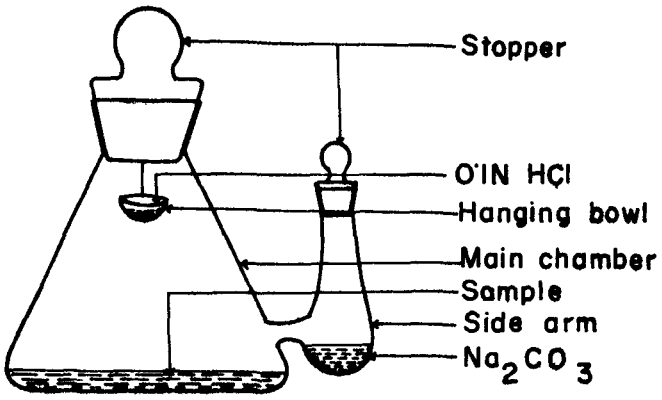
Blood sampling: Blood was collected directly from the heart in **A. testudineus** and **C. punctatus** and from the caudal vein in **A. cuchia, H. fossilis** and in **C. batrachus** with a heparinized syringe. It was centrifuged immediately at 7,000 x g for

10 min to separate the cells. 0.5 ml of the supernatant (plasma) was deproteinised with the addition of 1.0 ml of ice-cold 10% trichloro acetic acid (TCA) and removal of precipitated protein by centrifugation. The supernatant was neutralised with 1N NaOH and used for the estimation of ammonia and urea.

Tissue preparation: Fishes were sacrificed regularly by decapitation immediately after collecting blood at 12 noon. Tissues such as liver, kidney, muscle, brain, gill and skin were quickly removed blotted dry and deep frozen at -20°C until used for estimations. All estimations were completed within two days of sampling. Each frozen tissue was thawed on ice and a 10% homogenate prepared with deionized distilled water with a Potter-Elvehjem type motor driven glass homogeniser with a teflon pestle. Protein was separated by treating the homogenate with equal volume of ice-cold 10% TCA and centrifuging out the precipitate. The deproteinised tissue supernatant was neutralised with 1N NaOH and was used for ammonia and urea estimation.

Estimation procedures:

(a) Ammonia: Ammonia was estimated by micro-diffusion following the method of Kawakubo **et al** (1983) with a suitably modified diffusion bottle (see figure). This method had 90-95% recovery of total ammonia and was linear with different concentrations of NH_4Cl used as standard.



DIFFUSION BOTTLE

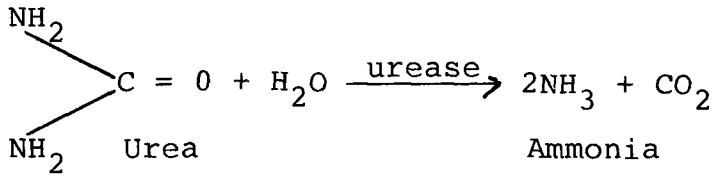
(i) Diffusion of ammonia: A suitable aliquot of each neutralised sample was taken in the main chamber of the diffusion bottle. 1.0 ml of saturated sodium carbonate (Na_2CO_3) was taken in the side arm. 0.6 ml of 0.1N hydrochloric acid (HCl) was taken in a small open glass bowl which was hanging in the ring from the stopper into the main chamber of the diffusion bottle. The main chamber and side arm were properly stoppered with glass stoppers with slight greasing for making the bottle air-tight. Na_2CO_3 from the side arm was carefully added into the sample in the main chamber and the incubation continued for 30 min in a water bath shaker maintained at 50°C with constant shaking (60 times/min). Ammonia diffused out from the sample due to alkalisation and was absorbed in the 0.1N HCl in the glass bowl. This HCl containing diffused ammonia was used for estimation of ammonia in the sample. The incubation time and temperature were standardised as sufficient to get 90-95% recovery of ammonia from the sample using ammonium chloride (NH_4Cl) as standard.

$$NH_4Cl + Na_2CO_3 \longrightarrow NaCl + NaHCO_3 + NH_3\uparrow$$

$$NH_3\uparrow + HCl \longrightarrow NH_4Cl$$

(ii) Estimation of ammonia: After the diffusion was complete 0.6 ml o.1N Sodium hydroxide (NaOH) was added to the 0.6 ml of 0.1N HCl in the open bowl taken in a test tube followed by 0.2 ml of ethanol to facilitate mixing. The solution was diluted with 1.0 ml water and 0.2 ml of 0.5% sodium hypochlorite (to form monochloramine), 1.0 ml 0.2N sodium hydroxide (NaOH) and 0.4 ml of 1-naphthol solution (5% w/v in acetone) were added in the above order. The solution was mixed thoroughly. After 5 min the O.D. was taken promptly in a spectrophotometer (Beckman Model 26) at 740 nm against a reagent blank. Maximum absorbance of the solution was observed at 740 nm instead of 720 nm used by Kawakubo **et al** (1983). The concentration of ammonia was calculated from a linear standard graph prepared with different concentrations of NH_4Cl (from 0.1 to 1.0 μ mole) following the same diffusion method.

(iii) Estimation of urea: Urea was estimated by converting urea to ammonia with urease and then estimating the ammonia formed from urea following the above ammonia estimation method.



$$2NH_3 + 2H_2O \longrightarrow 2NH_4OH$$

A suitable aliquot from each neutralised sample was treated with 20 units of urease (Sigma Type - IV) prepared in 0.1M potassium phosphate buffer (pH 7.5). That was incubated for 30 min at 30°C to convert completely all the urea present

to ammonia. The concentration of ammonia produced from urea was determined from the difference of ammonia concentration [Total ammonia (fluid ammonia + ammonia formed from urea) - fluid ammonia]. The actual concentration of urea was calculated from the standard graph prepared for ammonia produced from different concentrations of urea (from 0.1 to 1.0 μ mole) treated with same amount of urease. The standard graph was linear indicating complete conversion of urea to ammonia and all the estimations were done within the range of standard graph.

Survival of fishes outside water: Ten fishes of each species were kept individually in an empty glass jar under the laboratory condition. Each jar was covered with bilayers of cheese cloth and had 70 - 80% humidity. The time of the death of each fish was recorded. A fish was declared dead when it showed no physical movement and no heart beat even after shaking. A cross check was made by transfering the fish immediately to water and the fish did not revive. Though this method was not very accurate, an approximate time range for their survival outside water was determined.

Expression of results: The rate of excretion of ammonia and urea were expressed as μ moles per g body weight per 12 hrs and 24 hrs. The concentration of ammonia and urea in different tissues were expressed as μ moles per g wet weight of the tissue and μ moles per ml of blood plasma. The levels of significance between two sets of data were calculated by

students 't' test (Croxton **et al**, 1982) and 'p' value above 0.05 were taken as non-significant (N.S.).

Chemicals: All the chemicals used were of analytical grade. Urease (Type IV) was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Deionized and double glass distilled ammonia free water was used in all estimations.

RESULTS

Physiological level of tissue ammonia and urea:

Maximum physiological level of ammonia in different tissues was found in **C. punctatus** followed by **A. testudineus, C. batrachus, H. fossilis** and **A. cuchia** in descending order (Table 1). However, the level of urea followed almost a reverse order with highest concentration in the tissues of **A. cuchia** (except in liver) followed by **C. batrachus, H. fossilis, A. testudineus** and **C. punctatus** (Table 2). In liver, urea level was maximum in **C. batrachus.** Ammonia level was very high in both liver and kidney of all the fishes studied followed by muscle, gill, skin, brain and plasma. However, urea level was highest in the liver followed closely by kidney in general in all fishes. Urea concentration was very less in most other tissues except for muscle and brain of **A. cuchia** where the level was almost half of the liver concentration. Urea could not be detected in skin of **A. testudineus** and **C. punctatus.**

The ratio of ammonia:urea was similar to the distribution pattern of ammonia with highest value in **C. punctatus** followed

by **A. testudineus, H. fossilis, C. batrachus** and **A. cuchia** (Table 3).

Diurnal pattern of ammonia and urea excretion:

Normal diurnal pattern and total (24 hrs) excretion of ammonia and urea in the five different species studied have been presented in Table 4. Ammonia was the major excretory product and was excreted about 10 times more than urea in **A. cuchia, H. fossilis, C. batrachus,** 25 times in **A. testudineus** and 33 times in **C. punctatus**. Excretion pattern of ammonia was found to be similar both during day and night in all the species studied. Total amount of ammonia excreted in 24 hrs was maximum in **C. batrachus** and **A. testudineus** and minimum in **A. cuchia**. However, total urea excretion was maximum in **C. batrachus** followed by **H. fossilis, A. cuchia, A. testudineus** and **C. punctatus**. **H. fossilis** and **A. testudineus** only showed significantly higher urea excretion during night compared to day.

Renal and extra-renal excretion in male **H. fossilis**:

Renal and extra-renal excretion of ammonia and urea (Table 5) and renal output of urine and quality of urine (Table 6) have been studied only in male **H. fossilis**. Ammonia and urea were both excreted mainly through extra-renal source(s). Ammonia excretion was 150-200 times and urea about 3 times higher through extra-renal than renal source. The relative proportions of ammonia and urea were different between renal and extra-renal excretion. Ammonia was excreted 13-17 times

TABLE 1. Physiological level of ammonia in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	Liver	Kidney	Muscle	Brain	Gill	Skin	Plasma
A. cuchia	11.57	10.22	8.77	5.22	Rudimentary	7.19	0.426
	±1.23	±1.36	±1.01	±0.78		±0.94	±0.042
H. fossilis	14.56	13.90	12.43	6.74	9.17	8.11	0.473
	±2.11	±1.22	±1.09	±0.81	±1.20	±0.89	±0.035
C. batrachus	16.21	14.56	11.78	7.89	10.66	8.57	0.468
	±2.11	±1.88	±1.45	±1.09	±2.04	±1.06	±0.032
A. testudineus	17.16	17.55	12.72	5.66	11.24	8.92	0.731
	±2.81	±2.42	±1.98	±0.87	±1.04	±0.99	±0.063
C. punctatus	18.62	19.11	13.44	7.12	14.27	10.16	0.889
	±3.14	±3.44	±2.11	±0.99	±1.27	±1.11	±0.10

TABLE 2. Physiological level of urea in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	Liver	Kidney	Muscle	Brain	Gill	Skin	Plasma
A. cuchia	8.77	7.16	4.19	4.11	Rudimentary	0.79	0.779
	±1.02	±0.88	±0.61	±0.43		±0.13	±0.083
H. fossilis	6.23	5.53	1.32	2.49	1.56	0.53	0.755
	±0.61	±0.97	±0.32	±0.37	±0.52	±0.16	±0.081
C. batrachus	9.44	6.38	1.69	3.19	1.61	0.64	0.610
	±0.88	±0.91	±0.37	±0.46	±0.61	±0.11	±0.075
A. testudineus	4.57	3.86	0.87	1.21	0.56	BLD	0.371
	±0.52	±0.41	±0.21	±0.23	±0.11		±0.039
C. punctatus	2.68	2.11	0.45	1.06	0.37	BLD	0.226
	±0.41	±0.32	±0.09	±0.10	±0.05		±0.025

BLD - Below the level of detection.

TABLE 3. Ratio of ammonia : urea concentrations in different tissues and in blood plasma of five species of freshwater air-breathing teleosts.

Species	Liver	Kidney	Muscle	Brain	Gill	Skin	Plasma
A. cuchia	1.32	1.43	2.09	1.27	-	9.11	0.55
H. fossilis	2.34	2.51	9.49	2.71	5.88	15.30	0.63
C. batrachus	1.72	2.28	6.97	2.49	6.62	13.39	0.77
A. testudineus	3.75	4.55	14.62	4.68	20.07	-	1.971
C. punctatus	6.95	9.06	29.87	6.80	38.57	-	3.93

TABLE 4. Diurnal pattern of excretion of ammonia and urea (μmoles/g body wt) by five species of freshwater air-breathing teleosts. (Mean ± S.D.)

	Ammonia			Urea			Ammonia / Urea
	12 hrs day	12 hrs night	24 hrs	12 hrs day	12 hrs night	24 hrs	24 hrs
A.cuchia	2.09 ±0.31	1.97 ±0.35	4.06 ±0.65	0.200 ±0.024	0.196 ±0.006	0.396 ±0.03	10.25
p	N.S.			N.S.			
H. fossilis	2.87 ±0.43	3.21 ±0.65	6.08 ±1.08	0.227 ±0.041	0.336 ±0.064	0.563 ±0.105	10.80
p	N.S.			<0.02			
C. batrachus	5.11 ±0.69	5.04 ±0.31	10.15 ±1.00	0.404 ±0.069	0.489 ±0.093	0.893 ±0.162	11.37
p	N.S.			N.S.			
A. testudineus	5.00 ±0.61	5.01 ±0.31	10.01 ±0.92	0.173 ±0.013	0.206 ±0.022	0.379 ±0.035	25.21
p	N.S.			<0.02			
C. punctatus	3.29 ±0.29	3.65 ±0.81	6.94 ±1.10	0.111 ±0.019	0.097 ±0.013	0.208 ±0.032	33.37
p	N.S.			N.S.			

TABLE 5. Diurnal pattern of renal and extra-renal excretion of ammonia and urea (μmoles/g body wt) in male **H. fossilis.** (Mean ± S.D.)

	Renal excretion			Extra-renal excretion			Total excretion		
	Ammonia	Urea	Ammonia / Urea	Ammonia	Urea	Ammonia / Urea	Ammonia	Urea	Ammonia / Urea
12 hrs day	0.013 ±0.002	0.053 ±0.01	0.25	2.81 ±0.15 (21515)*	0.163 ±0.025 (208)*	17.24	2.82 ±0.15	0.216 ±0.035	13.06
12 hrs night	0.019 ±0.004 (46.2)**	0.085 ±0.029 (60.4)**	0.22	2.97 ±0.22 (15532)* (5.70)**	0.230 ±0.020 (171)* (41.1)**	12.91	2.99 ±0.22 (6.0)**	0.315 ±0.049 (46.3)**	9.49
p (day vs. night)	<0.005	<0.01		N.S.	<0.001		N.S.	<0.001	
24 hrs	0.031 ±0.002	0.130 ±0.021	0.24	5.58 ±0.44 (17900)*	0.362 ±0.051 (179)*	15.19	5.62 ±0.41	0.492 ±0.072	11.42

* % increase of extra-renal excretion than renal excretion.

** % increase of ammonia and urea excretion in the night than day.

TABLE 6. Diurnal variation in urine output (ml/g body wt) and concentration of ammonia and urea (μmoles/ml) in urine. (Mean ± S.D.)

	Urine output	Concentration in urine		
		Ammonia	Urea	$\frac{\text{Ammonia}}{\text{Urea}}$
12 hrs day	0.08 ± 0.005	0.156 ± 0.027	0.66 ± 0.15	0.24
12 hrs night	0.114 ± 0.006 (+42.5%)	0.173 ± 0.027 (+10.9%)	0.739 ± 0.23 (+11.97%)	0.23
p (day vs. night)	<0.001	N.S.	N.S.	

TABLE 7. Relative survival time (hrs) for five species of freshwater air-breathing teleosts kept outside water at 70-80% humidity.

Species	Survival time (hrs)
A. cuchia	90 - 100
H. fossilis	60 - 70
C. batrachus	60 - 70
A. testudineus	24 - 36
C. punctatus	8 - 12

more than urea through extra-renal source(s) and urea was 4-4.5 times more than ammonia through renal source. Significant increase in renal excretion of both ammonia and urea, and extra-renal excretion of urea were observed at night. However, total excretion of only urea was significantly higher at night than day. There was no difference in total ammonia excretion in 24 hrs. Urine output was significantly higher at night (Table 6). Urea concentration was more than 4 times higher than ammonia in the urine of both day and night. The ratio of ammonia:urea in the urine, therefore, did not vary significantly between the day and night.

Survivility outside water:

Maximum survival time for the five species of freshwater air-breathing teleosts when kept outside water at 70-80% humidity has been presented in Table 7. **A. cuchia** survived for longest period of time for 90-100 hrs while **H. fossilis** and **C. batrachus** survived for 60-70 hrs, **A. testudineus** 24-36 hrs and **C. punctatus** 8-12 hrs.

DISCUSSION

Physiological level of ammonia (Table 1) and urea (Table 2) in various tissues of the five different species of freshwater air-breathing teleosts showed direct correlation with their rate of air-breathing capacity and capacity for surviving outside water. Among the five species studied **C. punctatus** survived outside water for only 8-12 hrs while **A. testudineus** survived for 24-36 hrs, **H. fossilis** and

C. batrachus for 60-70 hrs and **A. cuchia** for 90-100 hrs at 70-80% humidity (Table 7). Maximum ammonia and minimum urea level in different tissues of **C. punctatus** and minimum ammonia and maximum urea level in **A. cuchia** with other three species in between suggest that the species having better survival capacity outside water retained more urea than ammonia in their tissues and vice-versa. The ratio of the concentration of ammonia:urea in different tissues (Table 3) also followed similar pattern as ammonia with highest value in **C. punctatus** followed by **A. testudineus, H. fossilis, C. batrachus** and least in **A. cuchia**. Ammonia level in the tissues of these fish were lower than those of purely aquatic freshwater ammoniotelic teleost, **Ictalurus punctatus** (Wilson & Poe, 1974). The lower level of ammonia in air-breathing teleosts could be due to its conversion to urea thus increasing their tissue urea level. The tissue distribution of ammonia was similar to that of other vertebrates with highest level in liver and kidney and least in brain. Liver and kidney are the main organs for ammonia production in teleost fish (Pequin & Serfaty, 1963; Vellas & Serfaty, 1974). Glutamate dehydrogenase (GDH), considered to be the nitrogen storage enzyme in absence of glutamine synthetase in the liver of salmonid fish (Webb & Brown, 1976), showed high activity in the liver and kidney of rainbow trout, **Salmo gairdneri** which were involved in the production of ammonia **in vivo** (Walton & Cowey, 1977). A similar distribution of the enzyme has been described in eel, **Anguilla rostrata** (McBean **et al**, 1966), carp, **Cyprinus carpio** (Pequin **et al**, 1970) and catfish,

I. punctatus (Wilson, 1973a). Brain avoids ammonia accumulation and keeps the ammonia level always low by converting it immediately to glutamine by the enzyme glutamine synthetase. The activity of glutamine synthetase has been reported to be very high in the brain of various fishes (Webb & Brown, 1976, 1980; Walton & Cowey, 1977). A similar function might be operating in the air-breathing teleosts studied to keep the ammonia level lower in the brain and higher in liver and kidney.

High level of urea observed in almost all tissues of at least four air-breathing species (except **C. punctatus**) has been a unique observation among freshwater teleosts. Wilson and Poe (1974) could detect small amount of urea only in liver and plasma of **I. punctatus**. Higher concentrations of urea and lower level of ammonia in different tissues observed give strong indication of active conversion of ammonia to urea in these freshwater air-breathing teleosts. Lower level of ammonia and urea in blood plasma indicated the presence of efficient excretory mechanisms in air-breathing teleosts during the aquatic life.

Ammonia was found to be the major excretory product in all the five species of air-breathing teleosts examined (Table 4) during their aquatic life resembling other freshwater teleosts, aquatic amphibians and freshwater stingrays (Smith, 1929; Wood, 1958; McLean & Fraser, 1974; Gregg & Cameron, 1981; Goldstein & Forster, 1971). The rate of excretion of ammonia was different in different species. In contrast

to the concentration of ammonia and urea in different tissues, the rate of excretion of both ammonia and urea showed no relation with their relative air-breathing capacity and survival capacity outside water. The effect of feeding and the type of food have been reported to influence the nitrogen excretion pattern in different fishes (Rychly & Marina, 1977; Atherton & Aitken, 1970; Ogino **et al**, 1980). However, these five species of air-breathing teleosts were fed same food (minced pork liver) and maintained under same environmental conditions. Hence, the variation in the physical activity of different species might have influenced the nitrogen excretion pattern in these fishes. These fishes were carnivorous and in carnivorous fishes the energy requirement has been reported to be met from the deamination of amino acids producing sufficient ammonia and keto acids (Walton & Cowey, 1977). Sluggish **A. cuchia** would require less energy and as a result less deamination of amino acids. Therefore, it might have produced and excreted least amount of both ammonia and urea. The rate of excretion of ammonia and urea was found maximum in **C. batrachus** which was a highly active fish and therefore, would require more energy leading to excess deamination of amino acids.

It was significant to note that the excretion of urea in these freshwater air-breathing fishes were in general very much higher than other freshwater teleosts reported (Olson & Fromm, 1971, Morii **et al**, 1978). The ratio of ammonia: urea excreted indicated that **A. cuchia, H. fossilis** and

C. batrachus excreted relatively more urea than ammonia compared to other two species. This was related to their survival capacity outside water and probably the level of o-u cycle enzyme activity. These fishes in general did not show any significant diurnal variation in their excretion pattern. Only **H. fossilis** and **A. testudineus** showed increased urea excretion at night than day. A satisfactory explanation cannot be given at this stage except that they might be more active at night.

Freshwater organisms excrete mainly ammonia by diffusion through extra-renal sources such as the gills of teleosts (Smith, 1929; Wood, 1958; Fromm, 1963; Fromm & Gillette, 1968; Payan & Matty, 1975) and skin of **Necturus** (Fanelli & Goldstein, 1964) while in **Xenopus** and ammoniotelic reptiles it was excreted in a copious urine. **H. fossilis** excreted major part of both ammonia ($>$ 99%) and urea ($\sim$ 75%) through extra-renal sources. The renal excretion constituted only 0.55% of the total ammonia and 26% of the total urea excreted. More excretion of urea than ammonia through skin and more ammonia than urea through gills were reported in two mudskipper fishes **Periophthalmus cantonensis** and **Boleophthalmus pectinirostris** (Morii **et al**, 1978). The smooth scale less mucoid skin might have supplemented its extra-renal excretion of ammonia and urea by diffusion through the gills which are less developed in **H. fossilis**. It is difficult to conclude whether urea excretion was more through the skin or gills in this air-breathing fish.

There was significant increase in urine output and a relatively higher (but non-significant) concentration of urea and ammonia in urine at night than day (Table 6). However, the ratio of ammonia:urea concentration in the urine did not change during day and night. Higher excretion of ammonia and urea and increased level of urine output at night tend to support the earlier suggestion that **H. fossilis** might be more active both physiologically and physically at night (Ramanujam **et al**, 1981). Urea excretion was also significantly higher at night in **A. cuchia** (Table 4). However, we have not come across any other report on the circadian pattern of metabolism or activity of freshwater air-breathing teleosts.

Higher level of urea in various tissues and higher rate of its excretion in four out of five species of freshwater air-breathing teleosts studied have been unique observations in freshwater teleosts. These observations suggest that the air-breathing freshwater teleosts might have the capability for converting ammonia to urea **in vivo** through an active o-u cycle as their physiological adaptation to spend sometime outside water successfully.

CHAPTER II

NORMAL UREOGENESIS

INTRODUCTION

Freshwater teleosts are known to be ammoniotelic excreting ammonia as the major nitrogen excretory product (Delaunay, 1931; Baldwin, 1964; Forster & Goldstein, 1969; Fromm, 1963). Presence of some urea as an excretory product (Smith, 1929; Wood, 1958) and low levels of urea in tissues and blood plasma (Denis , 1913-'14, Holmes & Donaldson, 1969) have been reported in several species of freshwater teleosts. However, the mechanism of synthesis of urea and its physiological significance in teleosts have not been well understood.

Urea production in vertebrates has been shown mainly through one or more of the three following pathways: (a) Ornithine-urea (o-u) cycle, (b) hydrolysis of dietary arginine, and (c) catabolism of purines (Fig.1 in General Introduction).

Active ureogenesis through o-u cycle 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 o-u cycle is reported to be incomplete as some of the enzymes of the cycle could not be detected in various teleost fishes (Krebs & Henseleit, 1932; Manderscheid, 1933; Brown & Cohen, 1960; Wilson, 1973). Brown and Cohen (1960) could not detect carbamylphosphate

synthetase (CPS) and ornithine transcarbamylase (OTC) activity out of the five enzymes of o-u cycle in several teleosts studied by them. Wilson (1973b) could not detect the arginine synthetase system (ARSS) activity in the liver and both CPS and ARSS activity in the kidney of channel catfish, **Ictalurus punctatus**. Brown and Cohen (1960) suggested the existence of a functional o-u cycle in the ancestral fish from which the freshwater teleosts evolved and during the process of evolution perhaps the genes responsible for some of these enzymes of o-u cycle whose activities could not be detected got deleted. This is known as the "deletion" hypothesis . They further suggested that gene deletion or repression took place at the level of emergence of the Actinopterygii. The extent Holostei and Teleostei are ammoniotelic and did not possess all the enzymes of the o-u cycle, whereas the Chondrichthyes, the Dipnoi and higher vertebrates (birds and reptiles apart) retained their ureotelic capacity. By implication, the Chondrichthyes and Crossopterigii would also have been ureotelic. A number of papers have provided evidence of the presence of the o-u cycle enzymes in several of these groups, i.e., elasmobranchs (Baldwin, 1958, 1960; Campbell, 1961; Schooler, 1964; Watts & Watts, 1966), Holocephali (Read, 1967); Dipnoi (Brown **et al**, 1966; Forster & Goldstein, 1966; Janssens & Cohen, 1966; Goldstein **et al**, 1967) and Coelacanthi (Brown & Brown, 1967). The convenience of excreting out ammonia by diffusion in freshwater might have gradually made the o-u cycle non-functional in the freshwater teleosts. The "deletion" hypothesis could not be confirmed by other

workers. This hypothesis appeared to be unsound when it has been pointed out that the genetic potential to synthesize the urea cycle enzymes was carried in the germ line. However, the use made of the cycle by various groups of animals in the evolutionary process would depend on the evolution of appropriate control systems. This system might vary even within a single group as a result of species adaptation to particular environment . This proved to be justified when Huggins **et al** (1969) reported a full complement of o-u cycle enzymes in a variety of freshwater and marine teleosts but with a very low activity. They proposed that the expression of the genes responsible for the synthesis of o-u cycle enzymes might have been altered due to an adaptational change in the freshwater teleosts where ammonia excretion by diffusion became easy and osmotic problem reversed from marine environment requiring no urea retention **in vivo**. They also suggested that o-u cycle could be one of the sources for ureogenesis in freshwater teleosts and reclassified teleosts as ureogenic (potential urea producer) even though they excreted primarily ammonia and very little amount of urea. High activity of o-u cycle enzymes has been reported in marine teleost, **Opsanus tau** (Read, 1971).

The full complement of o-u cycle enzymes have also been found in liver of a Chimaeroid, **Hydrolagus colliei** (Read, 1967) and the African aestivating lungfish **Protopterus aethiopicus** (Janssens, 1964; Brown, 1965; Janssens & Cohen, 1966; Forster & Goldstein, 1966) which were able to synthesize urea from ammonia and bicarbonate **in vitro**. In a better pre-

served sample of Coelacanth liver, activities of all the enzymes of o-u cycle were measured by Goldstein **et al** (1973) and found to be comparable to those in elasmobranchs.

Cvancara (1969b, 1971) suggested that dietary arginine could be a major source of urea in teleost fishes as arginase activity was predominantly found in the liver (Hunter, 1929; Cvancara, 1969b,1971), kidney and heart (Hunter, 1929; Cvancara, 1969b), and to a lesser extent in spleen; gills, ovaries, testes and muscle of some teleosts (Cvancara, 1969b).

Another source of urea in teleosts could be purine degradation or uricolytic pathway which was reported first by Brunel (1937). Adenine and guanine produce uric acid as a catabolic product which further breakdown in a three step uricolytic pathway to produce urea in ureotelic/ureogenic animals. Goldstein and Forster (1965) studied the activity of uricolytic pathway (uric acid ⟶ urea) in liver slices of several teleosts and found the conversion of uric acid to urea in all teleosts. The rate of production of urea ranged from 5 μ moles/g/hr in the goosefish, **Lophius americanus** to 23 μ moles/g/hr in winter flounder, **Pseudopleuronectes americanus.** They could also detect the activities of allantoicase in eighteen species of teleosts and suggested that uricolytic pathway could be the major source of urea in freshwater teleosts. Cvancara (1969a) could find relatively high activity of uricase in nineteen species of freshwater teleosts. He suggested that degradation of purines and nucleic acids,

might account for the urea production at the levels of which it is found in the blood and excreted in teleosts.

Among several species of freshwater air-breathing teleosts, five species such as **Heteropneustes fossilis, Clarias batrachus, Anabas testudineus, Channa punctatus** and **Amphipnous cuchia** were used in the present study. High physiological level in the tissues and more excretion of urea by the five species described in Chapter I gave an indication that ureogenesis is active in these fishes. Therefore, the normal level of activity of o-u cycle enzymes were assayed in different tissues of these five species of freshwater air-breathing teleosts. The activity of uricolytic pathway enzymes were studied in the different tissues of one of the five species, **H. fossilis.**

Plan of Work: This chapter aims at finding out the pathways of urea production in the air-breathing fishes by studying the activity of o-u cycle and uricolytic pathway enzymes. The work was planned as follows.

(1) The activity of all the five enzymes of o-u cycle such as carbamyl phosphate synthetase (ammonia dependent) (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) in the liver and kidney of the five species of freshwater air-breathing teleosts (**H. fossilis, C. batrachus, A. testudineus, C. punctatus** and **A. cuchia**) were assayed spectrophotometrically during their aquatic

life to find out the functional distribution and physiological level.

(2) The activity of all the three enzymes of uricolytic pathway such as uricase, allantoinase and allantoicase in the liver, kidney, muscle and brain were assayed spectrophotometrically in one of the five species, **H. fossilis** which showed maximum activity of o-u cycle enzymes and higher excretion rate and tissue urea among the five species studied. The results would indicate the relative contribution of the two major pathways for urea production.

MATERIALS AND METHODS

Animals: **Heteropneustes fossilis, Clarias batrachus, Anabas testudineus, Channa punctatus** and **Amphipnous cuchia** acclimatized to the laboratory conditions as mentioned in Chapter-I were used for enzyme estimations. Five fishes from each species were sacrificed 24 hrs after the last feeding. They were killed by decapitation and various tissues such as liver, kidney, muscle and brain were removed, blotted dry and immediately deep frozen at -20°C. All the enzymatic assays were completed within two days of collecting the tissues. The activity of o-u cycle enzymes were assayed in the liver and kidney of all the five species. Uricolytic pathway enzymes were assayed in all the tissues collected only from **H. fossilis.**

Preparation of tissue extract: The frozen tissues (liver and kidney) were thawed on ice and a 20% homogenate of each

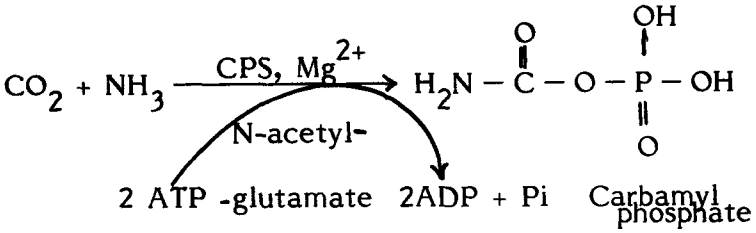
was prepared in 0.1% cetyltrimethyl ammonium bromide (CTB) with a Potter Elvehjem type motor driven glass homogenizer with a teflon pestle. Each homogenate was centrifuged at 600 x g at 0 ± 2°C for 15 min to remove the nuclei and cell debris. The supernatant was used for assaying the o-u cycle enzymes such as carbamyl phosphate synthetase (ammonia dependent) (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG).

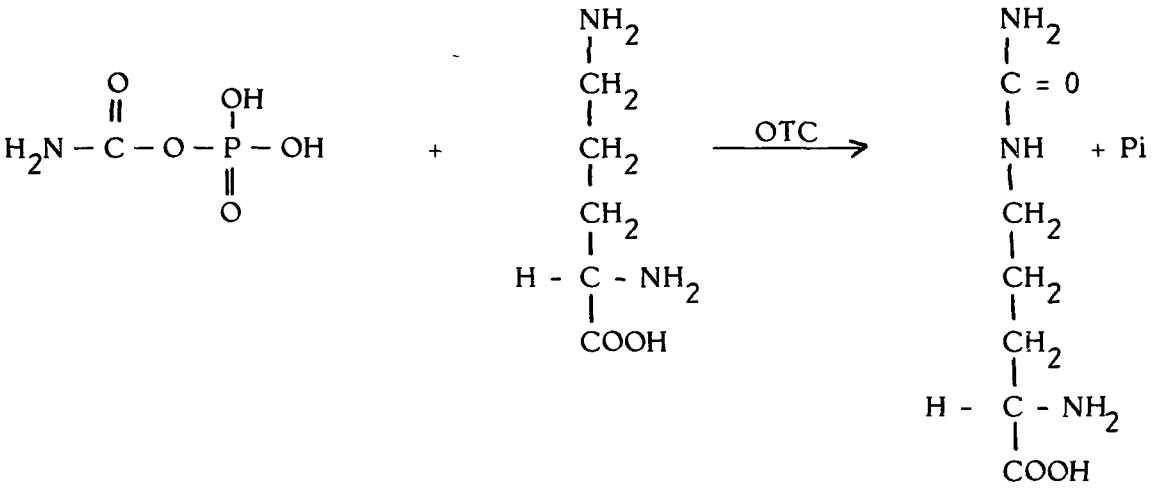
For uricolytic pathway enzyme assay a 10% homogenate of each frozen tissue was prepared in ice cold distilled water following the same procedure as mentioned above. Each homogenate was centrifuged at 600 x g at 0 ± 2°C for 15 min and the supernatant used for assaying the enzymes such as uricase, allantoinase and allantoicase.

Enzyme assay:

Carbamyl phosphate synthetase (E.C. 2.7.2.5):

CPS activity was assayed following the method of Brown and Cohen (1959). The carbamyl phosphate so formed by CPS during the period of incubation was converted further to citrulline in presence of excess of OTC and L-ornithine. Resultant citrulline was estimated for expressing the activity of CPS.





Carbamyl phosphate L-Ornithine L-Citrulline

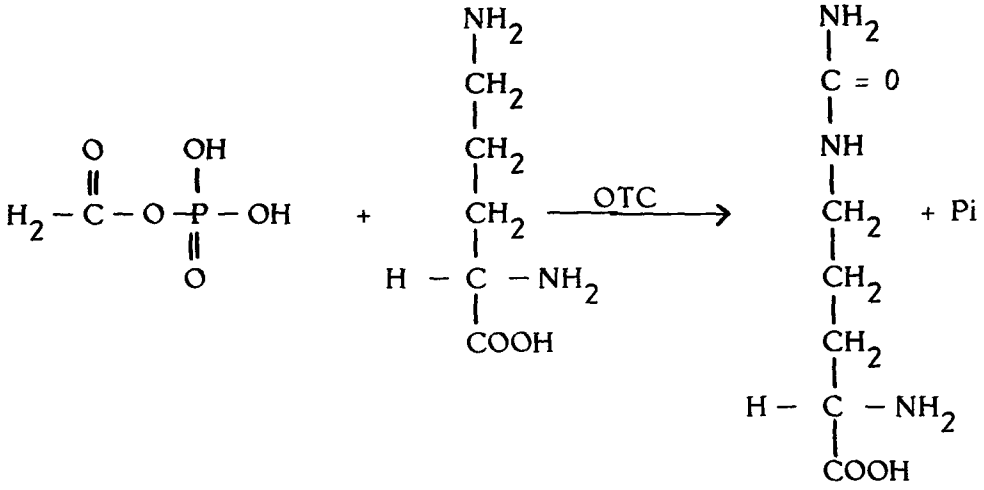
The assay mixture in a final volume of 1.0 ml contained:

Ammonium bicarbonate	50 µmoles
ATP	5 µmoles
L - ornithine	5 µmoles
N - acetylglutamate	5 µmoles
$MgSO_4$	10 µmoles
OTC	10 units
Tissue extract	0.3 ml

The assay mixture without the tissue extract was pre-incubated for 5 min at 37°C. The reaction was started with the addition of the tissue extract. After 30 min of incubation at 37°C, the reaction was stopped by the addition of 0.5ml

of 10% perchloric acid (PCA). A tissue blank was prepared for each assay with addition of PCA to the assay mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The citrulline formed was estimated in the supernatant following the method of Moore and Kauffman (1970). 1.0 ml of suitably diluted supernatant was treated with 2.5 ml of acid mixture (prepared by mixing 300 ml H_3PO_4, 100 ml H_2SO_4, 0.237 g $MnSO_4$ in 398 ml distilled water and 1.8 ml 0.1 M $FeCl_3$) and 0.25 ml of diacetylmonoxime (3% w/v in water). The reaction mixture was mixed thoroughly, boiled in dark for 30 min in hot water bath. The O.D. was read after cooling at 490 nm in a spectrophotometer (Beckman Model 26) against the tissue blank. The amount of citrulline present was calculated from the standard graph prepared using different concentrations (0.04 to 0.1 µmole) of citrulline. One unit of CPS activity was expressed as that amount of enzyme which catalysed for the formation of 1 µmole of citrulline per hr at 37°C.

Ornithine transcarbamylase (E.C. 2.1.3.3.): The activity of OTC was assayed following the method described by Brown and Cohen (1959) by estimating the product (citrulline) formed.



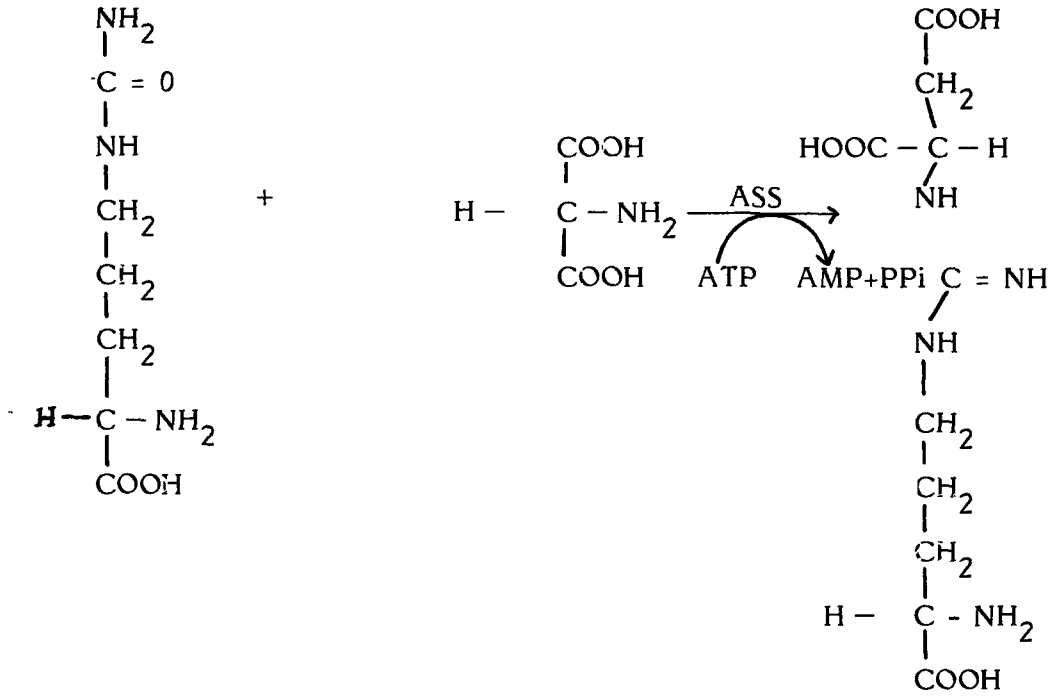
Carbamyl phosphate L-Ornithine L-Citrulline

The assay mixture (pH 8.3) in a final volume of 2.0 ml contained:

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

The assay mixture without the tissue extract was preincubated for 5 min at 37°C. The reaction was started with the addition of the tissue extract. After incubating for 20 min at 37°C, the reaction was stopped by the addition of 0.5 ml of 10% PCA to the reaction mixture. A tissue blank was prepared for each assay with the addition of PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. Citrulline formed during the incubation time was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above in CPS assay. One unit of OTC activity was expressed as that amount of enzyme which catalysed for the formation of 1 µmole of citrulline per hr at 37°C.

Argininosuccinate synthetase (E.C. 6.3.4.5.): ASS activity was assayed following the method of Ratner (1955) with the following modification. In the assay medium 20 units of urease (Sigma Type IV) were taken to convert all the urea present or formed to ammonia to avoid interference with citrulline estimation. The amount of citrulline utilized per unit time was used to express ASS activity.



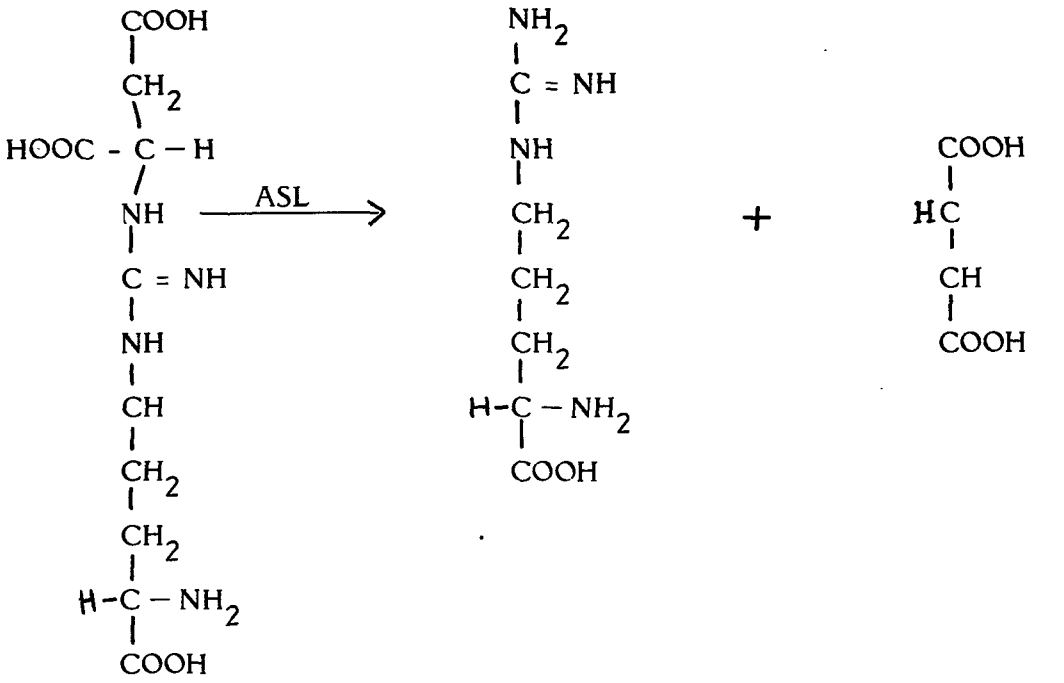
L-Citrulline L-Aspartic acid L-Argininosuccinic acid

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

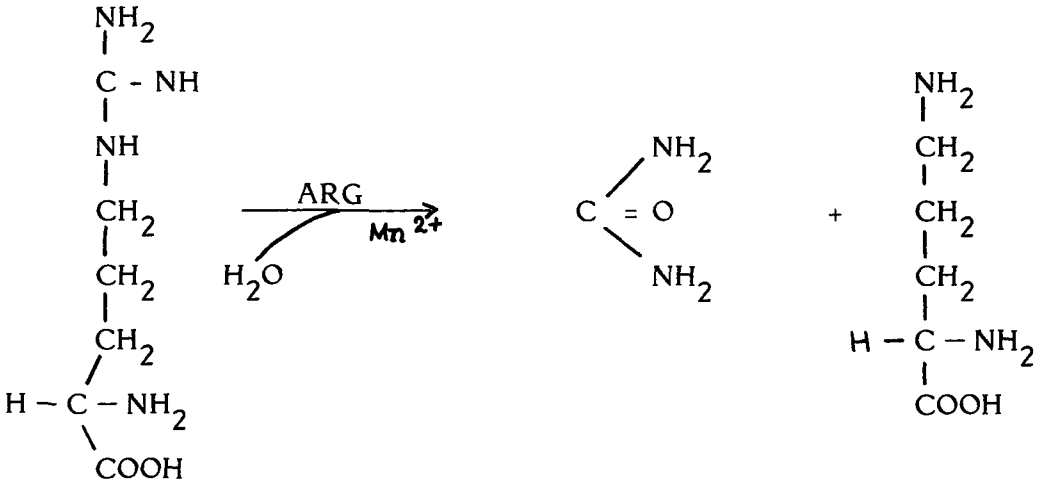
Potassium phosphate buffer (pH 7.0)	50 µmoles
L-Citrulline	3 µmoles
L-Aspartic acid	5 µmoles
$MgSO_4$	8.75 µmoles
ATP	5 µmoles
Urease	20 units
Tissue extract	0.2 ml

The reaction mixture without citrulline was pre-incubated for 5 min at 37°C. The reaction was initiated with the addition of citrulline and incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 10% PCA. In the tissue blank PCA was added in the reaction mixture before the addition of citrulline just before stopping the reaction. The precipitated protein was separated out by centrifugation. The amount of citrulline utilized during the incubation period was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above in CPS assay. One unit of ASS activity was expressed as that amount of enzyme which catalysed the utilization of 1 µmole of citrulline per hr at 37°C.

Argininosuccinate lyase (E.C. 4.3.2.1.): ASL activity was assayed following the method of Brown and Cohen (1959) with some modifications. The concentration of L-argininosuccinic acid was increased from 2 µmoles to 4 µmoles and 20 units of arginase (from Sigma) was added in each reaction mixture. Arginine formed by ASL activity in the tissue was converted to urea in presence of excess arginase and the amount of urea formed was estimated to express ASL activity.



L-Argininosuccinic acid | L-Arginine | Fumaric acid



L-Arginine | Urea | L-Ornithine

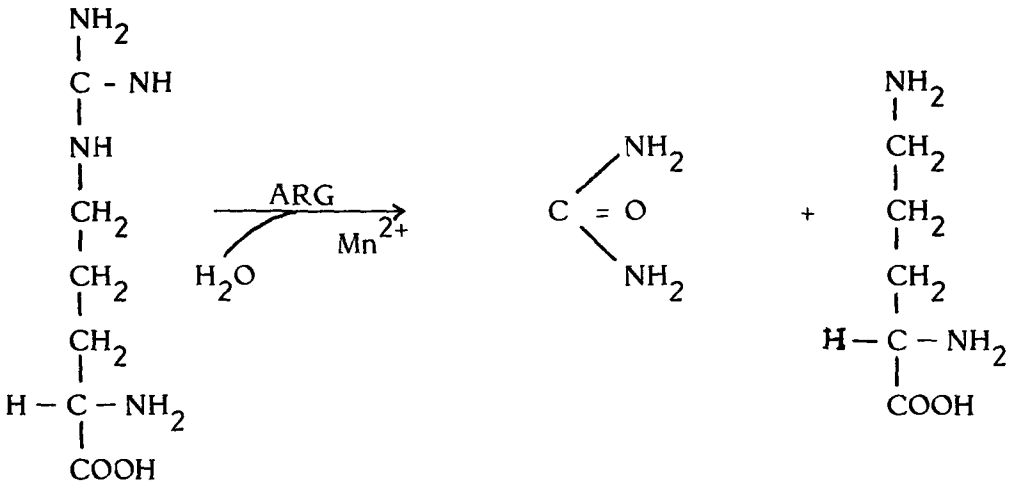
The assay mixture (pH 7.3) in a final volume of 1.0 ml contained:

Potassium phosphate buffer (pH 7.3)	50 µmoles
L-Argininosuccinic acid	4 µmoles
Arginase	20 units
Tissue extract	0.2 ml

The reaction mixture without the tissue extract was preincubated for 5 min at 37°C. The reaction was initiated with the addition of the tissue extract and incubated for 30 min at 37°C. The reaction was stopped by addition of 0.5ml of 10% PCA. A tissue blank was prepared with each assay by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The supernatant was used for urea estimation. Urea was estimated following the method of Moore and Kauffman (1970). The method was same as described for citrulline estimation except that the O.D. was taken at 478 nm. The concentration of urea was calculated from a linear standard graph prepared with different concentrations (0.01 to 0.05 µmoles) of urea. One unit of ASL activity was expressed as that amount of enzyme which catalysed the formation of 1 µmole of urea per hr at 37°C.

Arginase (E.C. 3.5.3.1.): ARG activity was assayed following the method of Brown and Cohen (1959) with the following modification. The concentration of L-arginine was increased to 50 µmoles per assay. The urea formed during the incubation

was estimated to express ARG activity.



L-Arginine Urea L-Ornithine

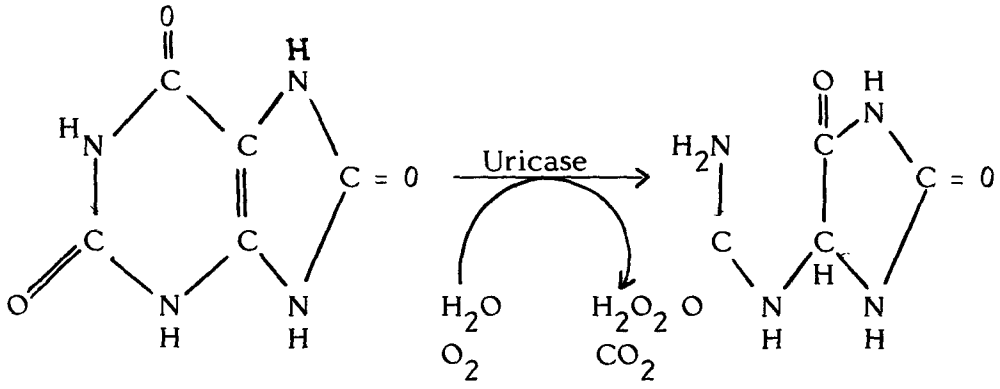
The assay mixture (pH 9.5) in a final volume of 2.0 ml contained:

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

The reaction mixture without L-arginine was preincubated for 10 min at 37°C. The reaction was started with the addition of L-arginine and incubated for 15 min at 37°C. The reaction was stopped by adding 1.0 ml of 10% PCA. A tissue blank was prepared with each assay by adding the PCA to the reaction mixture prior to the addition of L-arginine. The precipitated protein was separated by centrifugation and the supernatant was used for urea estimation. The amount of urea formed during the incubation period was estimated following the method

of Moore and Kauffman (1970) as described above in ASL assay. One unit of arginase activity was expressed as that amount of enzyme which catalysed the formation of 1 μmole of urea per hr at 37°C.

Uricase (E.C. 1.7.3.3.): The activity of uricase was assayed following the method of Kalckar (1947).



Uric acid Allantoin

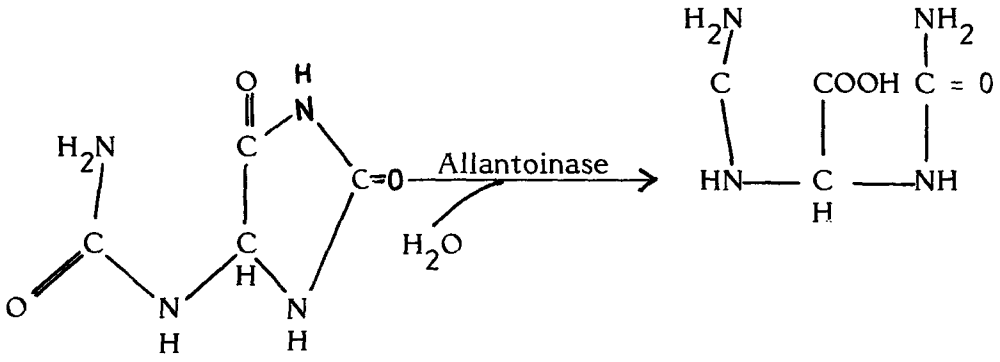
The assay mixture (pH 9.7) in a final volume of 3.0 ml contained:

Sodium glycinate buffer (pH 9.7)	200 μmoles
Uric acid	0.18 μmoles
Tissue extract	0.1 ml

The assay mixture was incubated in a rectangular quartz cuvette having 1 cm light path at 37°C directly in uv-visible spectrophotometer (Beckman Model - 26). The decrease in O.D. at 292 nm due to conversion of uric acid to allantoin was recorded at every 30 sec and the period of linear decrease was used for calculation. The amount of uric acid utilized per hr was calculated taking 12,300 as molar extinction coefficient for uric acid. One unit of uricase activity was expressed as that amount of enzyme which catalysed

the utilisation of 1 μmole of uric acid per hr at 37°C.

Allantoinase (E.C. 3.5.2.5.): Allantoinase activity was assayed following the method of Streamer (1980). The allantoic acid formed was converted to urea and glyoxylic acid by endogenous allantoicase. The amount of glyoxylic acid formed was determined by NADH oxidation in presence of LDH following the method of Brown **et al** (1966) described in detail for allantoicase assay below. Endogenous allantoicase was about 800 times more than allantoinase, thereby completely converting all allantoin formed during the assay to glyoxilic acid.



Allantoin Allantoic acid

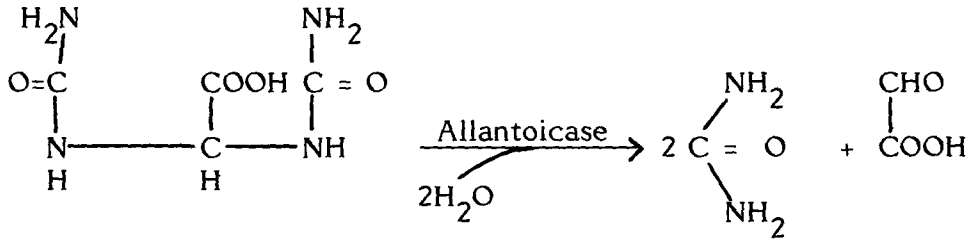
The assay mixture (pH 7.5) in a final volume of 3.0 ml contained:

HEPES buffer (pH 7.5)	125 μmoles
Allantoin	30 μmoles
NADH	0.6 μmoles
LDH	25 units
Tissue extract	0.2 ml

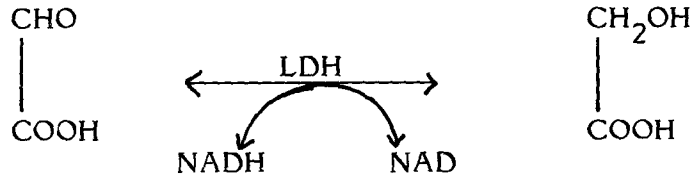
The assay mixture was incubated in a rectangular quartz cuvette having 1 cm light path at 37°C directly in

a uv-visible spectrophotometer (Beckman Model 26). The decrease in O.D. at 340 nm was recorded at every 30 sec interval and the period of linear decrease was used for calculation. The amount of allantoic acid formed during the assay period was calculated from NADH oxidation as described in detail for allantoicase assay below. One unit of allantoinase activity was expressed as that amount of enzyme which catalysed the oxidation of 1 μmole of NADH per hr at 37°C.

Allantoicase (E.C. 3.5.3.4.): The activity of allantoicase was assayed following the method of Brown **et al** (1966).The glyoxylic acid formed was converted to glycolic acid in presence of excess of lactate dehydrogenase (LDH) with the oxidation of NADH in an enzyme coupled reaction.



Allantoic acid — Urea — Glyoxylic acid



Glyoxylic acid — Glycolic acid

The assay mixture (pH 7.5) in a final volume of 3.0 ml contained:

HEPES buffer (pH 7.5)	125 µmoles
Allantoic acid	30 µmoles
NADH	0.6 µmoles
LDH	25 units
Tissue extract (suitably diluted)	0.05 ml

The assay mixture was incubated in a rectangular quartz cuvette having 1 cm light path at 37°C directly in a uv-visible spectrophotometer (Beckman Model 26). The decrease in O.D. was recorded at 340 nm at 30 sec interval and the period of linear decrease was used for calculation. The amount of substrate utilized per hr was calculated taking 6.3×10^3 as molar extinction coefficient for NADH. One unit of allantoicase activity was expressed as that amount of enzyme which catalysed the utilization of 1 µmole of NADH per hr at 37°C.

Protein: Protein was determined in each tissue extract following the method of Lowry **et al** (1951) using crystalline bovine serum albumin as the standard.

Expression of results: Enzyme activities were expressed as Mean ± S.D. of both total activity - units per g wet wt of tissue and as specific activity - units per mg protein.

Chemicals: All the enzymes, coenzymes and substrates were obtained from Sigma Chemical Company, St. Louis, Missouri,

U.S.A.. Other chemicals used were of analytical grade obtained from indegenous sources. Deionized double glass distilled ammonia free water was used in all preparations.

RESULTS

Activity of ornithine-urea cycle enzymes: The total and specific activity of o-u cycle enzymes were determined in the liver and kidney of the five species of freshwater air-breathing teleosts.

Liver: Total and specific activities in liver have been shown in Table 8 and 9 respectively. All the five enzymes of o-u cycle such as CPS (ammonia dependent), OTC, ASS, ASL and ARG could be detected in four out of the five species of freshwater air-breathing teleosts studied. ASS activity could not be detected in **C. punctatus** under the given assay condition. Hepatic CPS activity (total) was found maximum in **H. fossilis** followed by **A. testudineus, C. batrachus, C. punctatus** and **A. cuchia.** The activity of OTC was found maximum in **C. batrachus** followed by **H. fossilis, A. testudineus, A. cuchia** and **C. punctatus.** The activity of ASS was found maximum in **H. fossilis** followed by **A. cuchia, A. testudineus** and **C. punctatus.** Total activity of ARG was found maximum in **H. fossilis** followed by **C. batrachus, A. testudineus, C. punctatus** and **A. cuchia. H. fossilis** had higher level of most of the enzymes of o-u cycle in the liver except OTC which was found maximum in **C. batrachus.**

The distribution of the specific activity of o-u cycle enzymes in the liver (Table 9) followed in general the same pattern as observed for total activity among the five species of freshwater air-breathing teleosts studied.

Kidney: The total and the specific activity of o-u cycle enzymes in the kidney of the five species of freshwater air-breathing teleosts have been presented in Table 10 and 11 respectively. All the five enzymes could be detected in the kidney of at least three out of five species studied. ASS activity could not be detected in **A. testudineus** and **C. punctatus** under the given assay condition. Total activity of CPS in the kidney was found maximum in **A. cuchia** followed by **H. fossilis, C. batrachus, C. punctatus** and **A. testudineus.** OTC activity was higher in **A. cuchia** followed by **C. batrachus** and **H. fossilis.** The activity of ASS was also maximum in **A. cuchia** followed by **H. fossilis** and **C. batrachus.** The activity of ASL was observed maximum in **H. fossilis** followed by **A. cuchia, C. batrachus, A. testudineus** and **C. punctatus.** The ARG activity was found maximum in **A. cuchia** followed by **H. fossilis, C. batrachus, C. punctatus** and **A. testudineus.** The activity of most of the enzymes of o-u cycle in the kidney was found maximum in **A. cuchia** except the ASS which was found slightly more in the kidney of **H. fossilis.**

The distribution of the specific activity of o-u cycle enzymes in the kidney (Table 11) followed in general, the same pattern as observed for total activity among the five species of freshwater air-breathing teleosts studied.

TABLE 8. Total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	CPS	OTC	ASS	ASL	ARG
A. cuchia	1.19 ±0.28	93.06 ±4.17	23.44 ±3.44	16.35 ±2.40	597.12 ±3.56
H. fossilis	4.56 ±0.51	252.27 ±22.71	28.51 ±2.83	27.09 ±2.98	7699.49 ±939.51
C. batrachus	2.35 ±0.42	311.59 ±58.72	12.96 ±2.49	21.93 ±2.70	6653.68 ±886.92
A. testudineus	2.83 ±0.23	113.31 ±11.70	19.70 ±2.21	12.76 ±1.67	3579.71 ±167.82
C. punctatus	2.08 ±0.39	77.94 ±17.92	BLD	6.05 ±0.67	2889.16 ±452.99

CPS - Carbamylphosphate synthetase (ammonia dependent);
OTC - Ornithine transcarbamylase
ASS - Argininosuccinate synthetase
ASL - Argininosuccinate lyase
ARG - Arginase
BLD - Below the level of detection

TABLE 9. Specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	CPS	OTC	ASS	ASL	ARG
A. cuchia	0.007 ±0.001	0.554 ±0.022	0.138 ±0.023	0.097 ±0.014	3.56 ±0.86
H. fossilis	0.029 ±0.003	1.64 ±0.15	0.191 ±0.002	0.18 ±0.02	49.51 ±6.45
C. batrachus	0.014 ±0.003	1.88 ±0.24	0.078 ±0.015	0.131 ±0.021	39.95 ±5.13
A. testudineus	0.017 ±0.002	0.65 ±0.08	0.111 ±0.01	0.072 ±0.009	20.02 ±1.23
C. punctatus	0.014 ±0.003	0.53 ±0.08	BLD	0.044 ±0.005	20.44 3.70

Abbreviations are same as Table 8.

TABLE 10. Total activity (units/g wet wt) of ornithine-urea cycle enzymes in the kidney of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	CPS	OTC	ASS	ASL	ARG
A. cuchia	4.32 ±0.26	109.66 ±6.78	35.22 ±4.39	21.10 ±5.48	2053.39 ±126.09
H. fossilis	2.98 ±0.32	43.08 ±3.32	19.31 ±2.49	22.85 ±1.71	1585.60 ±103.54
C. batrachus	1.84 ±0.24	97.28 ±3.75	6.36 ±1.50	17.19 ±1.41	1578.13 ±132.57
A. testudineus	1.12 ±0.01	66.02 ±2.28	BLD	9.04 ±1.93	886.56 ±122.83
C. punctatus	1.52 ±0.27	65.01 ±6.22	BLD	3.09 ±0.60	1245.29 ±172.58

Abbreviations are same as Table 8.

TABLE 11. Specific activity (units/mg protein) of ornithine-urea cycle enzymes in the kidney of five species of freshwater air-breathing teleosts. (Mean ± S.D.)

Species	CPS	OTC	ASS	ASL	ARG
A. cuchia	0.026 ±0.002	0.671 ±0.047	0.216 ±0.029	0.129 ±0.04	12.54 ±1.38
H. fossilis	0.02 ±0.003	0.28 ±0.024	0.124 ±0.02	0.147 ±0.013	9.43 ±0.74
C. batrachus	0.011 ±0.001	0.594 ±0.054	0.039 ±0.01	0.105 ±0.008	9.66 ±1.36
A. testudineus	0.009 ±0.001	0.491 ±0.047	BLD	0.067 ±0.013	6.58 ±1.34
C. punctatus	0.011 ±0.002	0.471 ±0.024	BLD	0.023 ±0.004	9.26 ±2.90

Abbreviations are same as Table 8.

TABLE 12. Comparison of total activity (units/g wet wt.) of ornithine-urea cycle enzymes in the liver of different groups of fish and an aquatic amphibia.

Species	CPS	OTC	ASS	ASL	ARG	References
TELEOSTS:						
(a) Freshwater						
Cyprinus carpio (carp)	0.42 ±0.03	1.0 ±0.2	0.5	0.4 ±0.1	58.0 ±8.0	Huggins et al (1969)
Rutilus rutilus (roach)	0.11 ±0.01	1.0 ±0.1	0.3	0.8	329 ±13	"
Tinca vulgaris (tench)	0.19 ±0.04	2.4	3.6	1.3 ±0.2	366	"
Siluris glanis (catfish)	BLD	-	9.4	0.3	3542	"
Ictalurus punctatus (")	2.97 ±0.45	220.6 ±102.9	BLD	-	5472 ±1487	Wilson (1973b)
(b) Freshwater air-breathing						
A. cuchia	1.19 ±0.28	93.06 ±4.17	23.44 ±3.44	16.35 ±2.40	597.12 ±3.56	Present work
H. fossilis	4.56 ±0.51	252.27 ±22.71	28.51 ±2.83	27.09 ±2.98	7699.49 ±933.48	"
C. batrachus	2.35 ±0.42	311.59 ±58.72	12.96 ±2.49	21.93 ±2.70	6653.68 ±886.92	"
A. testudineus	2.83 ±0.23	113.31 ±11.70	19.70 ±2.21	12.76 ±1.67	3579.71 ±167.12	"
C. punctatus	2.08 ±0.39	77.94 ±17.92	BLD	6.05 ±0.67	2889.16 ±452.99	"
(c) Marine						
Opsanus tau (tood fish)	9.8	10,210	14.6	184	31800	Read (1971)
LUNGFISHES:						
(a) Purely aquatic						
Neoceratodus forsteri	0.3	154	4.3	8.5	1048	Goldstein et al (1967)
(b) Aestivating						
Protopterus aethiopicus	23.8-37.7	1530-1820	5.5-7.7	25.4-88.3	32,000-37,600	Janssens & Cohen (1966)
Protopterus aethiopicus	31.2	1675	6.6	56.8	34,800	Goldstein et al (1967)
Protopterus aethiopicus	22	1133 ±24	10.1	12.3 ±1.3	4023 ±402	Huggins et al (1969)
AQUATIC AMPHIBIA:						
Xenopus laevis	13	77	11	37	9943	"

Abbreviations are same as Table 8.

TABLE 13. Total activity (units/g wet wt) and specific activity (units/mg protein) of uricolytic pathway enzymes in different tissues of **H. fossilis.** (Means ± S.D.)

Tissue	Uricase		Allantoinase		Allantoicase	
	Total	Specific	Total	Specific	Total	Specific
LIVER	22.69 ±2.33	0.151 ±0.016	6.54 ±0.56	0.042 ±0.004	4598.30 ±504.06	29.09 ±2.78
KIDNEY	5.86 ±0.96	0.035 ±0.006	BLD	BLD	1255.37 ±193.77	7.51 ±1.32
MUSCLE	BLD	BLD	BLD	BLD	2680.47 ±321.68	27.44 ±3.41
BRAIN	BLD	BLD	BLD	BLD	1821.70 ±177.79	21.52 ±2.55

BLD - Below the level of detection.

CPS was found to be the rate limiting enzyme in both liver and kidney of all the species studied. The available data on o-u cycle enzyme activity in various fishes and in aquatic amphibians have been presented in Table 12 for comparison with the present results.

Uricolytic pathway enzymes: The activities (total and specific) of uricolytic pathway enzymes were studied in the liver, kidney, muscle and brain tissues of **H. fossilis** and have been presented in Table 13. All the three enzymes of uricolytic pathway such as uricase, allantoinase and allantoicase could be detected only in liver. Allantoinase activity in kidney and uricase and allantoinase in muscle and brain could not be detected under the given assay conditions. Allantoicase activity was found very high in all the tissues. It was about 800 times more than allantoinase in liver of **H. fossilis.** Allantoinase activity was observed only in the liver and was the rate limiting enzyme with very low activity.

DISCUSSION

The problem of ureogenesis in freshwater teleosts remained as an enigma because most of the species studied showed either absence or low activity of the enzymes related with urea synthesis. As a result, some eroneous ideas were suggested regarding the changes which might have occured in the process of ureogenesis during the evolution of freshwater teleosts. Brown and Cohen (1960) were perhaps logical at that time to suggest the deletion hypothesis as they could

not detect the activity of some of the enzymes of o-u cycle in several species of freshwater teleosts studied by them. However, Huggins **et al** (1969) reported the existence of most of the o-u cycle enzymes with very low activity in some species of freshwater teleosts. Although the findings could not justify active ureogenesis through o-u cycle, at least the evidence for its genetic existence was acceptable. They had also made a thoughtful proposition that "it would be most interesting to study species where an environmental pressure towards modification of ammoniotelism appears to be present, as in the case of the Indian tree fishes for example, to see whether adaptational changes in nitrogen metabolism have taken place." There are many species of freshwater air-breathing teleosts available in India besides the tree fish (**Anabas**). The habit and habitat of five commonly available species which we studied for their ureogenic capability have been described in detail in the General Introduction. All the o-u cycle enzyme activity could be detected in the liver of four and in kidney of three out of the five species (Table 8, 9, 10, 11). The cycle was incomplete in both liver and kidney of **C. punctatus**. **A. testudineus** had the functional o-u cycle only in the liver. The activities of all the enzymes were found generally higher in liver than kidney of the fishes studied. **A. cuchia**, however, showed higher enzyme activity in kidney than liver. Kidney might be the major ureogenic organ in **A. cuchia** whereas liver in other four species studied. The enzyme activities observed in these fishes (Table 8-11) were very high compared to those of purely freshwater teleosts (Brown & Cohen, 1960; Huggins

et al, 1969; Wilson, 1973b) and even freshwater sting rays (Goldstein & Forster, 1971) and permanently aquatic lungfish, **Neoceratodus forsteri** (Goldstein **et al**, 1967) reported earlier (Table 12). The enzyme activities were similar or nearer to those of aestivating lungfish **Protopterus aethiopicus** (Janssens & Cohen, 1966; Goldstein **et al**, 1967; Huggins **et al**, 1969), marine toadfish **Opsanus tau** (Read, 1971) and even aquatic amphibia **Xenopus laevis** (Huggins **et al**, 1969) where urea synthesis through o-u cycle have been confirmed (Table 12). Huggins **et al** (1969) while studying the activity o-u cycle enzymes in teleosts suggested that the basic difference between the Elasmobranchii and Teleosti was the manner of expression, rather than in the possession of the structural genes for the o-u cycle enzymes. Among the five species studied, **H. fossilis** showed higher activity for most of o-u cycle enzymes in its liver. Higher concentration of urea in plasma and urine (Chapter I) and higher o-u cycle enzyme activity observed in **H. fossilis** were similar to those of marine toad fish, **Opsanus tau** (Marshall & Graffin, 1932; Read, 1971). Ramaswamy and Reddy (1983) reported higher excretion rate and more accumulation of urea in the blood during 5 to 10 hrs aerial exposure of two different species of freshwater air-breathing teleosts, **A. scandens** and **C. gachua.** African lungfish **Protopterus** has been reported to be ammoniotelic while in water, excreting major part of its nitrogenous waste as ammonia (Smith, 1930; Sawyer, 1966; Goldstein **et al**, 1967). However, during aestivation **Protopterus** actively converted the accumulated ammonia to urea through o-u cycle (Janssens,

1964; Janssens & Cohen, 1966). O-u cycle activity in the liver of aestivating African lungfish, **P. dilloi** was several times higher than that of the permanently aquatic Australian lungfish, **Neoceratodus forsteri** in their primary aquatic habitat. The South African toad, **X. laevis** is primarily aquatic; and never leaves the water. Sometimes during the dry season when its habitat gets dehydrated, **Xenopus** burrows into the mud waiting for the water supply to be restored. **Xenopus** has been reported to be ammoniotelic, excreting 70-80% of its waste nitrogen as ammonia and 20-30% as urea in aquatic habitat (Balinsky & Baldwin, 1961). During aestivation, instead of accumulation, the toxic ammonia was actively converted to non-toxic urea through o-u cycle in **X. laevis.** The shift from ammoniotelism to ureotelism has been induced artificially in the laboratory by keeping **Xenopus** in moist peat or in hyper-osmotic medium (Balinsky **et al**, 1961). The increased synthesis and accumulation of urea was accompanied by a two fold increase in the activity of the o-u cycle enzymes in its liver (Mc Bean & Goldstein, 1967). The activity of o-u cycle at least in aestivating lungfish **Protopterus** and aquatic **Xenopus** were fully dependent on the environmental factors as suggested by Huggins **et al** (1969). In aqueous phase when ammonia excretion by diffusion was easy the o-u cycle enzymes were inactive and during ammonia toxicity **in vivo** due to blocking of ammonia excretion the cycle got activated to convert accumulated ammonia to urea. The five species of freshwater air-breathing teleosts studied were also primarily

ammoniotelic while in water (Chapter I), but still have high o-u cycle enzyme activity at least in four species similar to lungfish **Protopterus** and aquatic amphibia **Xenopus**. These observations are strong enough to suggest active ureogenesis through o-u cycle in four out of the five species of freshwater air-breathing teleosts studied. Possibly the functioning of the o-u cycle could be regulated by different environmental factors such as water deprivation , hyper-ammonia medium and osmotic stress.

Dietary arginine was suggested to be a potential source of urea since high activity of arginase occurred in the liver of most teleosts (Cvancara, 1969b). The activities of arginase were also quite high in all the five species of freshwater air-breathing teleosts studied. However, it is unlikely that arginine being an essential amino acid in fishes (Wilson & Poe, 1974) would be available in such a large quantity for degradation to account for the high physiological level and excretion of urea in these air-breathing teleosts.

Purine degradation or uricolytic pathway has been reported as an alternate source of urea production in teleosts (Goldstein & Forster, 1965; Cvancara, 1969a; Vellas & Serfaty, 1974). All the three enzymes of uricolytic pathway could be detected in the liver of **H. fossilis**. Florkin and Duchateau (1943) found high level of uricolytic enzymes in fish liver. Goldstein & Forster (1965) suggested that this pathway probably accounted for the small amount of urea formed in teleost

fish. While comparing the amount of contribution of urea by o-u cycle and uricolytic pathway in elasmobranchs Schooler **et al** (1966) found that urea production through uricolytic pathway was 10 times more **in vivo** and 100 times more **in vitro** than through o-u cycle. The activity of liver uricase was found to be lower in **H. fossilis** than in other freshwater teleosts (Goldstein & Forster, 1965; Kinsella **et al**, 1985).

CPS in both liver and kidney (4.56 and 2.98 units/g tissue respectively) and allantoinase (6.54 units/g tissue) in liver of **H. fossilis** were found to be rate limiting enzymes in o-u cycle and uricolytic pathway respectively. Hence, the formation of urea through o-u cycle would be approximately 4.56 μmoles/g/hr in liver and 2.98 μmoles/g/hr in kidney. Taken together total urea production via o-u cycle would be 4.56 + 2.98 = 7.54 μmoles/hr. The rate limiting step in uricolytic pathway would allow production of about 6.54 x 2 = 13.08 μmoles of urea/hr, since from one molecule of uric acid or allantoin two molecules of urea are formed. If we assume that the above enzymatically determined rates approximate the relative rates of urea formation **in vivo** by the two routes, then the formation of urea from uric acid would be approximately (13.08/7.54)=1.73 times higher than that produced by o-u cycle. This was reported to be about 23 times in **Protopterus aethiopicus**, the aestivating lungfish (Brown **et al**, 1966). However, the actual contribution of the two pathways could be different depending on the availability of their substrates **in vivo**. The contribution of urea by

the two different pathways in other species of freshwater air-breathing teleosts could not be discussed as the uricolytic pathway was studied only in **H. fossilis** as a model with higher o-u cycle enzyme activity.

The ability to synthesize urea by two distinct pathways in **H. fossilis** and the presence of complete o-u cycle (with high enzyme activity) in addition to the presence of uricolytic pathway (which is commonly expected in fresh water teleosts) for the synthesis of urea in other three species studied such as **A. cuchia, C. batrachus** and **A. testudineus** are unique findings among freshwater teleosts. They resemble more with their marine ancestors and aestivating dipnoan lungfish **Protopterus.** The observations on the high activity of o-u cycle enzymes in at least four out of five species of fresh-water air-breathing fishes studied during their aquatic life indicate that among the freshwater teleosts some of these fishes still retain functional o-u cycle besides uricolytic pathway for active ureogenesis. The functional o-u cycle might well be regulated by environmental factors to give them potential for resisting ammonia toxicity and osmotic stress.

CHAPTER III

HYPER-AMMONIA STRESS

INTRODUCTION

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

Ammonia toxicity has been primarily attributed to the un-ionized form NH_3 (free base) with the ionized form (NH_4^+) being relatively less toxic. The proportion of un-ionized ammonia increases with increase in pH and temperature (Emerson, 1975).

The effects of environmental ammonia on fish have been studied extensively. Acute toxicity studies were done on cutthroat trout **Salmo clarki** (Thurston **et al**, 1978) and channel catfish **Ictalurus punctatus** (Knepp & Arkin, 1973; Colt & Tchobanoglous, 1976; Robinette, 1976). Extensive proliferation and consolidation of gill lamellae and generative tissue damage in kidney were observed in salmonids exposed

to ammonia (Burrows, 1964; Reichenbach-Klinke, 1967; Olson & Fromm, 1971; Smart, 1976; Thurston **et al**, 1978). Sublethal exposure to ammonia reduced the oxygen carrying capacity of hemoglobin (Sousa & Meade, 1977), increased oxygen consumption, respiratory rate and heart rate (Smart, 1978), increased urine output (Lloyd & Orr, 1969) and hyper-excitability (Wuhrman & Woker, 1949; McCay & Vars, 1950; Fromm & Gillette, 1968; Olson & Fromm, 1971) in fish. Sousa and Meade (1977) proposed that the mechanism of ammonia toxicity involved stimulation of glycolysis by the ammonium ion (NH_4^+) and the simultaneous suppression of the Krebs cycle due to the depletion of α-ketoglutarate which removed ammonia by amination to form glutamate→ glutamine. These two concurrent actions would result in an increase of acidic metabolites, from glycolysis and early Krebs cycle, which would lower blood pH. The resulting acidemia would shift the oxygen dissociation curve (Bohr effect) to reduce maximal oxygen saturation of hemoglobin, and cause death by suffocation. Tomasso **et al** (1980) suggested that the toxic action of ammonia might involve an osmoregulatory disturbance in channel catfish as it has been reported to increase the permeability to tissues to water (Dennis, 1966; Lloyd & Orr, 1969). Another site of action could be inhibition of ATP production due to the uncoupling of oxidative phosphorylation by ammonium ion as suggested by Smart (1978). All these toxic mechanisms, besides any unknown mechanism, might be acting on the fish individually or simultaneously. The actual contribution of each to the total stress would depend on the concentration and form (ionized or un-ionized) of ammonia

present (Tomasso **et al**, 1980).

Reports on the effect of higher ambient ammonia on nitrogen excretion pattern in teleosts are very much limited. Fromm and Gillette (1968) demonstrated that an increase in ambient ammonia from 0 to 8 µg/ml caused an increase in blood ammonia of trout from 40 to 70 µg/ml. At some critical level of blood ammonia, if the fish has to survive, must either decrease its sensitivity to ammonia or convert the ammonia to a less toxic nitrogenous compound which can either be excreted immediately or stored temporarily until conditions are favourable for excretion. Olson and Fromm (1971) found that goldfish, **Carassius auratus** subjected to increased ambient ammonia level showed increase in urea excretion rate. Conversion of greater part of ammonia to urea and a shift towards ureotelism have been reported during water shortage and aestivation in amphibians (Janssens & Cohen, 1968; McBean & Goldstein, 1970; Balinsky, 1970, 1981), African lungfish (Janssens, 1964; Goldstein **et al**, 1967), and in certain mudskipper fish (Gordon **et al**, 1969, 1978). However, conversion of ammonia to urea was not known in freshwater teleosts where the functioning of o-u cycle was doubtful. Although Huggins **et al** (1969) could detect the activities of o-u cycle enzymes in some species of freshwater teleosts, the activities were too low to convert a significant portion of ammonia to urea.

Higher excretion rate and tissue level of urea in five freshwater air-breathing teleosts compared to other freshwater teleosts were observed during the present study (Chapter-I).

Presence of functional o-u cycle was also found in four out of five species studied (Chapter II). It was therefore, proposed that ureotelism could be a physiological adaptation for nitrogen metabolism in the freshwater air-breathing teleosts which are otherwise predominantly ammoniotelic in their primary aquatic habitat. In aquatic amphibians, increased tissue ammonia concentration has been suggested to trigger the transition to ureotelism (Janssens & Cohen, 1968; Janssens, 1972; Balinsky, 1981).

The obligatory air-breathing freshwater teleosts tolerate temporary dehydration for several hours (Chapter I) and some of them grow well in sewage fed water bodies where ammonia concentration is usually very high. Some of these species are used for culture in agricultural paddy fields where ammonia level remains high being used as fertiliser. It might be that the freshwater air-breathing teleosts have adaptational capacity to higher ambient ammonia due to the presence of regulatory shifting mechanism towards ureotelism. It was, therefore, planned to study the effect of different concentrations of ammonia on the metabolism and excretion pattern of ammonia and urea in a freshwater air-breathing teleost, **Heteropneustes fossilis**, where the activities of o-u cycle enzymes were found to be very high among the five species studied (Chapter II). The results obtained were viewed to find out (i) ammonia tolerance limit of the fish, (ii) tissue specific ammonia tolerance limit, (iii) alteration in the accumulation/excretion pattern of ammonia and urea, and (iv) the possibility of inducing

urea synthesis for detoxification of ammonia through o-u cycle.

Plan of work:

1. Survival capacity of **H. fossilis** was studied in different concentrations of NH_4Cl (25, 50, 75, 100, 150, 200mM).
2. Alterations in the excretion/absorption of ammonia and urea were studied at 48 hrs interval in **H. fossilis** treated with different concentrations of NH_4Cl for 28 days.
3. Concentration of ammonia and urea in different tissues such as liver, kidney, muscle, brain and in blood plasma were estimated at 7 days interval for 28 days in **H. fossilis** treated with diffrent concentrations of NH_4Cl.
4. Activities of o-u cycle enzymes such as CPS (ammonia dependent), OTC, ARSS and ARG were assayed in the liver and kidney at 7 days interval for 28 days in **H. fossilis** treated with 50 mM NH_4Cl.

MATERIALS AND METHODS

Animal: **Heteropneustes fossilis** weighing 25-35 g acclimatised to the laboratory conditions as described in Chapter I were used for experimentation.

Experimental set up: The experiment was carried out under the same environmental conditions to which the fishes were acclimatised. Fishes of similar size were used a day after

the last feeding and no food was given during the experimental period. They were treated in groups of five in plastic buckets containing different concentrations of NH_4Cl (25, 50, 75, 100, 150 and 200 mM) in 2 l bacteria free filtered tapwater containing streptopenicillin (20 mg/l) to stop microbial growth in the medium. The behavioural responses and rate of mortality were recorded in the treated fish. Complete mortality was observed within 48 hrs at 100, 150 and 200 mM of NH_4Cl. Hence. further experiments were conducted only at 25, 50 and 75 mM NH_4Cl. The treatments were continued for 28 days. Controls were run side by side with groups of five fishes being kept in plastic buckets containing 2 l of bacteria free filtered tapwater treated with streptopenicillin (20 mg/l). The medium was replaced in each bucket at every 48 hrs after collecting samples for estimation of total ammonia and urea in the medium. The increase or decrease in the amount of ammonia in the medium was expressed as excretion or absorption respectively of ammonia by the fish. The amount of urea estimated in the medium was calculated as total urea excreted by the fish. Control buckets with various concentrations of NH_4Cl (no fish) were used to find out the change in ammonia concentration in the medium during the experimental period for correcting the data. Concentration of ammonia and urea in different tissues such as liver, kidney, brain and muscle and in blood plasma of the fish were estimated after 7, 14, 21 and 28 days of exposure to NH_4Cl. The alteration in the activity of o-u cycle enzymes in liver and kidney was studied after 7, 14, 21 and 28 days of treatment of the fish to only 50 mM NH_4Cl.

Blood sampling: Blood was collected from the caudal vein with a heparinized syringe and were processed in the same way as mentioned in Chapter I for estimation of ammonia and urea level.

Tissue preparation: Fishes were sacrificed by decapitation immediately after collecting the blood at 12 noon. Tissues such as liver, kidney, muscle and brain were quickly removed blotted dry and deep frozen at -20°C until used for estimations. All estimations were completed within two days of sampling.

Tissues were processed for estimation of their ammonia and urea level as described in Chapter I.

Estimation procedure: Estimation of ammonia and urea in the sample was done following the methods described in Chapter-I.

Enzyme assay: The activity of o-u cycle enzymes such as carbamyl phosphate synthetase (ammonia dependent) (CPS), ornithine transcarbamylase (OTC) and arginase (ARG) were assayed following the methods described in detail in Chapter II. However, argininosuccinate synthetase and argininosuccinate lyase were assyed together to give the overall reaction as arginine synthetase system (ARSS) following the method of Brown and Cohen (1959).

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

Potassium phosphate buffer (pH 7.0)	50 µmoles
L-Citrulline	5 µmoles
L-Aspartic acid	5 µmoles
ATP	5 µmoles
$MgSO_4$	5 µmoles
Arginase	20 units
Tissue extract	0.2 ml

The reaction mixture without the tissue extract was pre-incubated for 5 min at 37°C. The reaction was initiated with the addition of the tissue extract. After incubation for 1 hr at 37°C, the reaction was stopped with the addition of 1.0 ml 10% PCA. A tissue blank was prepared with each assay by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The supernatant was used for urea estimation following the method of Moore and Kauffman (1970) as mentioned in Chapter II. One unit of the enzyme was defined as that amount which catalysed the production of 1 µmole of urea per hr at 37°C.

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

Expression of data: The rate of excretion or absorption of ammonia and urea during the experimental period were expressed as µmoles per g of body wt per 48 hrs. The concentration of ammonia and urea in tissues were expressed as µmoles per g wet wt and in blood plasma as µmoles per ml. The enzyme

activity were expressed both as total activity - units per g wet wt of tissue and as specific activity - units per mg protein.

Data were analysed statistically and expressed as mean ± S.D.. The level of significance between two sets of data were calculated by student 't' test and 'p' value above 0.05 were taken as non-significant (N.S.).

Chemicals: All the enzymes, coenzymes and substrates were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.. Other chemicals used were of analytical grade obtained from indigenous sources. Deionized double glass distilled ammonia free water was used in all preparations.

RESULTS

Survival of **H. fossilis** in NH_4Cl solution:

H. fossilis were exposed to NH_4Cl solution at different concentrations ranging from 25-200 mM. The fishes did not show any behavioural change in 25 mM NH_4Cl. The fishes became hyper excitable at higher concentrations of NH_4Cl and this increased with increasing concentration. Any disturbance in the experimental bucket resulted in violent disoriented escape attempts. All the fishes died at 100, 150 and 250 mM NH_4Cl within 48, 12 and 2 hrs of exposure respectively. These concentrations, therefore, could not be used for longterm experimentation. The hyper-excitability calmed down after 24 hrs in 50 mM and after 48 hrs in 75 mM.

Excretion of ammonia and urea:

Ammonia: Absorption from the medium dominated over excretion of ammonia during the exposure of the fishes to different concentrations of NH_4Cl (Table 14; Fig.3). This was concentration dependent with maximum absorption in 75 mM and minimum in 25 mM. After 8-10 days of treatment either the absorption of ammonia decreased or the excretion gradually increased to return to normal level after 24 days only in 25 mM NH_4Cl. In other two concentrations, although the above trend continued, the excretion was lower than absorption even by 28th day when the treatment was terminated. In control fishes there was a decrease in ammonia excretion upto 6 days after which it got stabilised in that lower level.

Urea: The excretion of urea slowly decreased upto 28 days in the control fishes (Table 15; Fig. 3). However, this decrease was not significant. Exposure to NH_4Cl showed, in general, concentration dependent alterations of urea excretion (Table 15; Figs. 3 & 4). There was significant suppression of urea excretion in all the concentrations upto 6-8 days of treatment. Excretion of urea increased significantly after 6 days in 25 mM and after 8 days in 50 and 75 mM. Urea excretion reached the highest rate around 12th to 14th day of treatment. Thereafter, the urea excretion decreased slowly during the later period of experimentation. However, it continued at a much higher rate than the control fish till the end of the experiment.

Tissue ammonia and urea level:

Ammonia: Ammonia accumulated significantly in different tissues and in blood plasma when the fishes were exposed to different concentrations of NH_4Cl (Tables 16 & 17; Figs. 5 & 6). The accumulation was maximum at 75 mM and minimum at 25 mM treatment. Accumulation of ammonia in most of the tissues reached maximum level within 7 days of treatment except in plasma where the peak was attained by 14th day. Thereafter, the concentration of ammonia **in vivo** either did not change or slowly decreased. The maximum ammonia accumulation was found in kidney (40.74) followed by liver (34), muscle (18) and brain (13.7 μmoles/g wet wt). In plasma it was 4.7 μmoles per ml. There was no significant change in tissue ammonia level in control fish althrough the experiment (Table 16; Fig. 5). The percent increase of tissue ammonia accumulation in treated fish was maximum in plasma (900%) compared to the control followed by kidney (220%), liver (150%), brain (145%) and muscle (70%) at 75 mM NH_4Cl treatment (Table 17; Fig. 6).

Urea: Urea concentration increased in all the tissues of the fish treated with all the three concentrations of NH_4Cl (Tables 18 & 19; Figs. 7 & 8). The accumulation of urea in the tissues was very high during the early period reaching the highest level by 14th day of treatment. The accumulation of urea increased with increasing concentration of ammonia in the medium. Control fish also showed an increase in urea level in their tissues except in the plasma where the level was unchanged (Table 18; Fig. 7). Maximum accumulation of urea in treated

fish was observed in the liver (27.8) followed by kidney (16.8), brain (9.6) and muscle (2.6 μmoles/g wet wt). In plasma the maximum level was 4.8 μmoles/ml. However, the percent increase in tissue urea in treated fish was highest in plasma (550%) followed by brain (260%), liver (165%), kidney (105%) and muscle (80%) (Table 19; Fig. 8).

Activity of o-u cycle enzymes in liver and kidney:

Liver: There was a significant induction of the activity (total and specific) of all the enzymes of o-u cycle (Tables 20 & 21; Figs. 9-12) except ARG in the fishes treated with NH_4Cl. Maximum increase of both total and specific activity was noticed almost in all the cases on 14th day of treatment except ARSS which showed maximum activity only by 28th day. Specific acivity of CPS showed a linear increase with increasing time. Maximum induction of about 190% was observed for ARSS activity while CPS was induced upto about 10-50% and OTC upto 30-40%.

Kidney: Significant induction of the activity of o-u cycle enzymes except ARG was also noticed in the kidney of **H. fossilis** treated with NH_4Cl (Tables 22 & 23; Figs. 9-12). CPS activity increased continuously with increasing period of treatment. However, ARSS activity reached the highest level by 7th day and the activities of OTC and ARG by 14th day of NH_4Cl treatment. The percent increase in enzyme activity was maximum for OTC followed by CPS, ARSS and ARG in the kidney of **H. fossilis** treated with 50 mM NH_4Cl (Tables 22 & 23; Figs. 10 & 12). Specific activity, in general, showed greater induction than

TABLE 14. Alterations in the rate (μmoles/g body wt /48 hrs) of excretion (+) or absorption (-) of ammonia by **H. fossilis** treated with different concentration of NH_4Cl. (Mean ± S.D.).

Days after treatment	2	4	6	8	10	12	14	16	18	20	22	24	26	28
H_2O (control)	+11.45 ±2.29	+9.18 ±1.44	+9.05 ±1.38	+8.33 ±3.44	+8.03 ±2.18	+7.89 ±1.45	+7.82 ±1.55	+7.78 ±1.67	+7.61 ±1.22	+7.66 ±1.33	+7.45	+7.51	+7.61	+7.53
25 mM NH_4Cl	-31.45 ±6.12	-28.44 ±6.75	-25.67 ±5.18	-26.11 ±5.56	-29.16 ±4.98	-24.44 ±4.44	-16.49 ±3.16	-15.79 ±3.09	-10.13 ±2.66	-5.19 ±1.18	-6.88	+12.38	+11.22	+10.67
50 mM NH_4Cl	-44.76 ±7.16	-40.23 ±6.87	-34.11 ±6.13	-39.19 ±5.89	-43.27 ±7.12	-40.69 ±7.23	-38.11 ±6.87	-37.12 ±6.23	-34.75 ±5.19	-30.15 ±5.22	-27.33	-23.19	-18.77	-16.26
75 mM NH_4Cl	-69.43 ±10.11	-67.22 ±9.87	-71.98 ±9.23	-66.23 ±8.86	-60.11 ±8.12	-62.19 ±6.67	-56.37 ±7.89	-48.22 ±7.36	-46.19 ±6.16	-41.33 ±6.76	-31.73	-28.16	-25.68	-23.11

TABLE 15. Alterations in the rate (μmoles/g body wt /48 hrs) of excretion of urea by **H. fossilis** treatment with different concentration of NH_4Cl. (Mean ± S.D.)

Days after treatment	2	4	6	8	10	12	14	16	18	20	22	24	26	28
H_2O	1.45 ±0.21	1.26 ±0.18	1.15 ±0.19	1.08 ±0.22	1.05 ±0.13	1.04 ±0.23	0.96 ±0.10	1.01 ±0.14	0.98 ±0.12	1.06 ±0.09	1.04	0.96	0.95	0.98
25 mM NH_4Cl	0.80 ±0.10	0.97 ±0.11	1.12 ±0.13	1.78 ±0.16	2.46 ±0.27	2.58 ±0.31	2.48 ±0.28	2.69 ±0.25	2.24 ±0.22	2.16 ±0.33	2.27	2.38	2.19	2.16
	(-44.8)	(-23.0)	(-2.6)	(+64.8)	(+134.3)	(+148.1)	(+158.3)	(+166.3)	(+128.6)	(+103.8)	(+118.3)	(+147.9)	(+130.5)	(+120)
50 mM NH_4Cl	0.67 ±0.06	0.61 ±0.05	0.82 ±0.06	1.18 ±0.09	2.96 ±0.31	3.24 ±0.33	2.82 ±0.32	2.98 ±0.34	2.66 ±0.26	2.44 ±0.21	1.98	2.05	2.11	1.92
	(-53.8)	(-51.6)	(-28.7)	(+9.3)	(+181.9)	(+211.5)	(+193.8)	(+195)	(+171.4)	(+130)	(+90.4)	(+114)	(+122)	(+96)
75 mM NH_4Cl	0.62 ±0.04	0.54 ±0.05	0.52 ±0.06	0.68 ±0.05	2.77 ±0.28	2.99 ±0.33	2.86 ±0.36	2.78 ±0.27	2.56 ±0.25	2.61 ±0.29	2.59	2.24	2.18	2.10
	(-57.2)	(-57.1)	(-54.8)	(-37.0)	(+164)	(+188)	(+198)	(+175)	(+161)	(+146)	(+149)	(+133)	(+130)	(+114)

% change compared to control are given in parentheses.

TABLE 16. Alterations in the concentration of ammonia in different tissues (μmoles/g wet wt.) and in blood plasma (μmoles/ml) of **H. fossilis** treated with different concentration of NH_4Cl. (Mean ± S.D.)

Tissue	NH_4Cl (mM)	Days after treatment				
		0	7	14	21	28
(control) LIVER	0	14.56 ± 1.15	13.33 ± 1.27	13.78 ± 0.81	13.98 ± 0.60	13.25 ± 0.78
	25		25.45 ± 1.51*	21.71 ± 1.27*	19.29 ± 1.14*	18.76 ± 1.12*
	50		30.50 ± 1.61*	27.99 ± 1.42*	23.68 ± 0.89*	23.15 ± 0.97*
	75		33.77 ± 3.04*	34.06 ± 2.66*	32.23 ± 1.83*	30.15 ± 2.68*
(control) KIDNEY	0	13.90 ± 1.22	12.60 ± 1.29	12.35 ± 0.43	13.02 ± 0.47	13.11 ± 1.23
	25		29.44 ± 2.29*	24.67 ± 1.72*	23.15 ± 1.18*	21.75 ± 1.78*
	50		37.62 ± 0.93*	36.27 ± 1.60*	36.65 ± 2.39*	34.35 ± 1.00*
	75		40.74 ± 1.01*	39.61 ± 3.63*	35.80 ± 2.22*	32.91 ± 2.92*
(control) MUSCLE	0	12.43 ± 1.09	10.73 ± 0.45	10.04 ± 0.22	10.35 ± 0.43	10.11 ± 0.44
	25		13.59 ± 0.86*	13.14 ± 0.71*	13.59 ± 0.55*	12.96 ± 0.38*
	50		16.80 ± 0.36*	14.78 ± 1.22*	14.20 ± 0.99*	14.02 ± 0.74*
	75		18.14 ± 0.91*	17.17 ± 0.60*	16.89 ± 0.45*	16.25 ± 0.89*
(control) BRAIN	0	6.74 ± 0.81	5.53 ± 0.24	5.47 ± 0.86	5.42 ± 0.45	5.51 ± 0.42
	25		8.51 ± 0.47*	9.14 ± 0.56*	8.63 ± 0.44*	8.24 ± 0.46*
	50		9.46 ± 0.41*	9.88 ± 0.74*	9.20 ± 0.73*	8.79 ± 0.51*
	75		13.68 ± 2.01*	11.82 ± 0.40*	10.45 ± 1.23*	10.22 ± 1.21*
(control) PLASMA	0	0.473 ± 0.04	0.496 ± 0.04	0.467 ± 0.07	0.458 ± 0.06	0.443 ± 0.04
	25		2.18 ± 0.21*	2.42 ± 0.23*	2.11 ± 0.19*	1.98 ± 0.13*
	50		3.84 ± 0.45*	3.94 ± 0.36*	3.14 ± 0.38*	3.04 ± 0.46*
	75		4.56 ± 0.56*	4.71 ± 0.51*	4.26 ± 0.47*	4.08 ± 0.51*

* p value calculated compared to control are significant at <0.001

TABLE 17. % increase in the concentration of ammonia in different tissues and in blood plasma of **H. fossilis** treated with different concentration of NH_4Cl.

Tissue	NH_4Cl (mM)	Days after treatment			
		7	14	21	28
LIVER	25	90.92	57.55	37.98	41.58
	50	128.81	103.12	68.38	74.72
	75	153.34	147.17	130.54	127.55
KIDNEY	25	133.65	99.76	77.80	65.90
	50	198.57	193.68	181.49	162.01
	75	223.33	220.73	174.96	151.03
MUSCLE	25	26.65	30.78	31.30	28.19
	50	56.57	47.21	37.20	38.67
	75	69.06	71.02	63.19	60.73
BRAIN	25	53.89	67.09	59.23	49.55
	50	71.07	80.62	69.74	59.53
	75	147.38	116.09	92.80	85.48
PLASMA	25	339.52	418.20	360.70	346.95
	50	674.19	743.68	585.59	586.23
	75	819.35	908.57	830.13	821.00

TABLE 18. Alterations in the concentration of urea in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** treated with different concentration of NH_4Cl. (Mean ± S.D.)

Tissue	NH_4Cl (mM)	Days after treatment				
		0	7	14	21	28
(control)	0	6.23 ± 0.61	7.31 ± 0.78$_b$	10.51 ± 0.60$_a$	12.72 ± 0.82$_b$	13.21 ± 1.11
LIVER	25		9.81 ± 0.87^b	21.86 ± 1.50^a	19.45 ± 2.19^b	18.34 ± 1.67^b
	50		12.26 ± 1.70^b	25.01 ± 3.85^a	23.93 ± 2.17^a	21.77 ± 2.05^a
	75		12.21 ± 0.97^a	27.80 ± 2.26^a	26.89 ± 2.45^a	25.44 ± 2.66^a
(control)	0	5.53 ± 0.97	6.48 ± 0.81$_d$	8.21 ± 0.61$_a$	10.82 ± 1.33$_e$	11.12 ± 0.89$_f$
KIDNEY	25		8.22 ± 0.89^d	12.76 ± 1.08^a	12.30 ± 1.26^e	12.11 ± 1.10^f
	50		10.43 ± 0.40^a	15.46 ± 2.29^a	15.37 ± 2.03^c	15.87 ± 1.70^b
	75		10.39 ± 0.61^a	16.74 ± 1.23^a	16.53 ± 1.77^b	16.79 ± 1.23^a
(control)	0	1.31 ± 0.32	1.22 ± 0.23$_f$	1.31 ± 0.28$_f$	1.37 ± 0.20$_e$	1.45 ± 0.27$_f$
MUSCLE	25		1.39 ± 0.25^f	1.79 ± 0.39^f	1.82 ± 0.33^e	1.78 ± 0.36^f
	50		1.89 ± 0.25^c	2.24 ± 0.22^b	2.30 ± 0.38^b	2.46 ± 0.28^c
	75		1.60 ± 0.31^f	2.16 ± 0.20^b	2.41 ± 0.41^b	2.62 ± 0.41^c
(control)	0	2.49 ± 0.37	2.87 ± 0.49$_b$	2.60 ± 0.33$_a$	2.91 ± 0.19$_a$	4.01 ± 0.38$_a$
BRAIN	25		4.26 ± 0.41^b	6.52 ± 0.45^a	7.06 ± 0.25^a	7.11 ± 0.30^a
	50		4.90 ± 0.31^a	9.43 ± 0.77^a	9.66 ± 0.46^a	9.45 ± 0.39^a
	75		5.05 ± 0.5^a	8.89 ± 0.55^a	9.12 ± 0.89^a	8.78 ± 0.46^a
(control)	0	0.755 ± 0.081	0.742 ± 0.033	0.737 ± 0.038	0.726 ± 0.036	0.71 ± 0.042
PLASMA	25		2.23 ± 0.38^a	2.54 ± 0.32^a	2.18 ± 0.27^a	2.16 ± 0.18^a
	50		3.16 ± 0.56^a	3.87 ± 0.48^a	3.54 ± 0.39^a	3.44 ± 0.32^a
	75		4.26 ± 0.71^a	4.82 ± 0.79^a	4.36 ± 0.56^a	4.41 ± 0.044^a

p value calculated compared to control are as a-<0.001; b-<0.005; c-<0.01; d-<0.025; e-<0.05; f- N.S.

TABLE 19. % increase in the concentration of urea in different tissues and in blood plasma of **H. fossilis** treated with different concentration of NH_4Cl.

Tissue	NH_4Cl (mM)	Days after treatment			
		7	14	21	28
LIVER	25	34.20	108.00	52.91	38.83
	50	67.72	137.96	88.13	64.80
	75	67.03	164.51	111.40	92.58
KIDNEY	25	26.85	55.42	13.68	8.90
	50	62.21	88.31	42.05	42.72
	75	60.34	103.90	52.77	50.99
MUSCLE	25	13.93	36.64	32.85	22.76
	50	47.54	71.00	67.88	69.66
	75	31.15	64.89	75.91	80.69
BRAIN	25	48.43	150.77	80.56	77.31
	50	70.73	262.69	147.06	135.66
	75	75.96	241.92	133.25	118.95
PLASMA	25	200.54	244.64	200.28	204.23
	50	325.88	425.10	387.60	384.51
	75	474.12	554.00	500.55	521.13

TABLE 20. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver of **H. fossilis** treated with 50 mM NH_4Cl. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	4.56 ±0.51		252.27 ±22.71		28.72 ±5.49		7699.49 ±933.48	
7	5.85 ±0.83 (±28.3)	<0.05	263.25 ±11.78 (+4.4)	N.S.	78.13 ±19.76 (+172.0)	<0.005	7193.40 ±495.44 (-6.6)	N.S.
14	6.42 ±0.84 (+40.2)	<0.01	327.92 ±29.45 (+30.0)	<0.05	82.06 ±11.40 (+185.7)	<0.001	8298.14 ±455.91 (+7.8)	
21	6.04 ±0.74 (31.9)	<0.02	305.85 ±27.20 (+21.2)	<0.02	75.85 ±15.45 (+164.1)	<0.001	7908.97 ±873.22 (+2.7)	N.S.
28	6.35 ±0.42 (+38.7)	<0.005	293.41 ±34.39 (+16.3)	<0.02	83.69 ±9.35 (+191.4)	<0.001	7507.24 ±356.74 (-2.5)	N.S.

CPS - Carbamyl phosphate synthetase
OTC - Ornithine transcarbamylase
ARSS - Arginine. synthetase system
ARG - Arginase
% change compared to control are given in parentheses.

TABLE 21. Alterations in the specific activity (units/g protein) of ornithine-urea cycle enzymes in the liver of **H. fossilis** treated with 50 mM NH_4Cl. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.029 ±0.003		1.62 ±0.16		0.21 ±0.04		49.51 ±6.45	
7	0.038 ±0.006 (+31.0)	<0.05	1.72 ±0.07 (+6.2)	N.S.	0.51 ±0.14 (+142.9)	<0.01	47.07 ±3.32 (-4.9)	N.S.
14	0.04 ±0.004 (+38.0)	<0.005	2.21 ±0.22 (+36.4)	<0.005	0.55 ±0.08 (+161.9)	<0.001	55.91 ±3.61 (+12.9)	N.S.
21	0.044 ±0.004 (+51.7)	<0.001	2.15 ±0.18 (+32.7)	<0.005	0.53 ±0.01 (+152.4)	<0.001	55.72 ±6.05 (+12.5)	N.S.
28	0.045 ±0.002 (+55.2)	<0.001	2.11 ±0.29 (+30.3)	<0.025	0.60 ±0.06 (+185.7)	<0.001	53.88 ±3.13 (+8.8)	N.S.

Abbreviations are same as Table 20.

% change compared to control are given in parentheses.

TABLE 22. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** treated with 50 mM NH_4Cl. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	2.98 ±0.32		43.08 ±3.32		21.21 ±3.62		1585.60 ±103.54	
7	4.39 ±0.43 (+47.3)	<0.005	81.62 ±2.22 (+89.5)	<0.001	47.73 ±7.61 (+125.0)	<0.001	1493.92 ±228.05 (-5.8)	N.S.
14	5.18 ±0.95 (+73.8)	<0.005	101.79 ±7.74 (+136.3)	<0.02	43.16 ±7.27 (+103.5)	<0.005	1757.55 ±221.93 (+10.8)	N.S.
21	5.11 ±0.19 (+71.5)	<0.001	114.31 ±24.00 (+165.3)	<0.001	39.89 ±4.81 (+88.1)	<0.001	1666.53 ±229.94 (+5.1)	N.S.
28	7.17 ±0.37 (+140.6)	<0.001	100.00 ±7.19 (+132.1)	<0.001	40.86 ±8.00 (+92.6)	<0.005	1531.58 ±224.57 (-3.4)	N.S.

Abbreviations are same as Table 20.

% change compared to control are given in parentheses.

TABLE 23. Alterations in the specific activity (units/g protein) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** treated with 50 mM NH_4Cl. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.02 ±0.003		0.28 ±0.024		0.137 ±0.021		9.43 ±0.74	
7	0.03 ±0.002 (+50.0)	<0.005	0.56 ±0.02 (+100)	<0.001	0.33 ±0.06 (+140.9)	<0.001	10.33 ±1.42 (+9.5)	N.S.
14	0.039 ±0.008 (+95)	<0.005	0.76 ±0.08 (+171.4)	<0.001	0.32 ±0.05 (+133.6)	<0.001	13.17 ±1.33 (+39.6)	<0.005
21	0.039 ±0.002 (+95.0)	<0.001	0.90 ±0.022 (+221.4)	<0.001	0.31 ±0.04 (+126.3)	<0.001	13.04 ±1.98 (+38.3)	<0.02
28	0.055 ±0.003 (+175.0)	<0.001	0.77 ±0.06 (+175.0)	<0.001	0.32 ±0.06 (+133.6)	<0.001	11.79 ±1.80 (+25.0)	<0.05

Abbreviations are same as Table 20.

% change compared to control are given in parentheses.

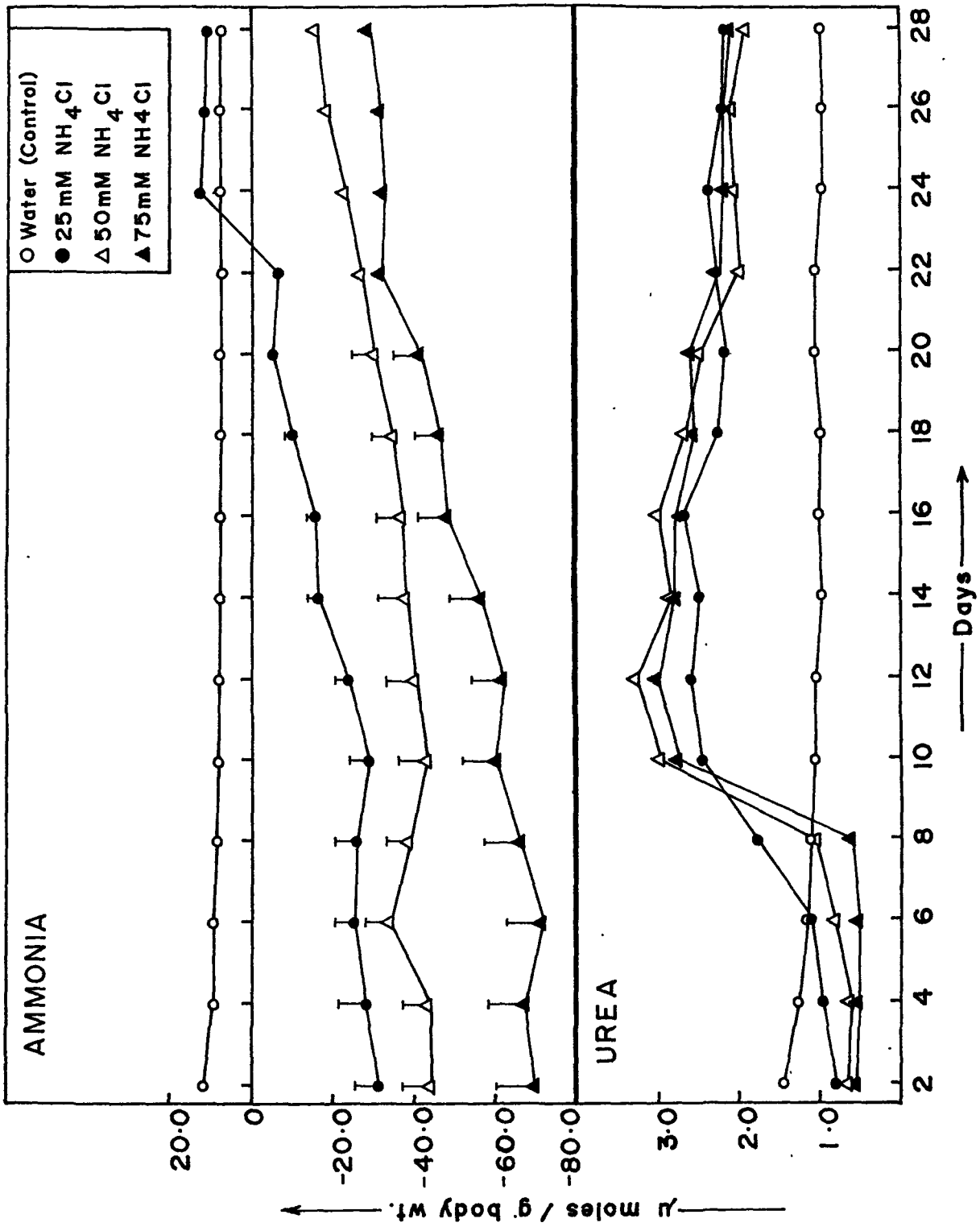


Fig. 3. Excretion (+)/absorption (-) of ammonia and urea (μmoles/g body wt /48 hrs) by **H. fossilis** treated with different concentration of NH_4Cl.

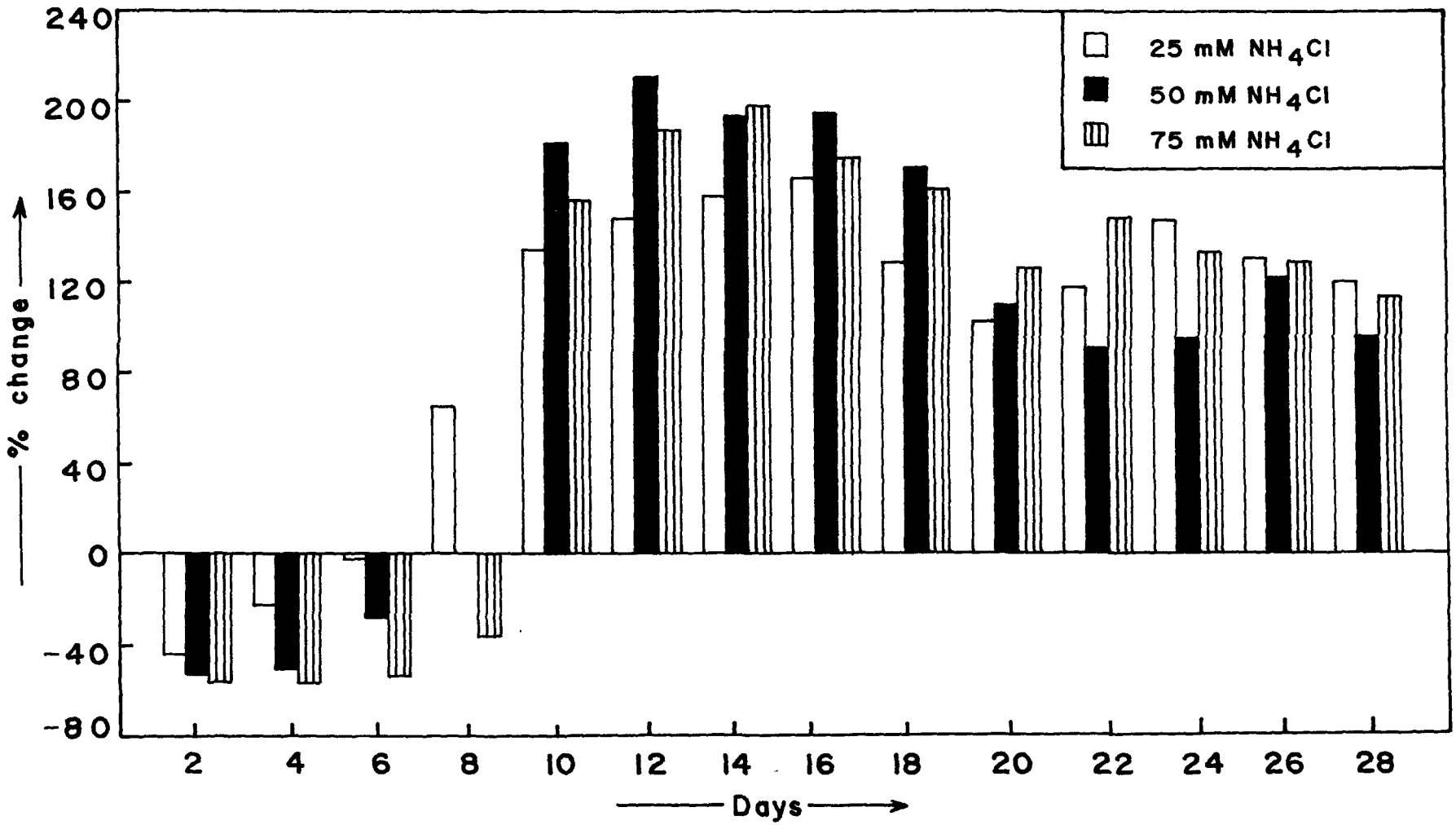


Fig. 4. % change of excretion of urea by **H. fossilis** treated with different concentration of NH_4Cl.

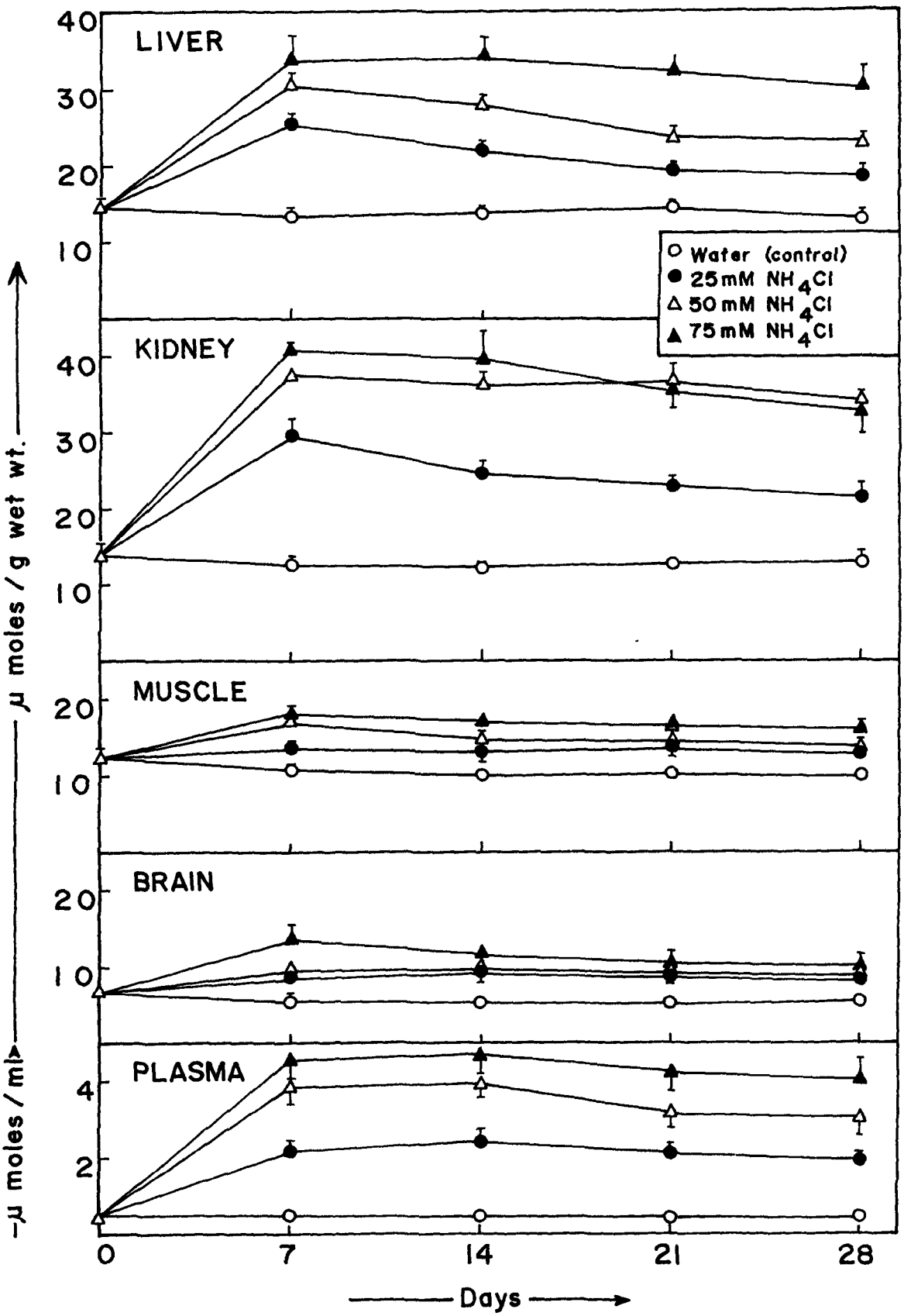


Fig. 5. Alterations in the concentration of ammonia in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** treated with different concentration of NH_4Cl.

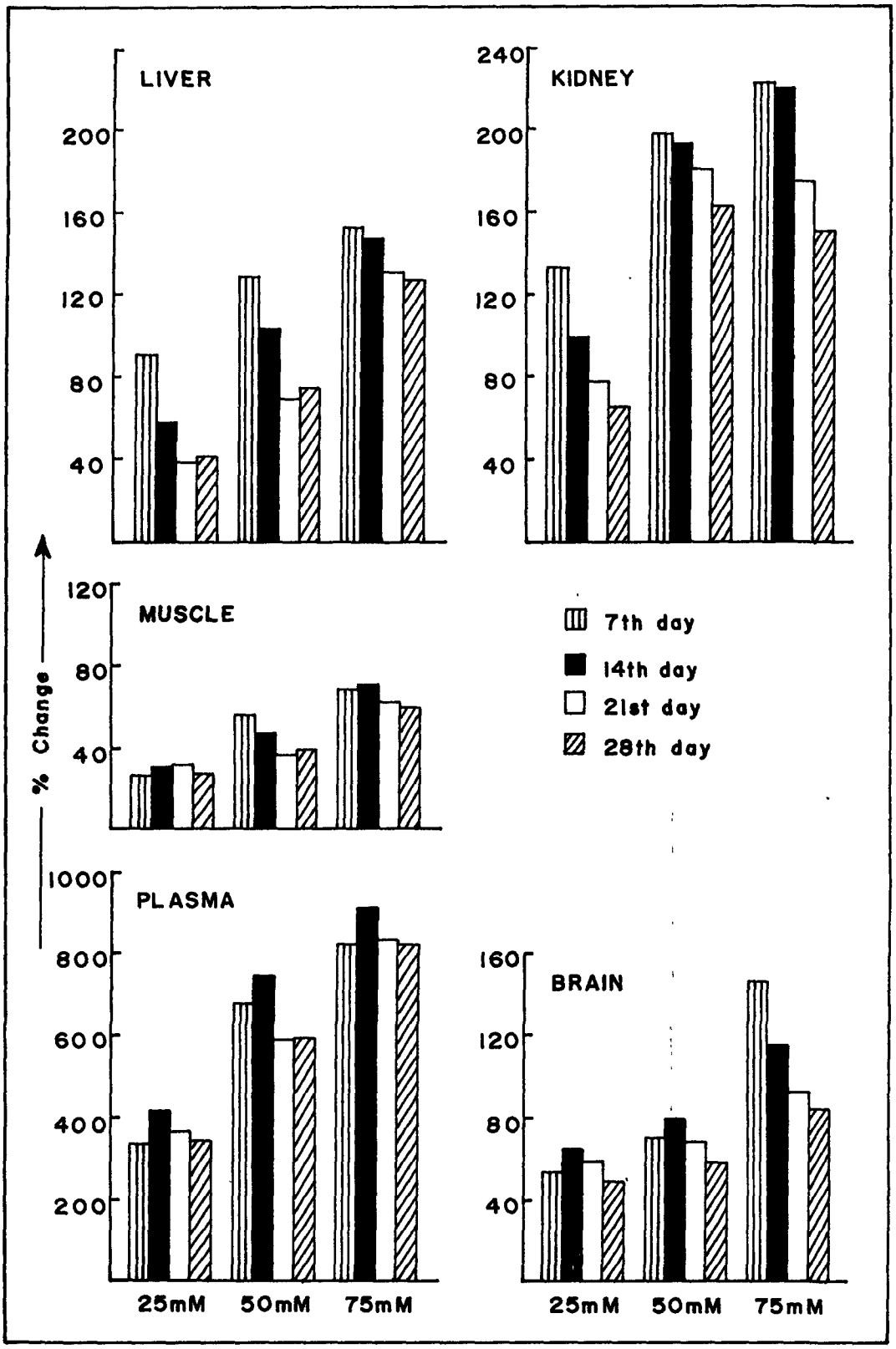


Fig. 6. % increase in the concentration of ammonia in different tissues and in blood plasma of **H. fossilis** treated with different concentration of NH_4Cl.

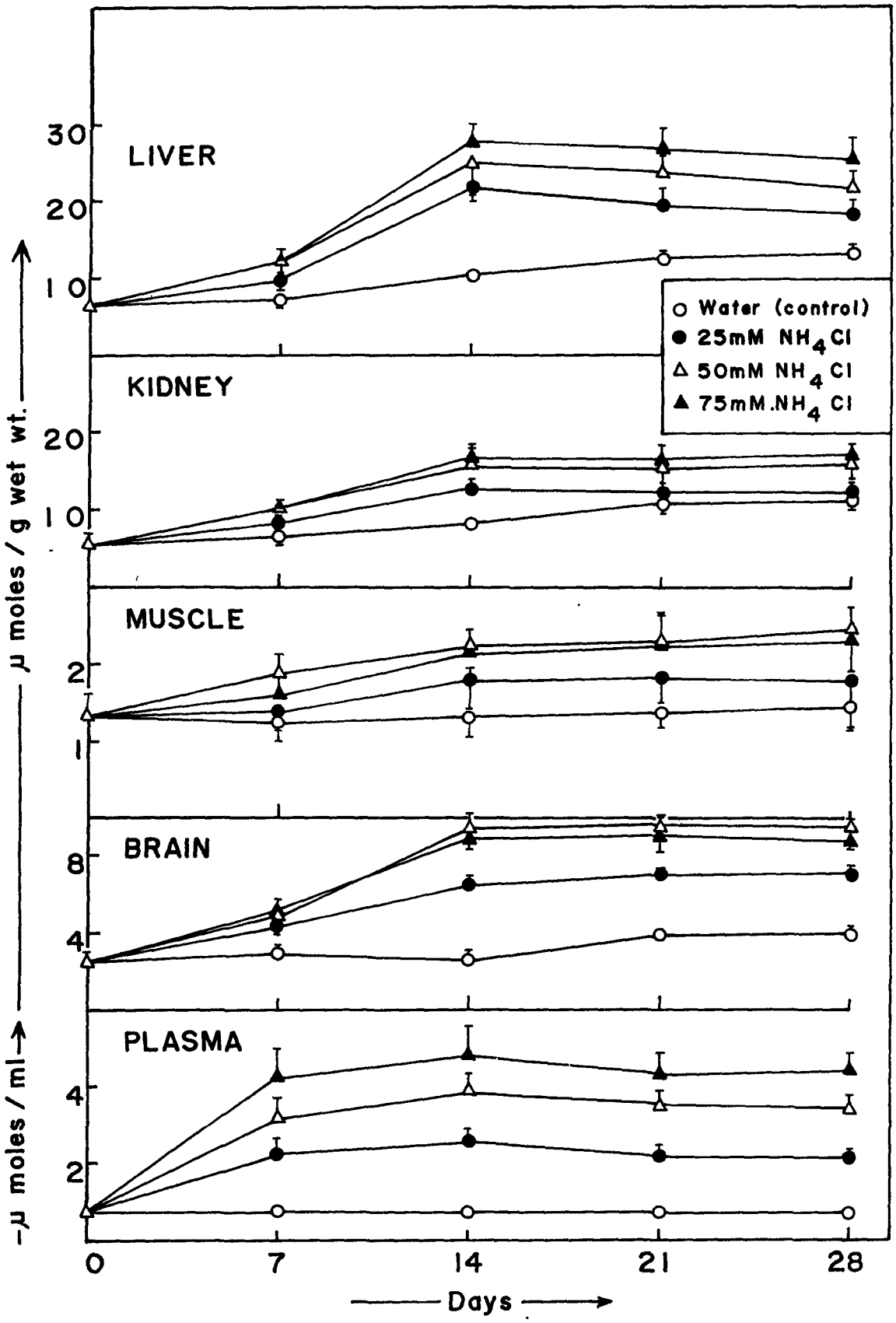


Fig. 7. Alterations in the concentration of urea in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** treated with different concentration of NH_4Cl.

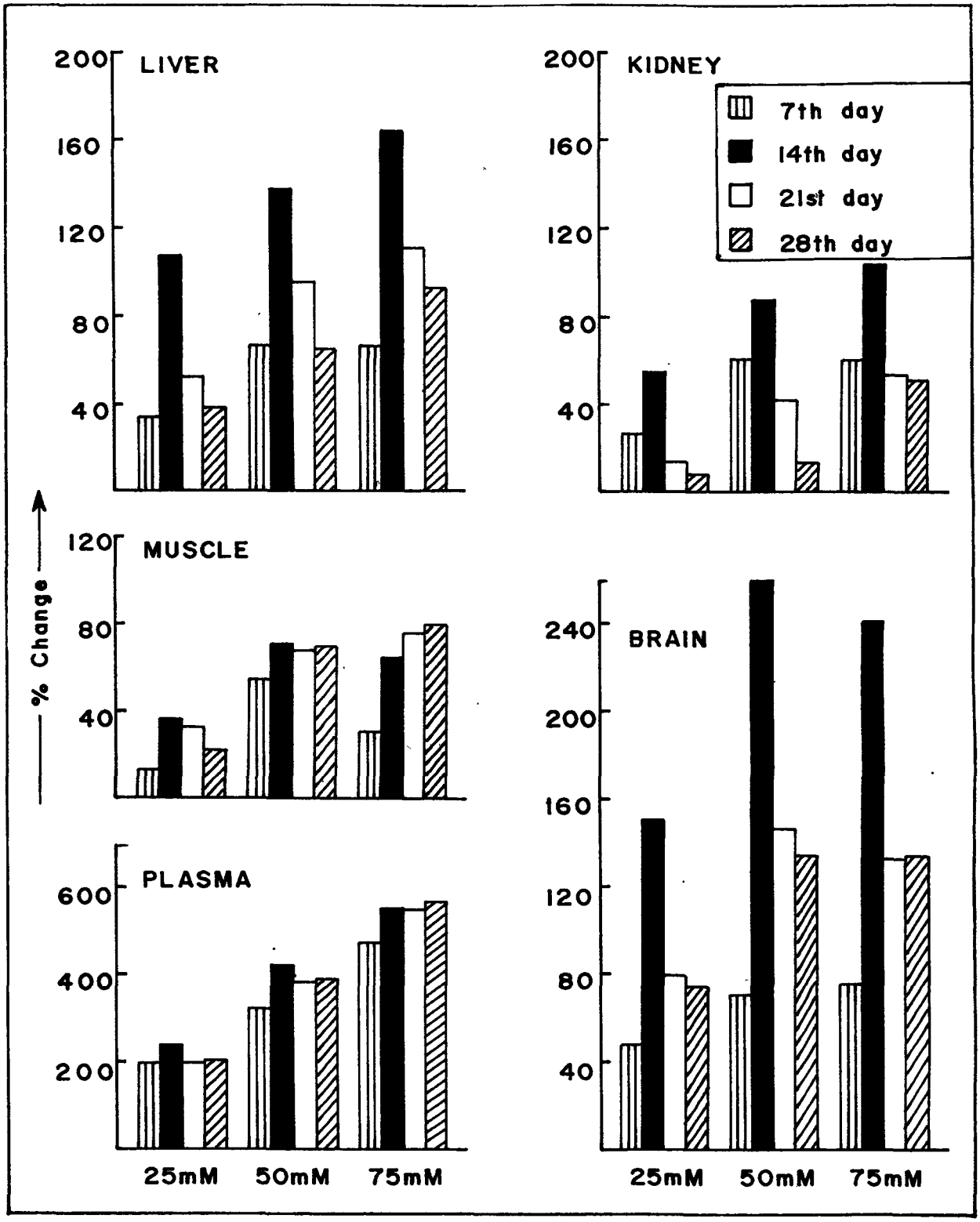


Fig. 8. % increase in the concentration of urea in different tissues and in blood plasma of **H. fossilis** treated with different concentration of NH_4Cl.

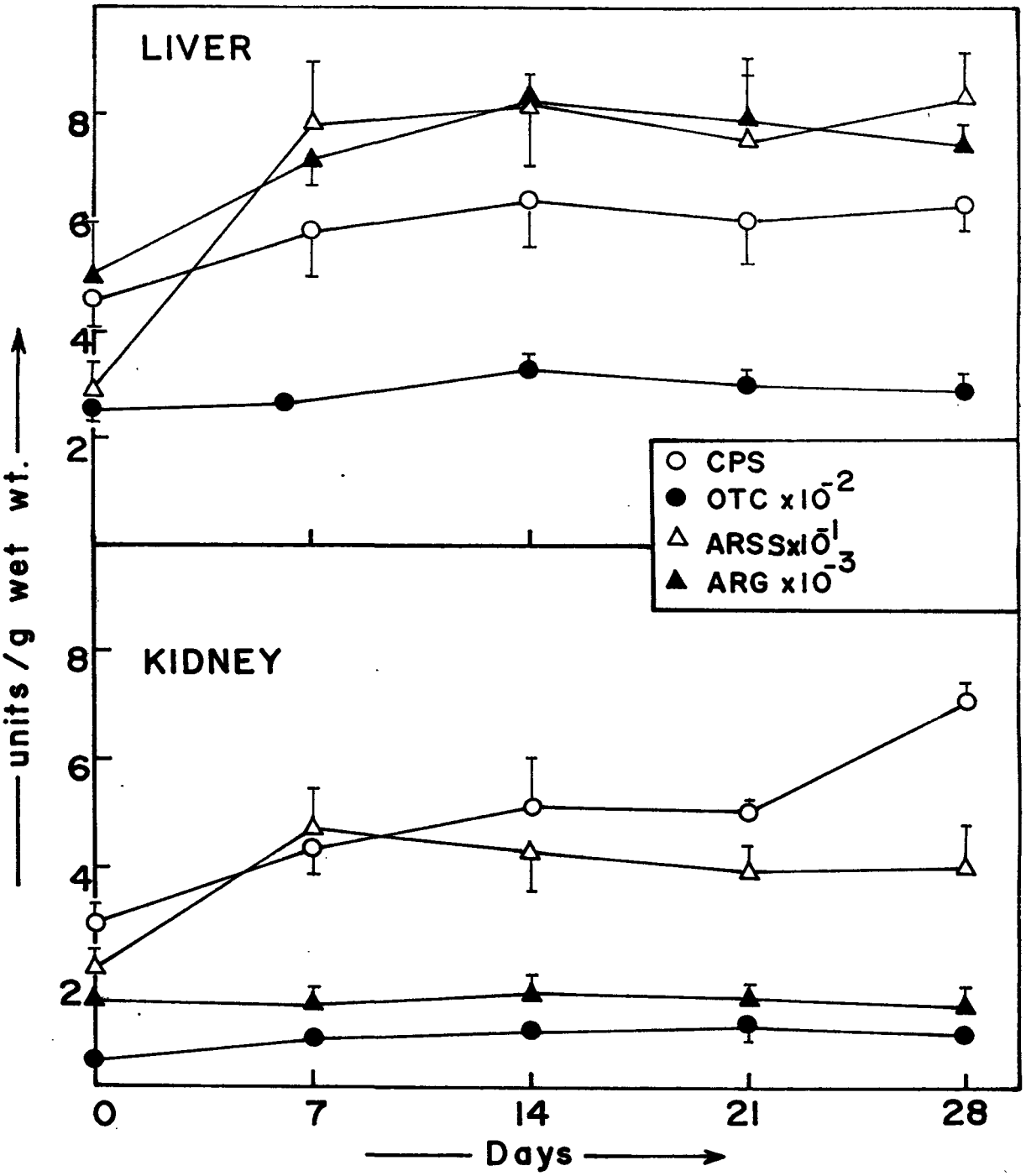


Fig. 9. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl.

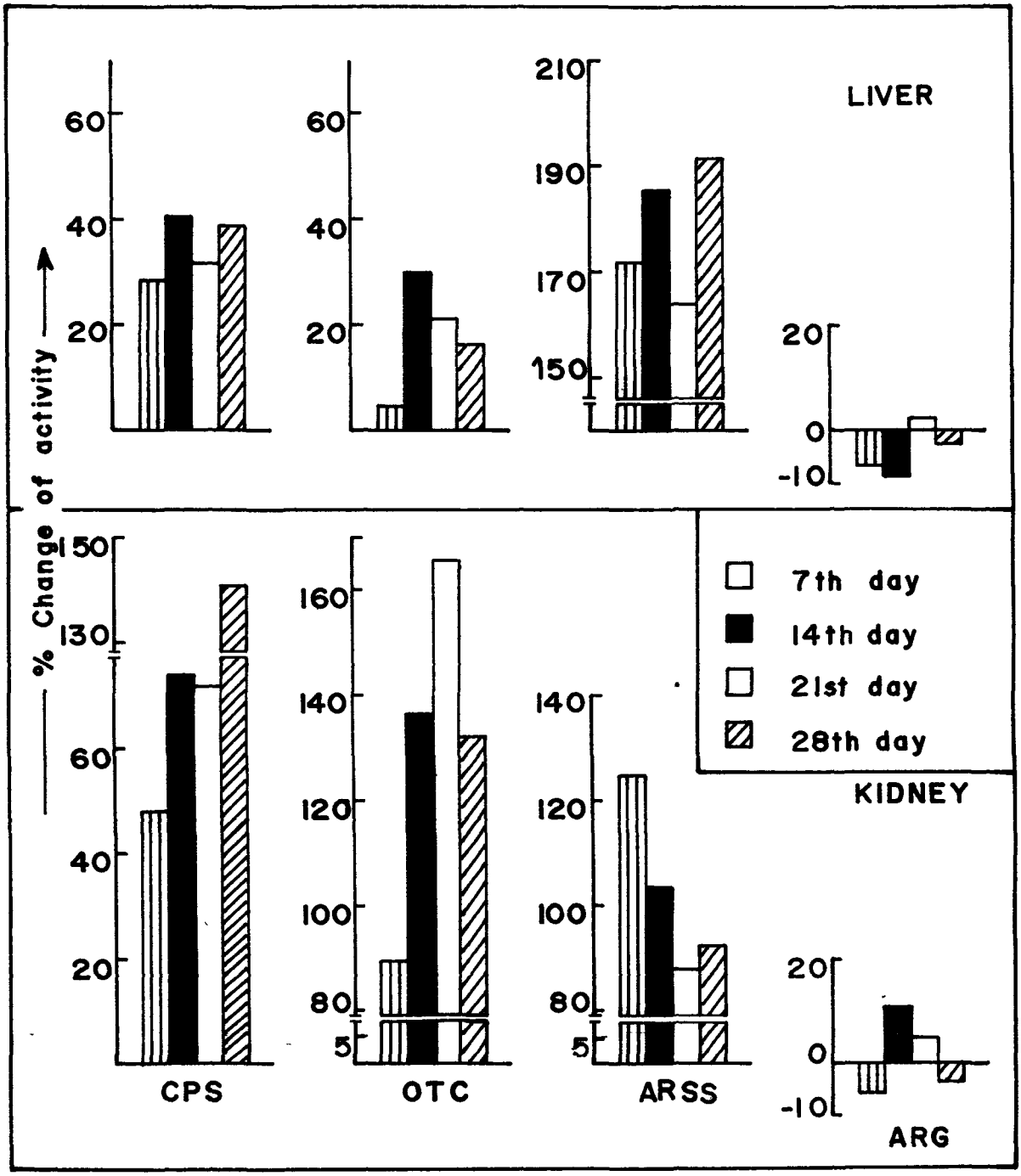


Fig. 10. % change of the total activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl.

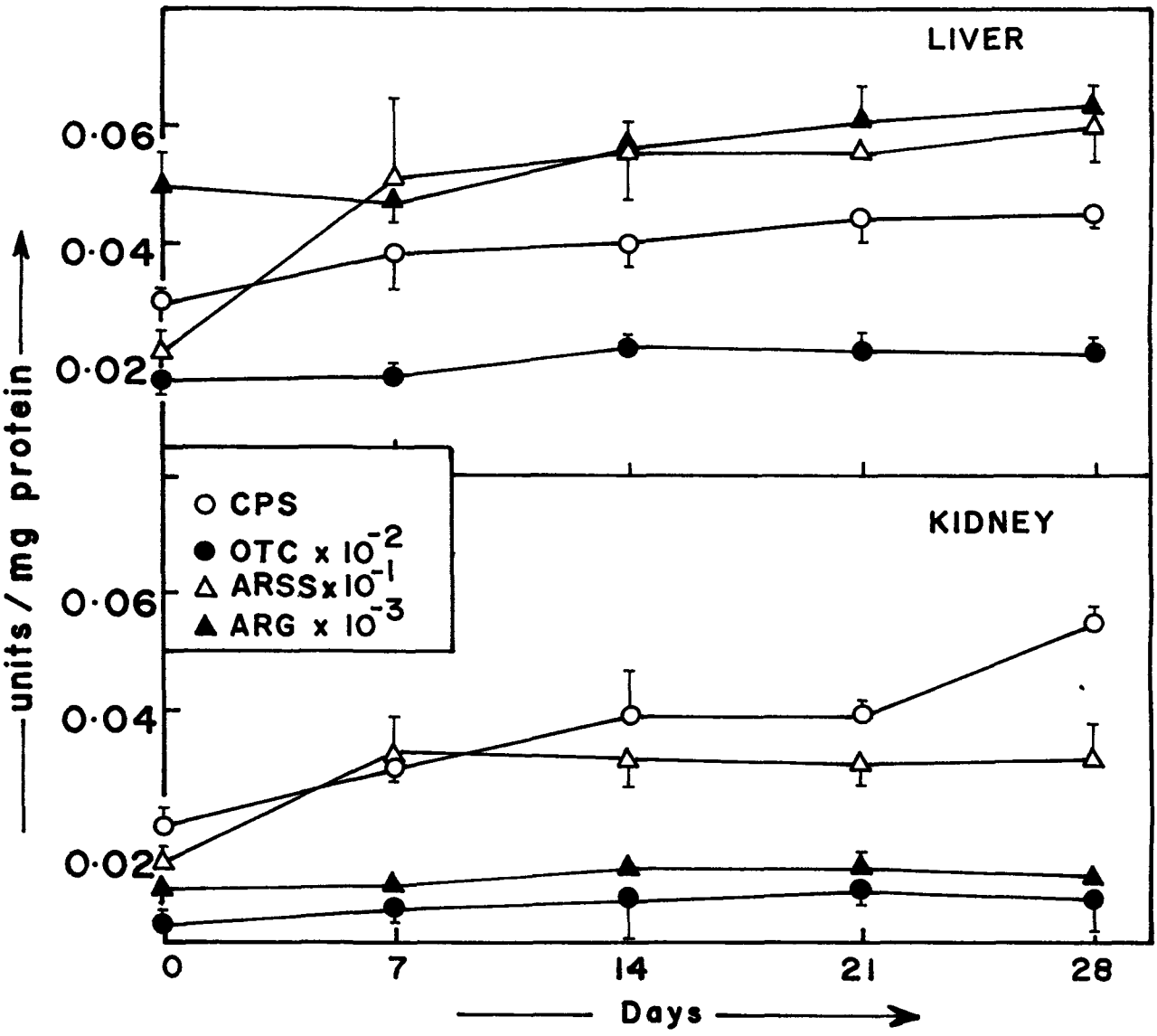


Fig. 11. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl.

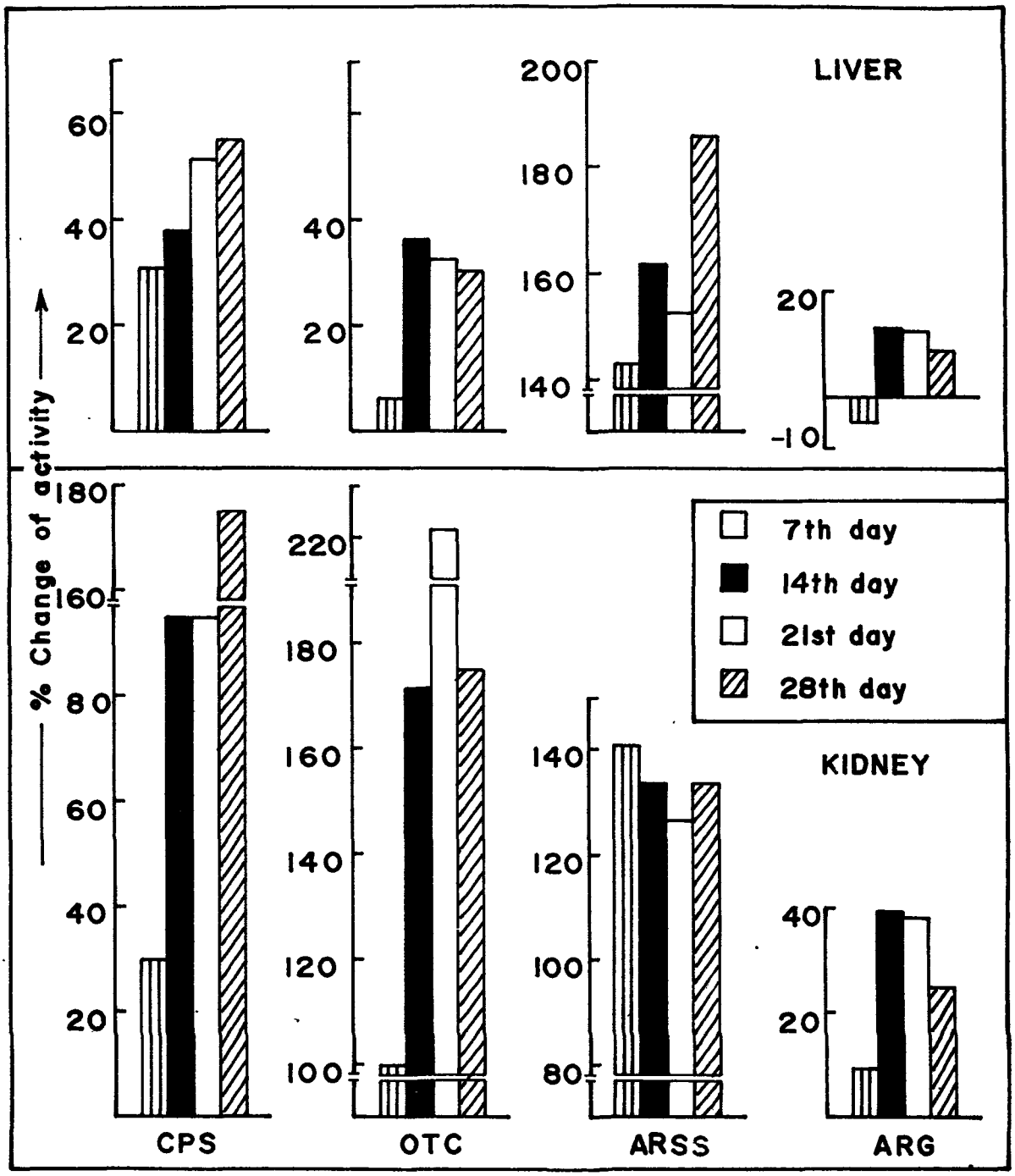


Fig. 12. % change in the specific activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl.

total activity in all cases.

The induction of o-u cycle enzyme activity was greater in kidney than in the liver of the treated fish.

DISCUSSION

Heteropneustes fossilis tolerated very high level of ambient ammonia compared to other freshwater teleosts and some aquatic amphibians. It survived well for 28 days even at 75 mM NH_4Cl solution without any visible sign of stress. Olson and Fromm (1971) reported a 50% mortality of trout, **Salmo gairdneri** in 24 hrs at 8 µg ammonia/ml (equivalent to 0.47 mM NH_4Cl). However, goldfish did not show any response at ammonia concentration as high as 25 µg/ml (equivalent to 1.47 mM NH_4Cl). Aquatic frog, **Xenopus laevis** did not survive beyond 1 hr at 10 mM NH_4Cl (Janssens, 1972). Among teleosts, goldfish was reported to have a better tolerance (10% mortality in 24 hrs at 40 µg/ml – 2.35 mM NH_4Cl) for ammonia toxicity (Schenone **et al**, 1982). However, the maximum tolerance limit (75 mM for 28 days) observed in **H. fossilis** for ammonia during the present study was several times higher than that of goldfish. Efficient physiological mechanism for detoxification of excess ammonia **in vivo** might be operating in the freshwater air-breathing teleosts such as **H. fossilis** providing its higher tolerance limit to ambient ammonia. Fishes exposed to higher concentration of NH_4Cl became immediately hyper-excitable. The excitability slowly calmed down over the period of time probably due to gradual acclimatization of the fish to the

changed environment. Olson and Fromm (1971) observed similar response in goldfish. Smart (1978) proposed a more direct mechanism of energy (ATP) depletion in the basilar region of the brain and pointed out that many of the symptoms of actute ammonia toxicity in mammals were present in fish (hyper-excitability, convulsion, coma) and the acute effects likewise were reversible. Increased ammonia concentration appeared to stimulate carbohydrate degradation to produce more keto acids for removal of ammonia by amination (Sousa & Meade, 1977; Kuhn **et al**, 1974). There occurred simultaneous suppression of citric acid cycle activity due to the diversion of keto acids such as α-ketoglutarate and oxaloacetate for removing ammonia by amination resulting into the formation of amino acids. The amination process is a highly energy utilizing process and thereby causes severe energy depletion. Utilization of energy or blocking of energy could be one of the major sources in addition to other deleterious effects, causing the death of **H. fossilis** treated with higher concentration of ambient ammonia.

Increased level of ambient ammonia has been reported to alter nitrogen excretion pattern in aquatic vertebrates. Decrease in excretion of total nitrogen and ammonia under eleveted ammonia stress has been reported in rainbow trout, **S. gairdneri** (Fromm & Gillette, 1968; Olson & Fromm, 1971). **H. fossilis** treated with different concentrations of NH_4Cl also showed an immediate suppression in ammonia and urea excretion. Ammonia was excreted primarily (99%) through the

extra-renal sources (gills and skin) and renal excretion of ammonia was negligible in **H. fossilis** (Chapter I). These surfaces (gills and skin) might have been used for absorption of ammonia into the body in favour of the concentration gradient from the hyper-ammonia external medium. Thus the ambient ammonia concentration decreased and tissue ammonia level increased immediately after the exposure of **H. fossilis** to higher concentration of NH_4Cl. The fish showed gradual recovery with relation to ammonia absorption and excretion and the time taken was proportionate to the concentration of NH_4Cl in the medium. Excretion of ammonia became normal by 24th day of treatment only with 25 mM NH_4Cl. In 50 and 75 mM treatment the excretion gradually increased but remained lower than the absorption even after 28 days of treatment.

Urea excretion which also primarily (about 75%) through the extra-renal sources in **H. fossilis** (Chapter I) was suppressed by about 50% immediately after treatment with NH_4Cl. Besides the inhibition of extra-renal urea excretion due to fast absorption of ammonia through those surfaces, renal excretion of urea also might have reduced to retain urea in the body for osmoregulation in treated fishes. Lloyd and Orr (1969) reported an increase in the rate of urine flow when rainbow trout were exposed to increased ammonia. However, this might not be true in the present case where the medium was also hyper-osmotic. A lower rate of urine flow for retention of water in the body has been reported in **Rana cancrivora** kept in hypertonic medium (Schmidt-Nielsen & Lee, 1962). Such a phenomenon might have occured in **H. fossilis** resulting in suppression of excretion

of ammonia and urea. Initial suppression and increased excretion of ammonia and urea after two weeks by another freshwater air-breathing teleost **Channa punctatus** exposed to ammonia solution has been reported (Arya, 1979). Urea excretion in **H. fossilis** increased after initial suppression by 2.5 to 3 fold from 10th day of treatment and continued at a much higher rate than the control till the end of the experiment. A similar 10 fold increase in urea excretion was reported in **X. laevis** immersed in 5 mM NH_4Cl (Janssens, 1972). The accumulation of urea in all tissues and blood plasma reached the maximum level by 14th day with no further increase during later period of treatment. Accumulation of both ammonia and urea in tissues was proportionate to the NH_4Cl concentration in the medium. Brain has been known to be highly susceptible to ammonia toxicity (Campbell, 1973). Hence, there was a tendency to maintain lower level of ammonia in the brain of **H. fossilis** exposed to higher concentration of NH_4Cl. There might be highly efficient ammonia detoxifying mechanism(s) in the brain. The glutamate is formed from one molecule of ammonia and α-ketoglutarate (from TCA cycle) in the presence of GDH. This again gets converted to glutamine utilizing another molecule of ammonia by glutamine synthetase to reduce further the ammonia toxicity. Glutamine traverse blood-brain barrier more easily than glutamate (Fromm & Gillette, 1968). High activity of glutamine synthetase has been shown in teleost brain (Webb & Brown, 1980). In our laboratory also very high activity of glutamate dehydrogenase (NADH dependent) and glutamine synthetase have been reported in the brain of **H. fossilis**

(unpublished observation). Liver and kidney have been shown to be the main organs in the fish for ammonia formation (Pequin & Serfaty, 1963; Vellas & Serfaty, 1974). Hence, they might have the capacity to tolerate the higher accumulation of ammonia. De Vooys (1969) observed an elevation in blood urea level after oral administration of NH_4Cl to perch, **Perch fluviatilis.** However, significant increase in both ammonia and urea level was observed in all the tissues studied in **H. fossilis** exposed to higher ambient ammonia. The level of accumulation of both ammonia and urea varied in different tissues. Each tissue might have its own tolerance limit depending on the physiology of the tissue. Ammonia and urea accumulated in different tissues to a much higher level in **H. fossilis** than those reported in other freshwater teleosts.

The increase in tissue ammonia and urea level in **H. fossilis** was accmpanied by the increase in the activity of o-u cycle enzymes both in liver and kidney. Activity of CPS and OTC were induced more in kidney than liver. Whereas, ARSS activity was induced maximally in liver. Accumulation of ammonia was very high in liver and kidney which might have led to the induction of o-u cycle enzymes in those two tissues. There are various reports on the induction of the activity of o-u cycle enzymes and more synthesis of urea with the increasing tissue ammonia level **in vivo** in amphibians (Balinsky **et al,** 1961; McBean & Goldstein, 1967; Janssens & Cohen, 1968; Janssens, 1972). McBean and Goldstein (1970) emphasized the importance of increase in ammonia level **in vivo** for the increased synthesis

of urea in **X. laevis** when subjected to hyper-osmotic stress. A ten fold induction of GDH activity was also recorded in the liver of **X. laevis** immersed in 5 mM NH_4Cl (Janssens, 1972). If the increased activity of CPS, which looks to be the rate limiting enzyme of o-u cycle, both in liver and kidney are summed up, a two fold increase of activity can be seen. This coincides with the increase in urea excretion rate from 10th day onwards at 50 mM NH_4Cl. However, it is difficult to say at present whether the increase of o-u cycle enzyme activity was due to the new synthesis of enzyme protein or the activation of pre-existing inactive enzyme molecules unless some specific studies are being conducted as suggested by Goldstein (1972).

Ammonia level increased in the tissues till 7th day of treatment after which the level gradually decreased. The o-u cycle enzymes might not have got induced immediately after immersion of the fish in NH_4Cl. The increased ammonia level must be one of the important factors to induce o-u cycle enzyme activity to synthesize more urea through o-u cycle thus decreasing ammonia level **in vivo**. This process might have kept the ammonia level below the toxic level **in vivo** and helped **H. fossilis** to tolerate such a high ambient ammonia compared to other teleosts. Other detoxifying pathways of ammonia such as glutamate $\longrightarrow$ glutamine synthetic pathway might be also operating in **H. fossilis** to supplement detoxification of ammonia through o-u cycle. Studies in this line are underway in our laboratory to clarify this point. However, induction of the activity of o-u cycle enzymes to convert excess ammonia to

urea under hyper-ammonia stress is a unique finding in any freshwater teleost where presence of o-u cycle was even doubted.

Uricolytic pathway which is reported to be present in this fish might have contributed some urea under the experimental condition. However, the production of urea through this pathway has been shown to be much lower in **H. fossilis** (Chapter II) than other teleosts and lungfishes (Goldstein & Forster, 1965; Brown **et al**, 1966). Though it is difficult to think of the induction of uricolytic pathway under hyper-ammonia stress, some amount of ammonia might have been detoxified through this pathway. This has to be investigated before drawing any definite conclusion.

However, there can not be any disagreement to the point that the o-u cycle reported in freshwater air-breathing teleosts is regulated by the environmental factors such as higher ambient ammonia at least in **H. fossilis.** This has made it possible for the fish to tolerate a very high concentration of ammonia both **in vitro** and **in vivo.**

CHAPTER IV
DEHYDRATION STRESS

INTRODUCTION

The success of a living organism depends on its ability to adapt to changes in its environment. Adaptation can occur at different levels. Adaptations at morphological level such as industrial melanism in moths were easily observed. However, adaptations at the molecular level, which were not easily marked, perhaps were more important during adaptations in living organisms. The significance of molecular or biochemical adaptations in different animals have been emphasized by several workers (Knox & Greengard, 1964; Goldstein, 1970; Prosser, 1973; Hochachka & Somero, 1973; Hoar, 1983; Schmidt-Nielsen, 1983).

One obvious method of adaptation at the molecular level has been found to be the changes in the activity of enzymes of different metabolic pathways. Some metabolic pathways were more sensitive and others more resistant to environmental fluctuations. The sensitive systems would respond easily and quickly for even minor variations in the environment. Somero and Hochachka (1971) classified biochemical adaptations into three categories depending upon the time course of adaptive change. (1) The changes that occurred very slowly over many generations on an evolutionary time scale. (2) The seasonal shifts in an organism's functional systems. (3) The immediate adaptive responses observed in an organism due to changes in its environment.

Gordon (1970) suggested that "nitrogen metabolism is one of the most sensitive physiological systems in its responses to environmental changes". One of the important enzymatic pathways involved in nitrogen metabolism is the ornithine-urea (o-u) cycle. The highly toxic nitrogenous metabolic end product ammonia is converted to urea through o-u cycle. Environmental factors such as temperature and water availability and diet (Millman, 1951; Mandelstam & Yudkin, 1952; Tillinghast **et al**, 1969; Nuzum & Snodgrass, 1971) have been shown to alter the activity of o-u cycle enzymes. The end product of nitrogen excretion is altered depending on the availability of water to the animals (Gordon, 1970). Purely aquatic animals excrete ammonia, animals with limited water supply converts ammonia to urea and insoluble uric acid is excreted by those animals with necessity to conserve metabolic water. Urea also has been important for osmoregulation in an hyper-osmotic medium in aquatic animals.

Aquatic animals which can tolerate temporary water deprivation or hyper-osmolar environment have been shown to convert ammonia to urea **in vivo** through o-u cycle. The effect of dehydration on o-u cycle enzymes and nitrogen excretion pattern has been studied by various workers in amphibians. Purely aquatic **Xenopus laevis** excreted predominantly ammonia as the major nitrogenous excretory product while in water. It accumulated large amount of urea when kept out of water or in dilute saline solutions (Balinsky **etal**,1961). Janssens and Cohen (1968) reported increased synthesis of urea in **X. laevis**

under conditions of water shortage. Both the rate of urea production and level of carbamyl phosphate synthetase activity were increased when **X. laevis** was desiccated by exposure to slightly hyper-osmotic saline solutions (McBean & Goldstein 1970). Balinsky (1970) also reported increased activity of (o-u) cycle enzymes in aestivating **X. laevis.** McClanahan (1972) reported the elevation of plasma concentration in **Scaphiopus cauchi** due to urea accumulation when the soil dried. Urea accumulation both in plasma and tissues was observed during their burrowing period inside the soil in other terrestrial amphibians such as, **Bufo viridis** (Katz, 1973; Rick **et al**, 1980; Degani **et al** 1981), **Ambystoma tigrinum** (Delson & Whitford, 1973), **Salamandra salamandra** (Degani, 1981b) and in **Pelobates syriacus** (Degani, 1982).

African lungfish **Protopterus** living in shallow water had to face seasonal evaporation. It excreted major part of its nitrogenous waste in the form of ammonia while in water. During aestivation instead of accumulation of ammonia the concentration of urea in the tissues increased sharply (upto about one per cent of the body weight) (Smith, 1930). Predominantly ammoniotelic lungfish became exclusively ureotelic during aestivation (Smith, 1930; Sawyer, 1966; Janssens & Cohen, 1968). Conversion of greater part of waste nitrogen to urea and the accumulation of urea inside the body by about five times during aestivation as compared to the concentration while in water has been reported in African lungfish, **Protopterus annectens** and **P. aethiopicus** (Janssens, 1964).

The accumulated urea was excreted out immediately when he lungfish returned to water (Janssens, 1964). Goldstein **et al** (1967) reported higher activities of o-u cycle enzymes in the liver of aestivating African lungfish **Protopterus dilloi** than that of the permanently aquatic Australian lungfish **Neoceratodus fosteri.** The amphibious mudskipper fish has been reported to excrete ammonia as the major excretory product while in water (Gregory, 1977; Morii **et al**, 1978). A shift towards ureotelism has been reported during its stay outside water for 12 hrs in **Periophthalmus sobrinus** (Gordon **et al**, 1969) and for 9.5 days in **Periophthalmus cantonensis** (Gordon **et al**, 1978). Morii (1979), however, could not find any such conversion of ammonia to urea in **P. cantonensis** and **Boleophthalmus pectinirostris** kept outside water. Gregory (1977) could not detect all the enzymes of o-u cycle in two mudskipper fish **Periophthalmus expeditionium** and **Periophthalmus gracilis.** The marine air-breathing teleost **Blennius pholis** excreted 81.4% ammonia and 18.6% urea in sea water and remained predominantly ammoniotelic even during the period of aerial exposure upto 24 hrs (Davenport & Sayer, 1986). Air-breathing teleosts, capable of remaining outside water for substantial period of time, face similar problem like amphibians and lungfishes in excreting and accumulating ammonia during their stay outside water. The freshwater air-breathing teleosts predominantly found in India, are also amphibious according to the definition of Gordon **et al** (1969): "amphibious fishes are those which spend periods of time out of water, on or above the ground surface as normal parts of their life histories."

It has not been clearly understood that how the freshwater air-breathing teleosts manage the problem of nitrogen excretion during their aerial exposure. Higher rate of excretion and high tissue level of urea, and higher level of activity of o-u cycle enzymes have been found at least in four out of the five species of freshwater air-breathing teleosts studied such as **H. fossilis, C. batrachus, A. testudineus, C. punctatus,** and **A. cuchia** during their aquatic life (Chapter I & II). **H. fossilis** among the five species showed maximum rate of urea excretion, high tissue urea and maximum activity of o-u cycle enzymes and also survived for 70-80 hrs outside water. A shift towards ureotelism might occur in **H. fossilis** during their aerial exposure to avoid ammonia toxicity and also to maintain the osmotic equilibrium. Therefore, **H. fossilis** was used as the model species of freshwater air-breathing teleost to study the effect of dehydration on the excretion pattern of ammonia and urea and the activity of o-u cycle enzymes.

Plan of work:

(1) Alterations in the rate of excretion of ammonia and urea by **H. fossilis** were determined after 6, 12, 18, 24, 36 and 48 hrs of aerial exposure.

(2) The water content of the muscle was determined after 6, 12, 18, 24, 36 and 48 hrs of dehydration.

(3) The concentration of ammonia and urea in different tissues and blood plasma of **H. fossilis** were estimated after 3, 6, 9, 12, 15, 18, 21, 24, 36 and 48 hrs of aerial exposure.

(4) Activity of o-u cycle enzymes were assayed in the

in the liver and kidney of **H. fossilis** exposed to air for 12, 24, 36 and 48 hrs.

MATERIALS AND METHODS

Animal: **Heteropneustes fossilis** weighing 25-35 g acclimatised to laboratory conditions as described in Chapter I were used for experimentation.

Experimental set up: Fishes of similar size were used a day after the last feeding. The experiment was started at 6 A.M.. Each fish was kept separately in a glass jar without water under the laboratory condition. The humidity around the fish was 70-80% as determined by a humidity meter. Each jar was covered with bilayers of cheese cloth.

Excretion of ammonia and urea by the experimental fishes were estimated after 6, 12, 18, 24, 36 and 48 hrs of dehydration. Alteration in the concentration of ammonia and urea in different tissues and in blood plasma of the fishes were estimated after 3, 6, 9, 12, 15, 18, 21, 24, 36 and 48 hrs of dehydration. Activities of o-u cycle enzymes were assayed in liver and kidney after 12, 24, 36 and 48 hrs of dehydration. Data from five fishes were taken into consideration for each point.

Excretion of ammonia and urea: Each fish was washed quickly with 100 ml of distilled water in the glass jar at the stipulated time as mentioned above and transferred to another dry glass

jar immediately. Amount of ammonia and urea excreted were estimated in the washings following the method described in Chapter I.

Blood sampling: Blood was collected from the caudal vein of each fish with a heparinized syringe as mentioned in Chapter I *for estimating ammonia and urea level in blood plasma.* Blood was sampled immediately after washing the fishes for determination of ammonia and urea excretion.

Tissue preparation: Fishes were sacrificed by decapitation immediately after collecting the blood. Tissues such as liver, kidney, muscle and brain were removed blotted dry and deep frozen at -20°C immediately. All the estimations were completed within two days of collecting the sample. Tissues were processed for determination of ammonia and urea following the method described in Chapter I.

Estimation procedure:

Ammonia and urea: Estimation of ammonia and urea were done following the method described in Chapter I.

Enzyme assay: The activities of the enzymes of ornithine-urea cycle such as carbamyl phosphate synthetase (ammonia dependent) (CPS), ornithine transcarbamylase (OTC) and arginase (ARG) were assayed following the method described in Chapter II and arginine synthetase system (ARSS) following the method described in Chapter III.

Protein: Protein concentration of the tissues were estimated following the method of Lowry **et al** (1951).

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

Expression of data: The rate of excretion of ammonia and urea was expresseqd as μmoles per g body weight per hr. The concentration of ammonia and urea in the tissues was expressed as μmoles per g wet weight of each tissue and in blood plasma as μmoles per ml plasma. The enzyme activity was expressed both as total activity - units per g wet weight and as specific activity - units per mg protein.

Data were presented as mean ± S.D. The level of significance between two sets of data were analysed statistically using student 't' test and the 'p' value more than 0.05 was taken as non-significant (N.S.).

Chemicals: All the enzymes, coenzymes and substrates used were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.. Other chemicals were of analytical grade and obtained indigenously. Deionized, double glass distilled ammonia free water was used for all preparations.

RESULTS

Excretion of ammonia and urea:

The alterations in the excretion of ammonia and urea by **H. fossilis** kept outside water for different period of time have been presented in Table 24 and Figs. 13 & 14. There was immediate suppression in the excretion of both ammonia and urea when **H. fossilis** was kept outside water. The decrease in ammonia excretion was about 75% within the first 6 hrs which further reduced to 86% between 24-48 hrs stay outside water (Table 24; Fig. 13). Urea excretion decreased by 42% within the first 6 hrs of emersion. However, there was increase in urea excretion with the recovery of inhibition to 10 to 14% between 24-48 hrs (Table 24; Fig. 14). The ratio of ammonia: urea excretion also decreased significantly from 10-12.5 in water to 5.27 within the first 6 hrs of emersion (Table 25). The ratio gradually decreased to 1.67 with increasing time of aerial exposure upto 36-48 hrs of emersion.

Water content in muscle:

There was linear decrease of water content of the muscle from 80% to 78% during 48 hrs of aerial exposure. The decrease was only about 3% but was statistically significant (Table 26).

Tissue ammonia and urea level:

Ammonia: There was significant accumulation of ammonia in all the tissues studied and blood plasma during aerial exposure of **H. fossilis** for 48 hrs (Table 27; Fig. 15). Ammonia level

in liver, kidney, muscle and brain increased within 3 hrs to a level which was maintained till 24 hrs of dehydration. It increased again from 24 hrs to 48 hrs of dehydration in these tissues (Fig. 15 & 16). In plasma a linear increase in ammonia level was noticed with increasing time (Fig. 15 & 16). Maximum accumulation of ammonia took place in kidney (32.16 µmoles/g wet wt) followed by liver (28.46), muscle (26.25) and brain (15.42 µmoles/g wet wt). Blood plasma accumulated 4.76 µmoles ammonia/ml of plasma. The relative increase of ammonia was found maximum in plasma (906.3%) followed by kidney (131.4%), brain (128.8%), muscle (111.2%) and liver (95.5%) (Table 27; Fig. 16).

Urea: Tissue urea level increased significantly and linearly in almost all the tissues of **H. fossilis** studied with increasing time of exposure to air (Table 28; Figs. 15 & 17). In brain the urea level doubled after 9 hrs of emersion as in other tissues and there after the increase was relatively slower in brain till 48 hrs. Urea accumulation was higher in all the tissues after 48 hrs of emersion. Liver and kidney accumulated more urea with 38.82 and 36.55 µmoles/g wet wt respectively. Brain had 6.14 and muscle 3.95 µmoles/g wet wt Plasma urea level reached maximum level of 6.78 µmoles/ml after 48 hrs of exposure to air. The percent increase in urea level was highest in plasma (798%) followed by kidney (602%), liver (486.6%), muscle (201.5%) and brain (146.6%) after 48 hrs of emersion (Table 28; Fig.17).

Activities of o-u cycle enzymes:

Liver: The alterations in the activity (total and specific) of o-u cycle enzymes in the liver of **H. fossilis** during dehydration have been presented in Tables 30 and 31 and Figs. 18-21. Both total and specific activity of all the enzymes showed similar changes with time of dehydration in the liver of **H. fossilis.** The enzyme activity increased linearly with increasing time of exposure to air outside water except for ARG where the activity remained unaffected. The activity of ARSS was induced by 2.5 times while that of OTC and rate limiting enzyme CPS were induced by about two times from the control level. Significant induction of CPS, OTC and ARSS activity was observed within 12 hrs of emersion. The maximum percent increase of enzyme activity was seen in ARSS (148%) followed by OTC (60.3%) and CPS (53.3%) with ARG showing insignificant variation (3.2%) (Table 30; Fig. 19).

Kidney: Alterations in the activity of o-u cycle enzymes in the kidney of **H. fossilis** kept outside water for 48 hrs have been presented in Tables 32 and 33 and Figs. 18-21. Activity of all the enzymes got gradually induced significantly under dehydration stress. Unlike liver, the activity of OTC was induced maximum and ARG which was unaffected in liver was significantly induced in kidney. However, ARG induction was apparent only after 36 hrs of dehydration and was induced by about 27% at 48 hrs of emersion. OTC induction was also apparent only by 24 hrs of dehydration and was quickly induced thereafter by 150% within another 12 hrs. The percent increase

TABLE 24. Alteration in the rate of excretion (μmoles/hr/g body wt) of ammonia and urea by **H. fossilis** kept outside water. (Mean ± S.D.)

		Time (hrs)					
		0 — 6	6 — 12	12 — 18	18 — 24	24 — 36	36 — 48
AMMONIA							
H_2O (control)		0.237 ±0.025	0.243 ±0.022	0.263 ±0.030	0.272 ±0.025	0.237 ±0.027	0.280 0.030
Outside water		0.058 ±0.006	0.066 ±0.010	0.064 ±0.008	0.032 ±0.003	0.032 ±0.003	0.040 ±0.005
	p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
		(-75.5)	(-72.8)	(-75.7)	(-88.2)	(-86.5)	(-85.7)
UREA							
H_2O (control)		0.019 ±0.002	0.019 ±0.003	0.028 ±0.003	0.028 ±0.003	0.019 ±0.002	0.028 ±0.003
Outside water		0.011 ±0.001	0.018 ±0.002	0.019 ±0.002	0.019 ±0.003	0.017 ±0.002	0.024 ±0.003
	p	<0.005	N.S.	<0.005	<0.01	N.S.	N.S.
		(-42.1)	(-5.3)	(-32.1)	(-32.1)	(-10.5)	(-14.3)

% change compared to control are given in parentheses.

TABLE 25. Ratio of ammonia:urea excreted by **H. fossilis** kept outside water.

	Time (hrs)					
	0 — 6	6 — 12	12 — 18	18 — 24	24 — 36	36 — 48
H_2O (control)	12.47	12.79	9.39	9.71	12.47	10.0
Outside water	5.27	3.67	3.37	1.68	1.88	1.67

TABLE 26. Alterations in the water content (% wet wt) in the muscle of **H. fossilis** kept outside water. (Mean ± S.D.)

Time spent outside water (hrs)	% water content	% change	p
0 (control)	80.55 ± 0.82		
6	79.18 ± 0.98	(-1.7)	N.S.
12	79.00 ± 0.89	(-1.92)	<0.05
18	78.85 ± 0.45	(-2.11)	<0.01
24	78.62 ± 0.63	(-2.40)	<0.01
36	78.45 ± 0.55	(-2.61)	<0.005
48	78.21 ± 0.60	(-2.91)	<0.005

TABLE 27. Alterations in the ammonia concentration in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** kept outside water. (Mean ± S.D.)

Tissue		Time spent outside water (hrs)										
		0	3	6	9	12	15	18	21	24	36	48
LIVER		14.56 ±1.15	19.13 ±1.95	21.26 ±0.98	19.56 ±1.94	20.88 ±2.95	23.82 ±3.12	24.11 ±3.25	22.21 ±2.56	19.37 ±1.99	25.74 ±3.60	28.46 ±2.29
	p		<0.01 (+31.4)	<0.001 (+46.0)	<0.005 (+34.3)	<0.01 (+43.4)	<0.005 (+63.6)	<0.005 (+65.6)	<0.005 (+52.5)	<0.01 (+33.0)	<0.005 (+76.8)	<0.001 (+95.5)
KIDNEY		13.90 ±1.22	22.86 ±5.46	26.08 ±1.37	21.18 ±0.63	24.82 ±1.52	25.36 ±2.18	21.37 ±2.44	22.34 ±1.95	23.85 ±1.68	24.68 ±2.83	32.16 ±2.66
	p		<0.02 (+64.5)	<0.001 (+87.6)	<0.001 (+52.4)	<0.001 (+78.6)	<0.001 (+82.5)	<0.005 (+53.7)	<0.001 (+63.7)	<0.001 (+71.6)	<0.001 (+77.6)	<0.001 (+131.4)
MUSCLE		12.43 ±1.09	13.43 ±0.77	13.19 ±0.66	12.98 ±0.78	14.82 ±1.51	15.21 ±1.78	14.24 ±1.90	14.90 ±1.21	14.65 ±1.12	25.74 ±2.64	26.25 ±2.76
	p		N.S. (+8.1)	N.S. (+6.1)	N.S. (+4.4)	<0.05 (+19.2)	<0.05 (+22.4)	N.S. (+14.6)	<0.02 (+19.9)	<0.025 (+17.9)	<0.001 (+107.1)	<0.001 (+111.2)
BRAIN		6.74 ±0.81	9.48 ±1.09	9.59 ±0.62	10.66 ±0.65	9.70 ±0.47	10.12 ±0.52	10.85 ±0.72	11.27 ±0.91	12.33 ±1.66	16.08 ±1.29	15.42 ±1.45
	p		<0.01 (+40.7)	<0.005 (+42.3)	<0.001 (+58.2)	<0.001 (+43.9)	<0.001 (+50.2)	<0.001 (+61.0)	<0.001 (+67.2)	<0.005 (+82.9)	<0.001 (+138.6)	<0.001 (+128.8)
PLASMA		0.473 ±0.035	0.846 ±0.045	1.18 ±0.09	1.45 ±0.12	1.67 ±0.12	1.98 ±0.14	2.33 ±0.16	2.69 ±0.13	3.45 ±0.15	4.16 ±0.17	4.76 ±0.18
	p		<0.005 (+78.9)	<0.001 (+149.9)	<0.001 (+206.6)	<0.001 (+253.1)	<0.001 (+318.6)	<0.001 (+392.6)	<0.001 (+468.7)	<0.001 (+629.4)	<0.001 (+779.5)	<0.001 (+906.3)

P value calculated compared to control;
% change compared to control are given in parentheses.

TABLE 28. Alterations in the urea concentration in different tissues (μmoles/g wet wt.) and in blood plasma (μmoles/ml) of **H. fossilis** kept outside water. (Mean ± S.D.)

Tissue		Time spent outside water (hrs)										
		0	3	6	9	12	15	18	21	24	36	48
LIVER		6.23 ±0.61	7.66 ±1.04	9.71 ±0.74	12.54 ±0.85	16.23 ±0.90	18.24 ±1.10	20.33 ±0.96	21.78 ±1.12	24.45 ±0.79	30.94 ±1.74	36.55 ±1.85
	p		<0.05 (+23.0)	<0.001 (+55.9)	<0.001 (+101.3)	<0.001 (+160.5)	<0.001 (+192.8)	<0.001 (+226.3)	<0.001 (+249.6)	<0.001 (+292.5)	<0.001 (+396.6)	<0.001 (+486.6)
KIDNEY		5.53 ±0.97	8.33 ±0.72	9.56 ±1.17	12.73 ±1.25	18.59 ±1.68	21.27 ±1.69	23.44 ±1.92	25.68 ±2.23	27.55 ±1.59	32.71 ±1.65	38.82 ±2.21
	p		<0.005 (+50.6)	<0.005 (+73.4)	<0.001 (+130.2)	<0.001 (236.2)	<0.001 (+284.6)	<0.001 (+323.8)	<0.001 (+364.4)	<0.001 (+398.2)	<0.001 (+491.5)	<0.001 (+602.0)
MUSCLE		1.31 ±0.32	1.44 ±0.26	1.55 ±0.19	2.50 ±0.36	2.83 ±0.13	2.92 ±0.41	3.11 ±0.36	3.15 ±0.32	3.22 ±0.41	3.56 ±0.48	3.95 ±0.52
	p		N.S. (+9.9)	N.S. (+18.3)	<0.005 (+90.8)	<0.001 (+116.0)	<0.001 (+122.9)	<0.001 (+137.4)	<0.001 (+140.5)	<0.001 (+145.8)	<0.001 (+171.8)	<0.001 (+201.5)
BRAIN		2.49 ±0.37	2.75 ±0.32	2.91 ±0.45	5.11 ±0.61	4.50 ±0.30	4.76 ±0.41	4.81 ±0.36	5.96 ±0.51	5.17 ±0.69	5.78 ±0.59	6.14 ±0.85
	p		N.S. (+10.4)	N.S. (+17.3)	<0.001 (+105.2)	<0.001 (+80.7)	<0.001 (+91.2)	<0.001 (+93.2)	<0.001 (+139.4)	<0.001 (+107.6)	<0.001 (+132.1)	<0.001 (+146.6)
PLASMA		0.755 ±0.081	0.987 ±0.075	1.35 ±0.07	1.78 0.07	2.22 0.16	2.68 0.14	2.95 0.13	3.25 0.25	4.21 0.32	5.25 0.36	6.78 0.41
	p		<0.005 (+30.7)	<0.001 (+78.8)	<0.001 (+135.8)	<0.001 (+194.0)	<0.001 (+255.0)	<0.001 (+290.7)	<0.001 (+330.5)	<0.001 (+457.6)	<0.001 (+595.4)	<0.001 (+798.0)

P value calculated compared to control
% change compared to control are given in parentheses.

TABLE 29. Ratio of ammonia:urea concentration in different tissues and in blood plasma of **H. fossilis** when kept outside water.

Tissue	Time spent outside water (hrs)										
	0	3	6	9	12	15	18	21	24	36	48
LIVER	2.34	2.50	2.20	1.56	1.29	1.31	1.19	1.02	0.79	0.83	0.78
KIDNEY	2.51	2.74	2.72	1.66	1.34	1.19	0.91	0.87	0.87	0.75	0.82
MUSCLE	9.49	9.33	8.51	5.19	5.24	5.21	4.58	4.73	4.55	7.23	6.65
BRAIN	2.71	3.45	3.30	2.09	2.16	2.13	2.26	1.89	2.38	2.78	2.51
PLASMA	0.63	0.86	0.88	0.81	0.75	0.74	0.79	0.83	0.82	0.79	0.70

TABLE 30. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver of **H. fossilis** kept outside water. (Mean ± S.D.)

Time spent outside water(hrs)	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	4.56 ±0.51		252.27 ±22.71		28.72 ±5.49		7699.49 ±933.48	
12	5.35 ±0.37 (+17.3)	<0.05	305.00 ±19.23 (+20.9)	<0.02	64.08 ±11.29 (+123.1)	<0.005	7821.09 ±530.03 (+1.6)	N.S.
24	6.50 ±0.74 (+43.5)	<0.005	337.43 ±19.11 (+33.8)	<0.005	63.42 ±9.49 (+120.8)	<0.001	7808.54 ±367.62 (+1.4)	N.S.
36	6.67 ±0.61 (+46.3)	<0.005	408.90 ±20.79 (+62.1)	<0.001	67.67 ±5.49 (+135.6)	<0.001	7946.63 ±422.47 (+3.2)	N.S.
48	7.22 ±0.78 (+58.3)	<0.005	404.42 ±22.36 (+60.3)	<0.001	71.23 ±10.21 (+148.0)	<0.001	7905.43 ±412.67 (+2.7)	N.S.

CPS - Carbamyl phosphate synthetase
OTC - Ornithine transcarbamylase
ARSS - Arginine synthetase system
ARG - Arginase
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 31. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver of **H. fossilis** kept outside water. (Mean ± S.D.)

Time spent outside water(hrs)	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.029 ±0.003		1.62 ±0.16		0.21 ±0.04		49.51 ±6.45	
12	0.034 ± 0.003	<0.05	1.92 ±0.12	<0.02	0.40 ±0.07	<0.005	49.22 ±3.02	N.S.
	(+17.2)		(+18.5)		(+90.5)		(-0.6)	
24	0.041 ±0.004	<0.005	2.12 ±0.11	<0.005	0.40 ±0.06	<0.005	49.14 ±2.79	N.S.
	(+41.4)		(+30.9)		(+90.5)		(-0.8)	
36	0.042 ±0.003	<0.001	2.59 ±0.09	<0.001	0.43 ±0.04	<0.001	50.37 ±3.14	N.S.
	(+44.8)		(+59.9)		(+104.8)		(+1.7)	
48	0.046 ±0.004	<0.001	2.56 ±0.10	<0.001	0.45 ±0.05	<0.001	50.03 ±4.67	N.S.
	(+58.6)		(+58.0)		(+114.3)		(+1.1)	

Abbreviations are same as Table 30.
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 32. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** kept outside water. (Mean ± S.D.)

Time spent outside water(hrs)	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	2.98 ±0.32		43.08 ±3.32		21.21 ±3.61		1585.60 ±103.54	
12	4.64 ±0.26 (+55.7)	<0.001	44.63 ±3.38 (+3.6)	N.S.	38.25 ±7.72 (+80.3)	<0.01	1657.12 ±241.48 (+4.5)	N.S.
24	5.10 ±0.90 (+71.1)	<0.005	107.92 ±5.62 (+150.5)	<0.001	35.96 ±4.53 (+69.5)	<0.005	1667.16 ±369.05 (+5.14)	N.S.
36	5.23 ±0.23 (+75.5)	<0.001	112.93 ±12.33 (+162.1)	<0.001	36.47 ±4.77 (+76.7)	<0.005	2222.05 ±289.69 (+40.1)	<0.01
48	5.37 ±0.45 (+80.2)	<0.001	119.27 ±12.44 (+176.9)	<0.001	38.11 ±6.99 (+79.68)	<0.005	2016.76 ±318.47 (+27.2)	<0.05

Abbreviations are same as Table 30.
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 33. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** kept outside water. (Mean ± S.D.)

Time spent outside water(hrs)	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.02 ±0.003		0.28 ±0.024		0.14 ±0.021		9.43 ±0.74	
12	0.032 ±0.002 (+60.0)	<0.001	0.31 ±0.03 (+10.7)	N.S.	0.27 ±0.05 (+92.9)	<0.005	11.57 ±1.72 (+22.7)	<0.05
24	0.036 ±0.007 (+80.0)	<0.005	0.76 ±0.04 (+171.4)	<0.001	0.25 ±0.03 (+78.6)	<0.005	11.74 ±2.65 (+24.5)	N.S.
36	0.034 ±0.002 (+70.0)	<0.001	0.80 ±0.09 (+185.7)	<0.001	0.26 ±0.09 (+85.7)	<0.001	15.81 ±2.21 (+67.7)	<0.005
48	0.037 ±0.004 (+85.0)	<0.001	0.77 ±0.11 (+175.0)	<0.001	0.27 ±0.06 (+92.9)	<0.005	14.28 ±2.85 (+51.4)	<0.02

Abbreviations are same as Table 30.
p value calculated compared to control
% change compared to control are given in parentheses.

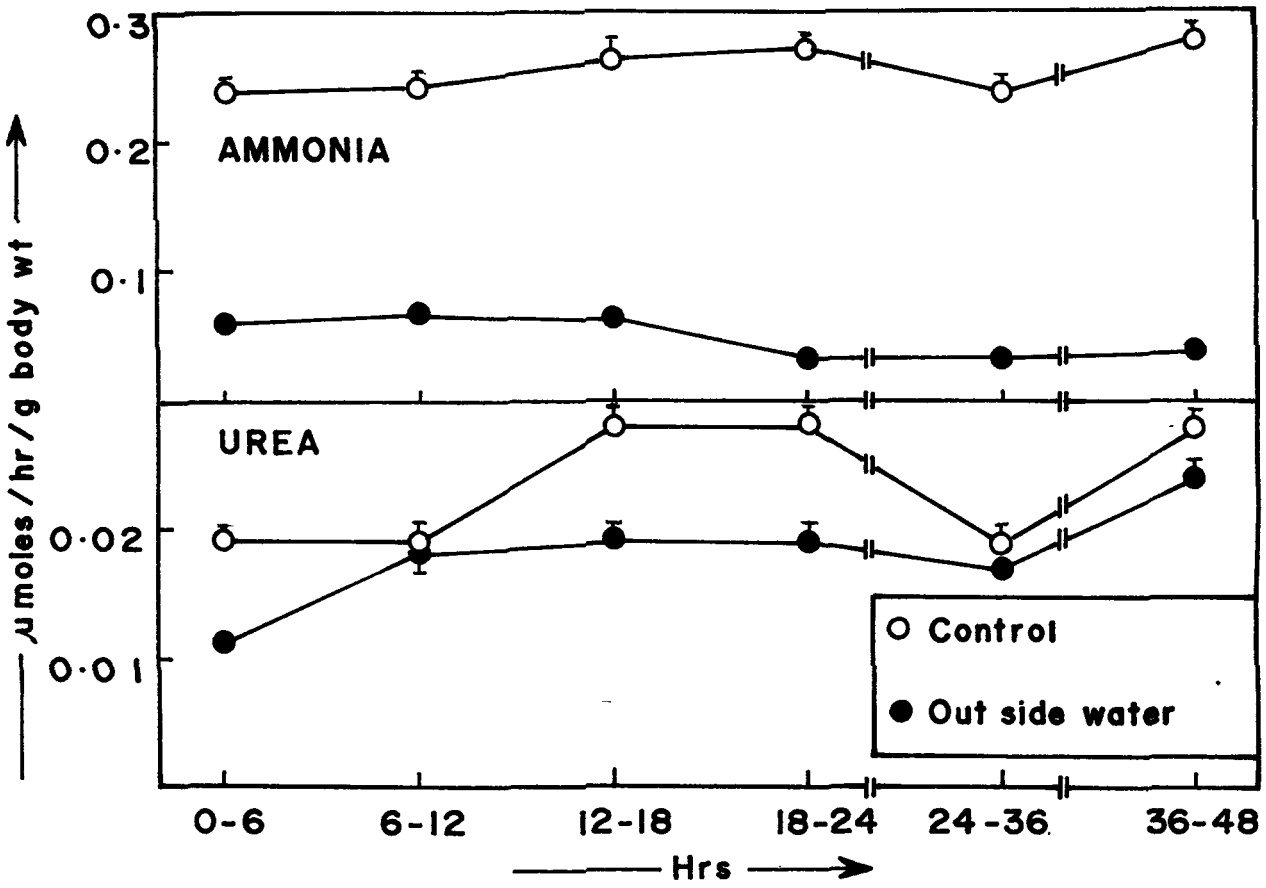


Fig. 13. Alterations in the rate of excretion (µmoles/hr/g body wt.) of ammonia and urea by **H. fossilis** kept outside water.

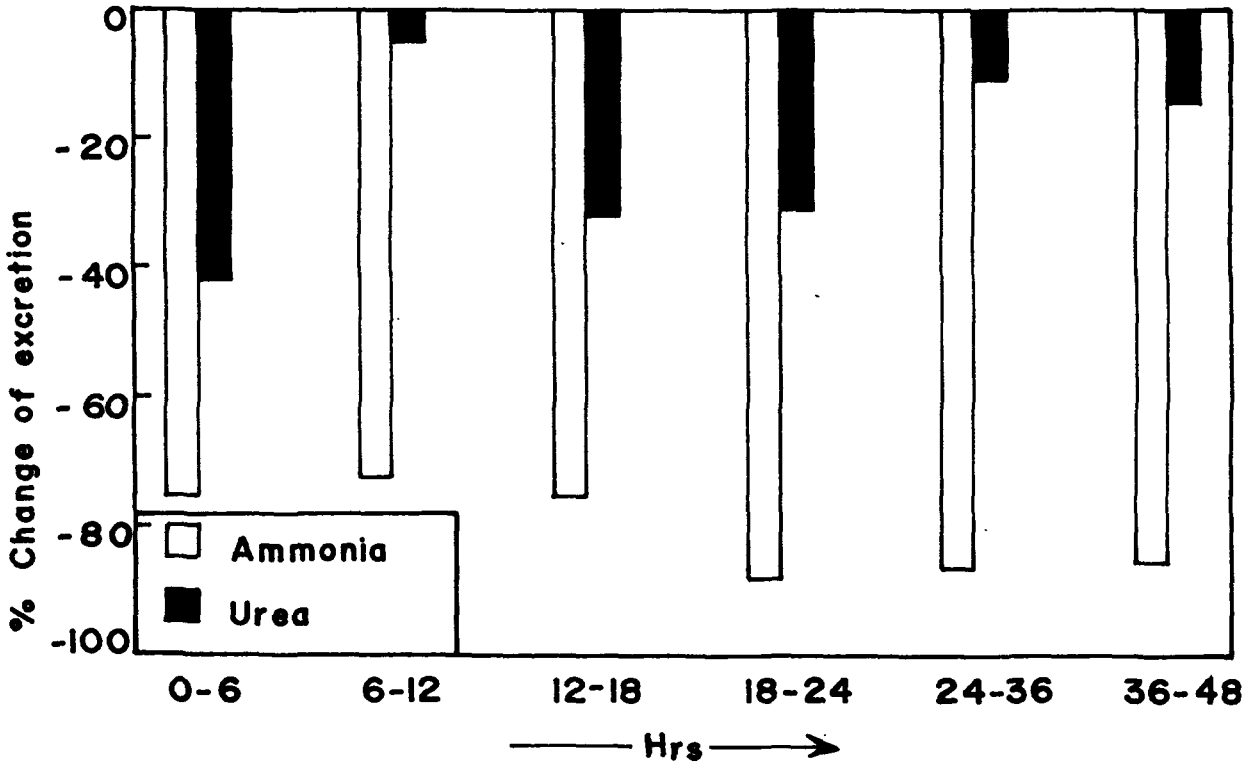


Fig. 14. % change in ammonia and urea excretion rate by **H. fossilis** kept outside water.

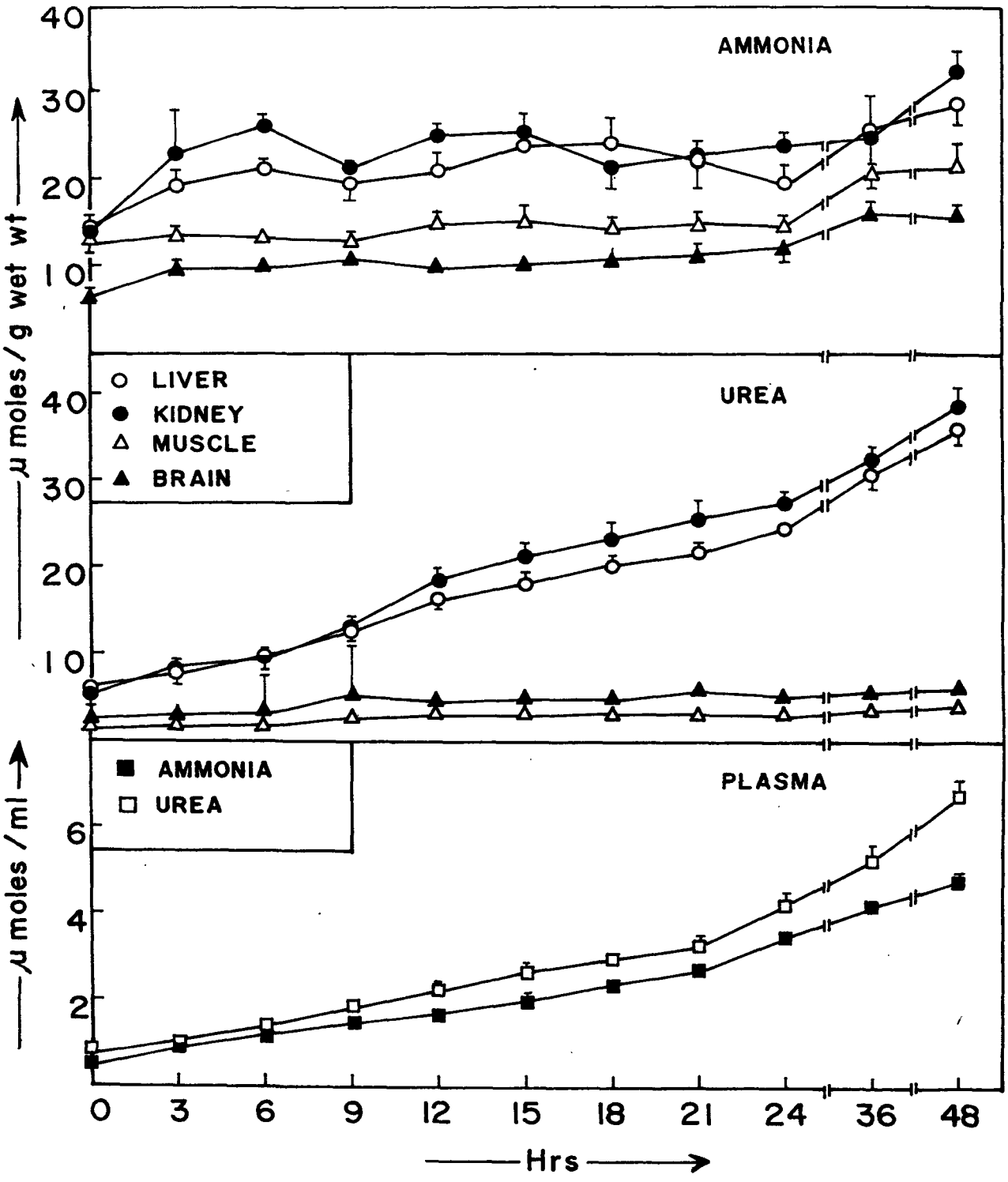


Fig. 15. Alterations in the ammonia and urea concentration in different tissues (μmoles/g wet wt.) and in blood plasma (μmoles/ml) of **H. fossilis** kept outside water.

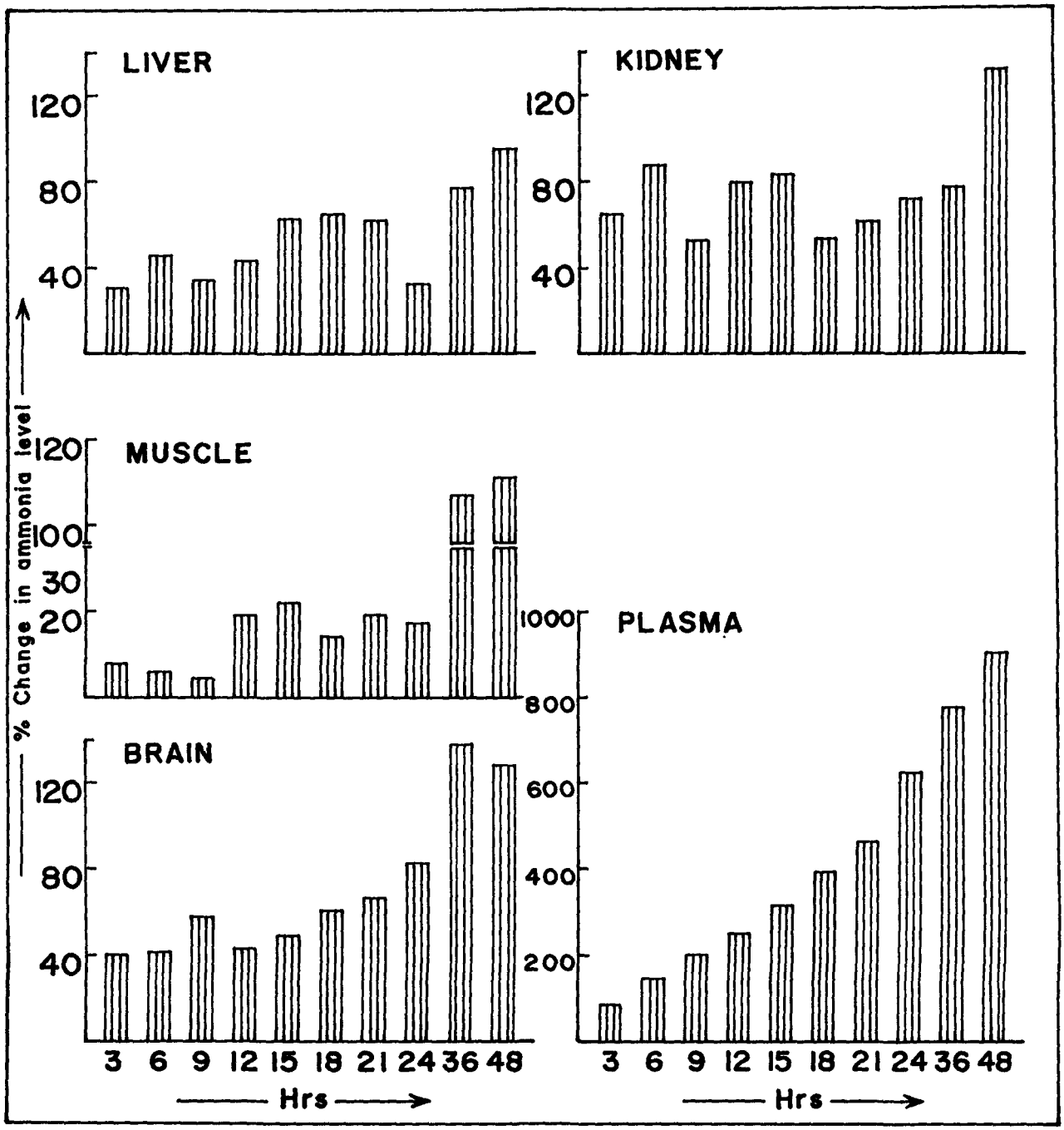


Fig. 16. % change in the concentration of ammonia in different tissues and in blood plasma of **H. fossilis** kept outside water.

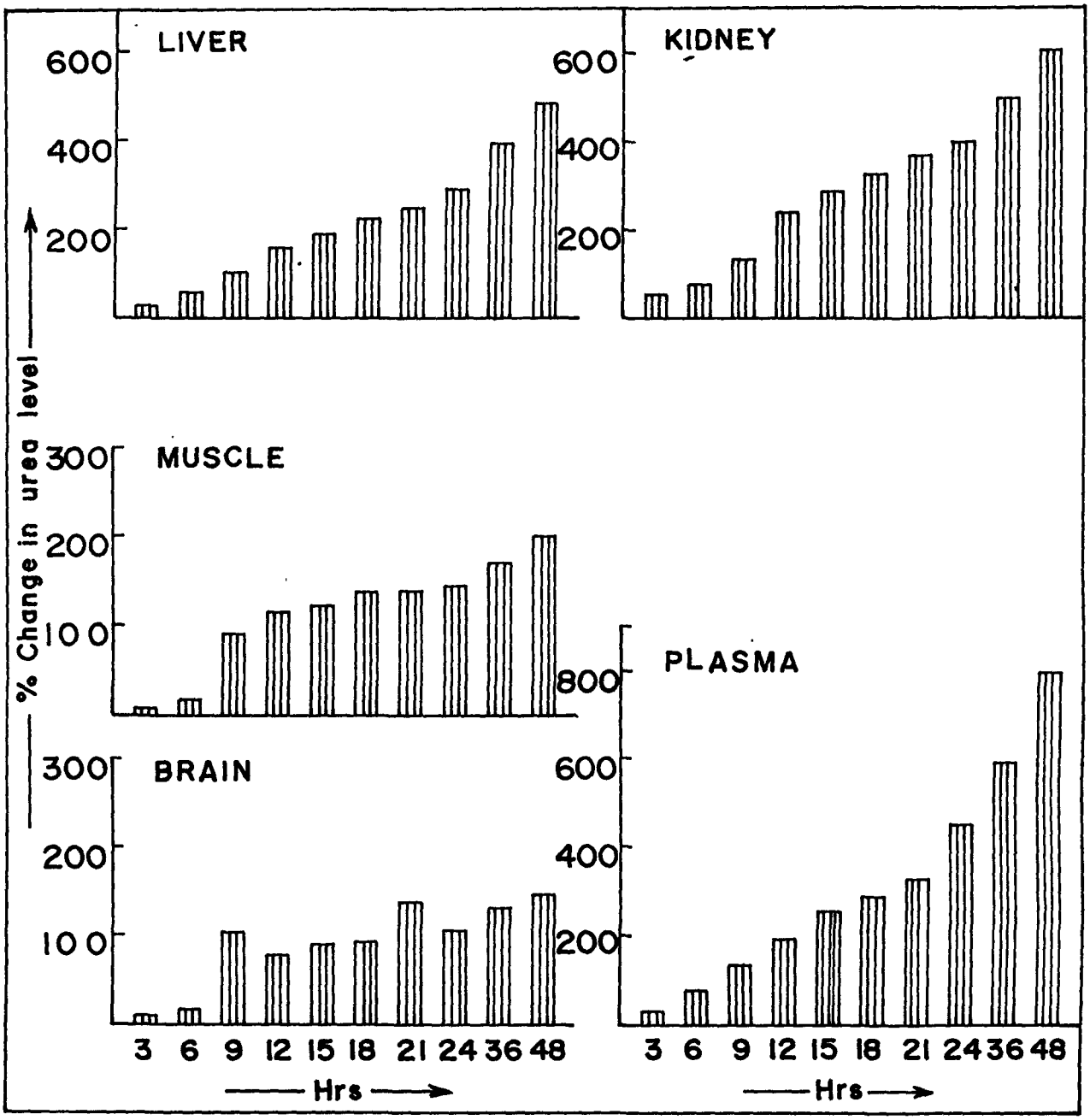


Fig. 17. % change in the concentration of urea in different tissues and in blood plasma of **H. fossilis** kept outside water.

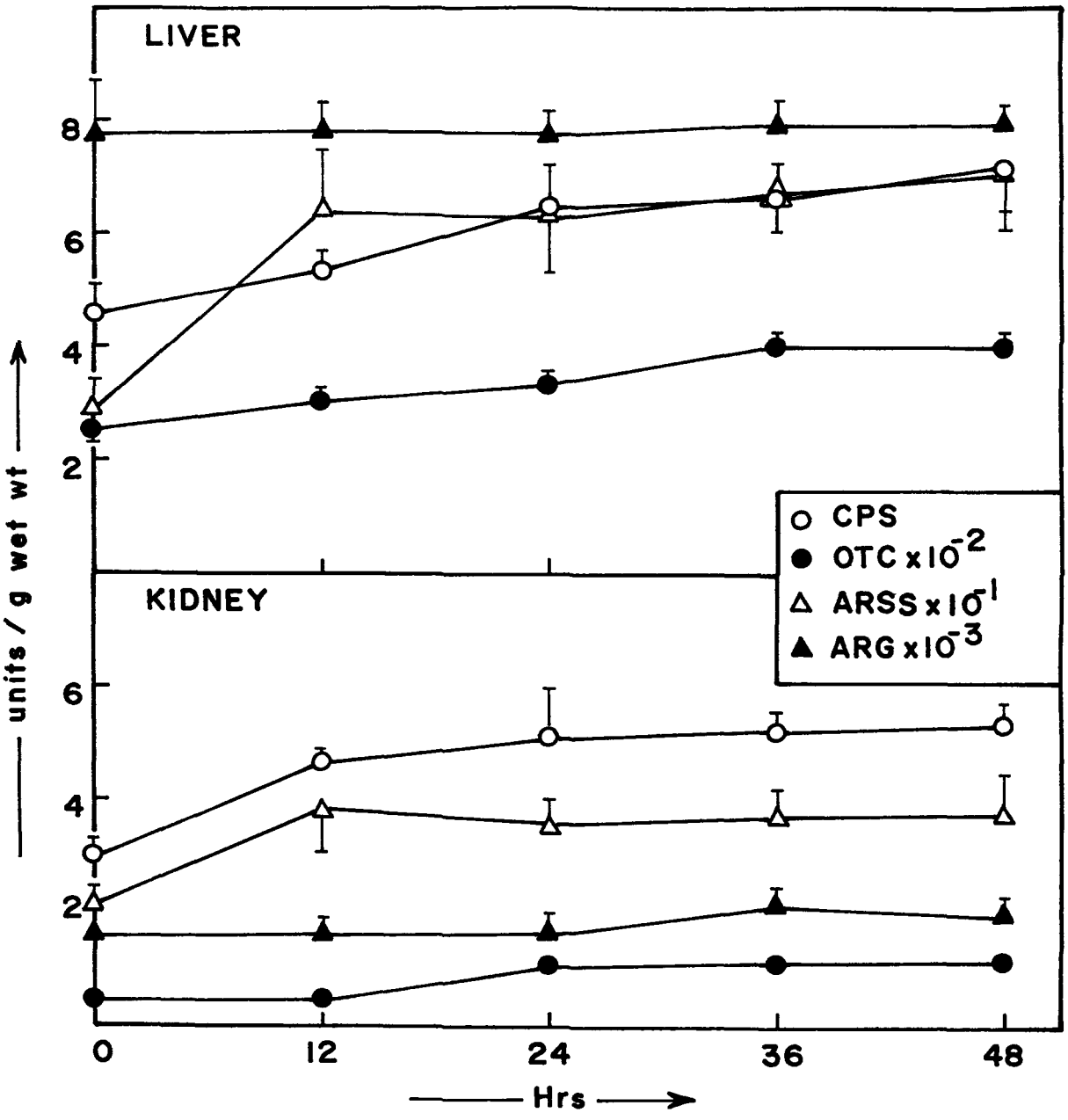


Fig.18. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** kept outside water.

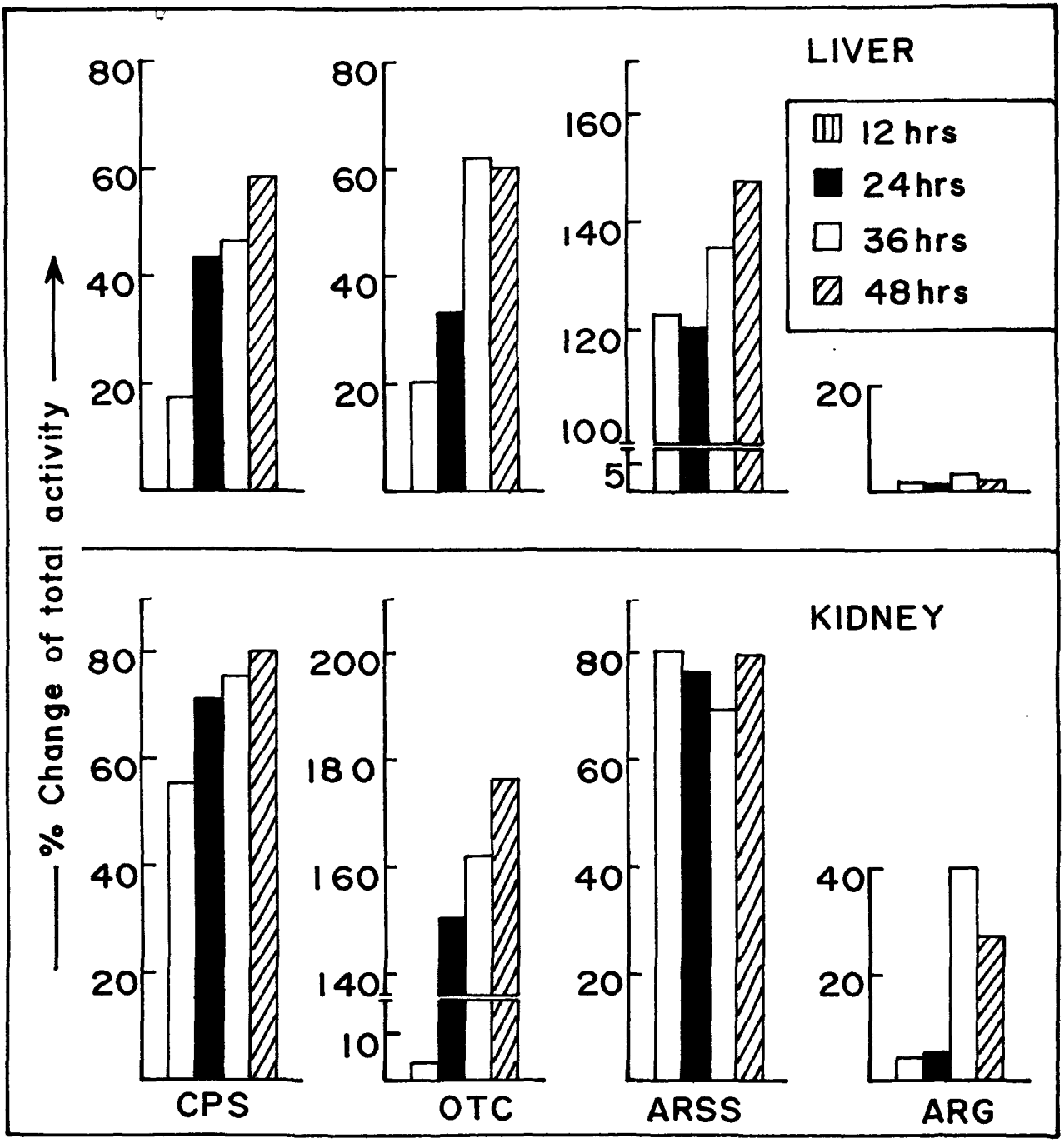


Fig. 19. % change in the total activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** kept outside water.

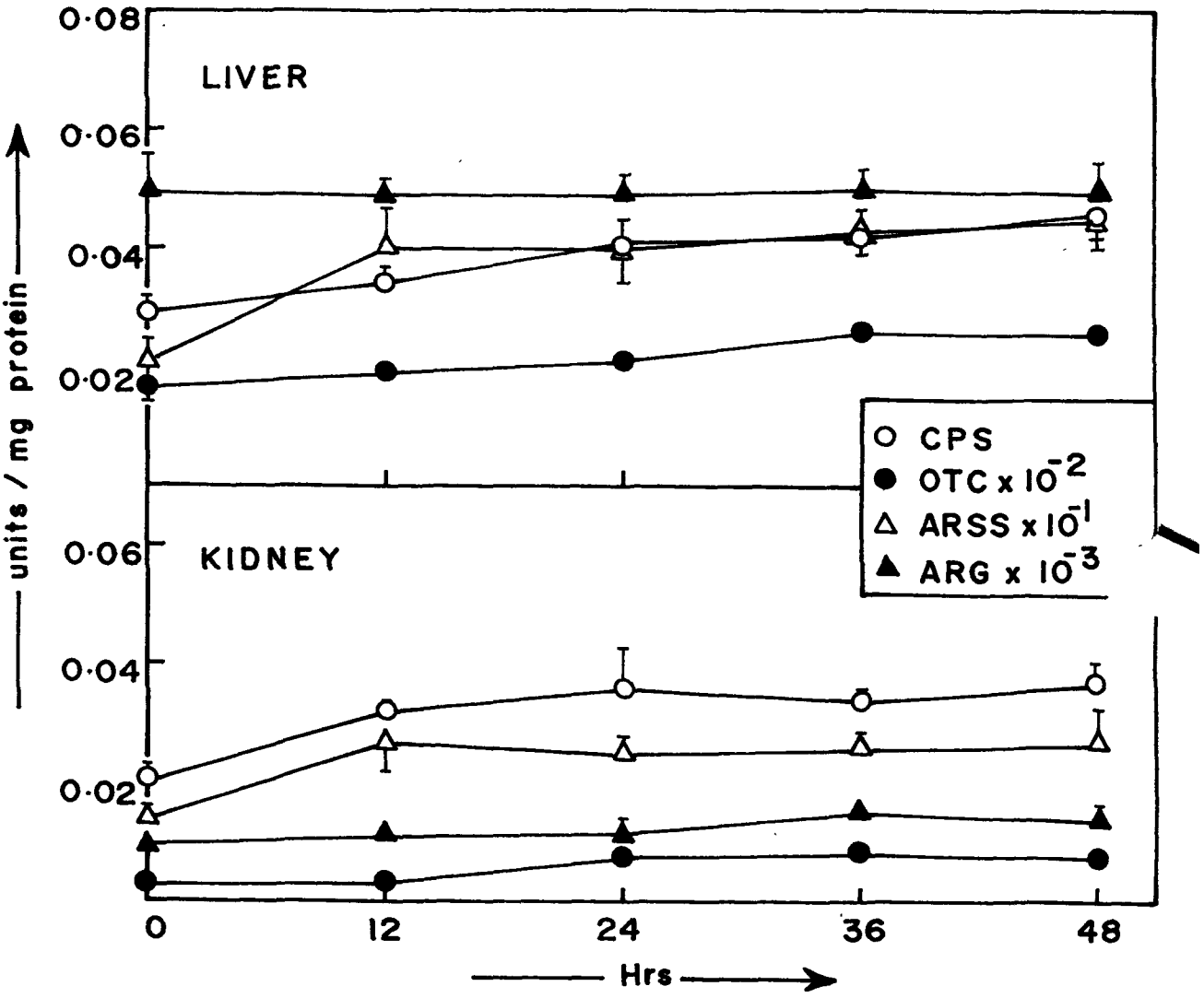


Fig. 20. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** kept outside water.

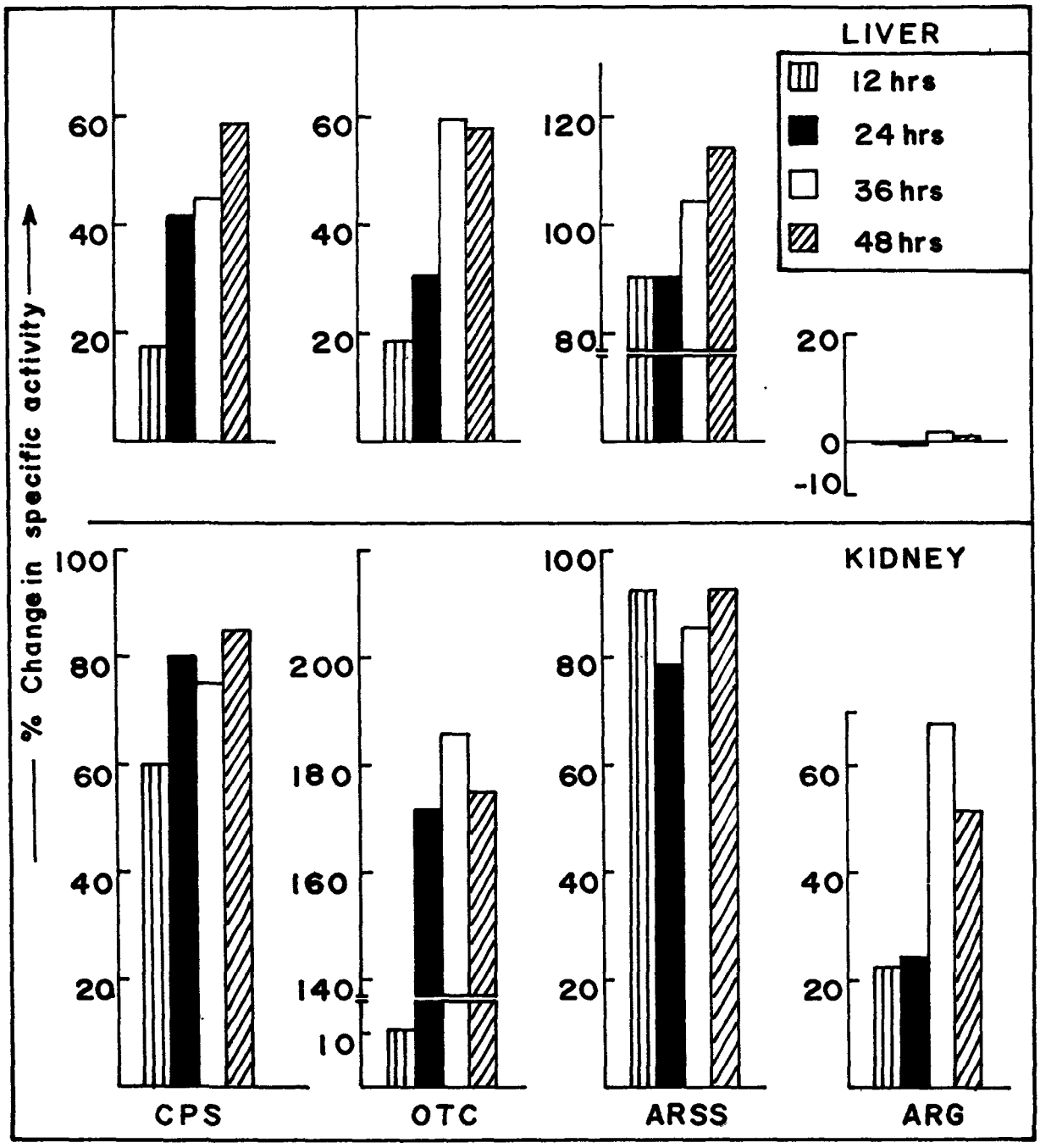


Fig. 21. % change in the specific activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** kept outside water.

was highest for OTC (176.9%) followed by CPS (80.2%), ARSS (79.7%) and ARG (40.1%), (Table 32; Fig. 13). The specific activity and total activity of kidney enzymes followed, in general, similar pattern of change during the 48 hrs exposure of **H. fossilis** to air.

DISCUSSION

The air-breathing freshwater teleost, **H. fossilis** excreted some amount of both ammonia and urea unlike the total suppression of nitrogen excretion reported in some other amphibious fishes during aerial exposure (Gordon **et al**, 1978; Ramaswamy & Reddy, 1983). Partial suppression in nitrogen excretion was reported in a marine air-breathing teleost, **Blennius pholis** exposed to air (Davenport & Sayer, 1986) resembling that of **H. fossilis**. Schmidt-Nielsen and Forster (1954) pointed out that during dehydration the glomerular filtration rate in the kidney of amphibians decreased resulting in decreased excretion of urea. Most of ammonia (99%) and majority of urea (75%) were excreted through extra-renal sources in **H. fossilis** (Chapter I). During aerial exposure ammonia and urea were excreted along with the mucus whose excretion was enhanced with increased period of exposure of **H. fossilis** to air. Mucus was secreted possibly from the gills and scaleless mucoid skin of the fish. Davenport and Sayer (1986) reported that most of the ammonia excreted in **Blennius pholis** was with the increased mucus secretion (whose actual source of secretion was not clear) through extra-renal sources. The excess mucus secretion with water loss during aerial exposure

might have caused the water loss in the muscle of **H. fossilis.** A quick covering of the body surface with mucus with increased osmolarity in the muscle due to accumulation of urea might have limited the water loss to only 3% even after 48 hrs of exposure. Accumulation of urea in the body might have decreased the urea excretion during the initial period of dehydration. It recovered gradually during the later part of the experimental period possibly after accumulation of sufficient urea. The decrease of ammonia excretion which was more pronounced (75%) than urea (42%) initially, further decreased to 85% during the later period of experimentation. The accumulated ammonia later might have got converted to urea thereby increasing urea excretion. Thus a shift from ammoniotelism towards ureotelism in **H. fossilis** with the increasing time of aerial exposure has been evident. Concentration of urea circulating in the plasma acts as a buffer against osmotic stress (Brown, 1970) and plays a major role in pH homeostasis (Atkinson & Bourke, 1984). Increase in body urea concentration has been reported during dehydration in various amphibious fishes and amphibians. Increased level of urea in plasma was reported in spadefoot toads undergoing dehydration (Mc Clanahan, 1967). Gordon (1970) suggested that the crab-eating frog, **Rana cancrivora** maintained elevated plasma concentration of urea as an adaptation to inhabit environments of high salinity. Increased plasma level of urea was also observed in **Xenopus laevis** during aestivation (Balinsky, 1970) and exposure to hyper-osmotic solution (McBean & Goldstein, 1967, 1970)

Conversion of greater part of accumulated ammonia to urea during stay outside water in mudskipper fish **Periophthalmus sobrinus** (Gordon **et al**, 1969) for 12 hrs and in **P. cantonensis** (Gordon **et al**, 1978) for 9.5 days have been reported. Ramaswamy and Reddy (1983) observed elevated plasma and tissue urea level in two species of freshwater air-breathing teleosts **Anabas scandens** and **Channa gachua** and suggested a probable shift towards ureotelism during their stay outside water. However, such a shift towards ureotelism could not be observed in marine air-breathing teleost **Blennius pholis** (Davenport & Sayer, 1986). Morii (1979) also could not detect any conversion of ammonia to urea in **P. cantonensis** and **Boleophthalmus pectinirostris** during their stay outside water and suggested that conversion of ammonia to urea was not performed in these two species of mudskipper fish. Instead, they reported decrease of nitrogen excretion and proposed a decrease in metabolic activity resulting in less ammonia production in the two species during aerial exposure. However, this suggestion has not yet been confirmed and our present results in **H. fossilis** exposed to air suggest both increased production of ammonia and also its conversion to urea during emersion.

Gregory (1977) reported an incomplete o-u cycle in the liver of mudskipper fishes and suggested that those fishes were incapable of synthesizing urea from ammonia through o-u cycle. Hence, their capability to shift towards ureotelism was remote. However, they had functional uricolytic pathway for ureogenesis. **H. fossilis** did possess a complete o-u cycle

with very high activities of all the enzymes in both liver and kidney compared to other freshwater teleosts. Besides, a functional uricolytic pathway was also recorded in the liver of **H. fossilis** (Chapter II). Therefore, it might be possible for **H. fossilis** to avoid accumulation of excess amount of toxic ammonia during aerial exposure by converting it to urea via o-u cycle.

There was an accumulation of both ammonia and urea in all the tissues and in blood plasma of **H. fossilis** when subjected to aerial exposure. The accumulation of ammonia increased quickly within 3 hrs of emersion after which no further or very less increase in ammonia accumulation took place almost in all the tissues. There might be either a decrease in the rate of ammonia production (as suggested by Morii, 1979) or the excess of ammonia produced was converted to urea through o-u cycle. The later mechanism seems to be more convincing at least in **H. fossilis** as the urea level in different tissues and in blood plasma increased gradually with increasing time of aerial exposure. The ratio of ammonia:urea was also found to be decreasing with increasing time almost in all the tissues indicating gradual increase in accumulation of urea than ammonia. The excretion of urea became normal after 24 hrs of emersion suggesting that urea synthesis was accelerated to maintain the higher tissue level and normal excretion rate.

The activity of the enzymes of o-u cycle was induced in the liver and kidney of **H. fossilis** at a rate which could

be parallel to the rate of ureogenesis during aerial exposure. Accumulation of ammonia to a toxic level due to the inhibition of its excretion and decrease in water content **in vivo** might have induced o-u cycle enzymes for enhanced urea synthesis in **H. fossilis**. This might be a physiological adaptation to avoid ammonia toxicity and also to maintain osmotic equilibrium by retaining urea inside the body during water deprivation. The maximum induction of activity has been for ARSS in liver (2.5 fold) followed by CPS and OTC (about 1.5 fold). However, in kidney the maximum induction was seen for OTC (2.8 fold) followed by CPS and ARSS (1.8 fold). Hepatic ARG activity was unaffected during the experimental period. It is difficult to say for sure whether this increase in enzyme activity was due to the synthesis of fresh enzyme proteins or the result of conversion of pre-existing inactive enzymes to their active forms without conducting specific experiments as suggested by Goldstein (1972).

However, it is the first definite report so far to show the induction of o-u cycle enzyme activity in any freshwater teleost under dehydration stress probably due to the accumulation of ammonia to a toxic level **in vivo**. Such adaptive modifications have been found in lungfish, **Protopterus** and aquatic amphibia, **X. laevis** during aestivation. Smith (1930) showed that **Protopterus** was capable of converting large quantities of ammonia into urea which comprised as much as 1% of the body weight at the end of one year of aestivation. Several workers have definitely shown that the major route for converting

accumulated ammonia to urea during aestivation was o-u cycle in **Protopterus** (Forster & Goldstein, 1966; Janssens & Cohen, 1968). However, in aestivating lungfish, there was no increase in the rate of incorporation of C^{14}-bicarbonate into urea in liver slices (Forster & Goldstein, 1966) and no elevation of CPS activity in the liver (Janssens & Cohen, 1968). They suggested that probably the o-u cycle continued to operate at the normal level also during aestivation as the normal level was already very high.

In contrast to the situation in **Protopterus**, there was an increased rate of urea synthesis in **X. laevis** subjected to water deprivation which was accompanied by five fold increase in CPS activity (Janssens & Cohen, 1968). There was not much change in the activity of other o-u cycle enzymes. However, Boernke (1973) found the increase in arginase activity in the liver and kidney of three species of amphibians (other than **X. laevis**) under dehydrated condition. Considering these observations, the induction of specific enzymes of o-u cycle in **H. fossilis** during dehydration seems to be unique in character. The induction was either due to ammonia toxicity or dehydration or both or might be some other unknown reasons.

It has been reported earlier (Chapter I) that **H. fossilis** survived outside water for upto 70-80 hrs. However, during this experiment the death rate increased significantly after 48 hrs outside water for which the experiment was terminated at 48 hrs. Though there is no direct information about the cause of death, several factors such as ammonia toxicity,

dehydration and depletion of energy could be possible causes of death. There was 75 to 85% decrase in ammonia excretion by **H. fossilis** during early period of aerial exposure resulting in an elevated level of ammonia **in vivo**. However, this level was lower than the maximum level of ammonia tolerated by **H. fossilis** during exposure to higher ambient ammonia for 28 days (Chapter III). The fasting fishes might have also faced the problem of energy depletion under severe stressful conditions. Balinsky (1970) pointed out that the production of urea was metabolically expensive since three molecules of ATP were necessary for every mulecule of urea. Therefore, a large amount of energy must have been utilised for increased ureogenesis. In addition to this, dehydration stress and secretion of excess mucus under dehydrated condition which also an energy linked process, might have resulted into the severe loss of energy under starved condition leading to the death of fish.

CHAPTER V

HYPER-OSMOTIC STRESS

INTRODUCTION

Availability of water was suggested as the major factor to decide the nature of end product of nitrogen metabolism in vertebrates (Needham, 1938; Baldwin, 1947; Gordon, 1970). There has been several studies on the excretion pattern and nitrogen metabolism in animals particularly aquatic having to withstand at any stage of their life cycle, change in the availability of environmental water. Besides dehydration, aquatic animals face the problem of water shortage during their sudden exposure to a hyper-osmotic medium. The animals enhance the osmolarity of body fluid to prevent exosmosis by accumulating some special substances such as amino acids, urea, TMAO etc.

Role of urea in osmoregulation has been well documented in various amphibians (Balinsky **et al**, 1961; Janssens, 1968; McBean & Goldstein, 1970; Colley **et al**, 1972, Balinsky, 1981) and in fishes particularly selacians (Alexander **et al**, 1968; Goldstein, 1972; Pang **et al**, 1977). Even during early stages of embryonic development urea has been reported as an osmotic effector (Price & Daiber, 1967; Read, 1968). 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 & Somero, 1978; Bowlus & 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 synthesized and retained urea primarily for maintaining osmotic equilibrium in hyper-osmotic environment such as sea water.

Smith and Smith (1931) emphasized the role of urea in osmoregulation in elasmobranchs and made the following observations.

(a) Urea, an end product of the combustion of protein, was not completely excreted in elasmobranchs, and partly retained in body fluids.

(b) The osmotic pressure of the body fluids of different fish varied depending on their habitat. It was highest in those living in seawater and lowest in freshwater. Both chloride and urea concentration followed the same pattern. Urea contributed about 50% to the total difference in osmotic pressure.

(c) Urea concentration in urine was lower than in serum, indicating its reabsorption in kidney.

(d) The extra-renal excretion of urea in the freshwater sawfish, **Pristis** was limited.

On the basis of the above they proposed the following:

(a) The internal osmotic pressure in elasmobranchs, unlike teleosts, was maintained higher than that of the external aquatic medium even in seawater where the difference

was small. This was achieved by retention of various substances, including salts and trimethylamine oxide, but the key contribution was that of urea.

(b) All elasmobranchs, thus, extracted water osmotically from the medium in which they live.

(c) Urea was produced at a constant rate through metabolism and its loss through the gills, though limited, was passive. Regulation of its concentration in the body was achieved by reabsorption in the kidney.

(d) The fish responded to a downward change in osmotic pressure of its environment by taking in more water and producing a more copious urine in which urea was lost. Thus, the internal osmotic pressure reached to an appropriate level at which water intake and urea loss were again stabilised. The reverse occurred when the fish changed to a hyper-osmotic environment.

(e) Having evolved this response, elasmobranchs could cope with any change in the osmotic environment, given time.

Very high concentration of urea and its osmoregulatory role in the body fluids of marine elasmobranchs and holocephaleans made many workers to search for the possible pathways of ureogenesis. All the enzymes necessary for urea synthesis via the o-u cycle have been demonstrated in elasmobranchs (Baldwin, 1960; Brown, 1964; Schooler, 1966; Read, 1968) and in the chimaeroid, **Hydrolagus colliei** (Read, 1967). All the

three enzymes of the uricolytic pathway were also reported to be present in elasmobranchs (Brunel, 1937). Schooler **et al** (1966) showed that the rate of urea formation via the o-u cycle was much more active than the purine pathway in **Squalus acanthias** both **in vivo** and **in vitro**. Goldstein and Forster (1971) found that the activities of o-u cycle and the rate of urea synthesis were much lower in freshwater ray, **Potamotrygon** compared to other marine elasmobranchs. Its renal tubules have been reported to have lost the ability to retain urea which was correlated with their freshwater habitat. Most of the terrestrial and aquatic amphibians appear to show an ability to adapt to a wide variety of osmotic environments. Accumulation of urea as a solute in intra- and extra-cellular fluid seems to play a critical role in amphibians for adaptation to high salinity (Gordon, 1962; Katz, 1973; Balinsky, 1981) or terrestrial life (McClanahan, 1972, 1975; Delson & Whitford, 1973; Degani, 1981a; Degani **et al**, 1981). High concentrations (300-900 mM) of urea have been reported in terrestrial amphibians such as **Scaphiopus couchi** (McClanahan, 1972), **Ambystoma tigrinum** (Delson & Whitford, 1973), **Salamandra salamandra** (Degani, 1981b), **Bufo viridis** (Degani **et al**, 1981, 1984). An increase in the level of urea in plasma was observed when **Bufo viridis** (Tercafs & Schoffeniels, 1962; Katz & Ben-Sasson, 1984; Katz, 1986), **Rana pipiens** (Scheer & Markel, 1962), **Rana temporaria** and **Rana esculanta** (Ackrill **et al**, 1969) were subjected to hyper-osmotic stress. Inrease in urea synthesis, excretion and accumulation in blood plasma was reported in

X. laevis subjected to water shortage by keeping either in moist peat or exposing to hyper-osmotic solution (Janssens & Cohen, 1968; Funkhouser & Goldstein, 1973).

Some fishes, which could survive outside water due to their air-breathing capacity such as African lungfish **Protopterus**, undergo aestivation during the dry season. Increase in plasma urea level during aestivation was reported in **Protopterus** (Smith, 1930; Sawyer, 1966; Janssens, 1964).

Studies on the effect of hyper-osmotic stress on the nitrogen metabolism of freshwater teleosts are limited. Gupta and Hanke (1982a) reported very less increase in urea as compared to K^+, Mg^{2+} and Ca^{2+} levels in plasma when **Cyprinus carpio** was treated with hyper-osmotic mannitol solution. They emphasized the importance of the cations for osmotic balance in blood plasma rather than urea. Conversion of greater part of ammonia to urea, accumulation and more excretion of urea during the stay outside water of mudskipper fishes are being reported (Gordon **et al**, 1969, 1978). Ramaswamy and Reddy (1983) also found more excretion of urea and more accumulation of urea in plasma of two freshwater air-breathing teleosts, **Anabas scandens** and **Channa gachua** during aerial exposure. Our present study in **H. fossilis**, has shown more synthesis, accumulation and excretion of urea under hyper-ammonia stress (Chapter-III) and more synthesis and accumulation of urea under dehydration stress (Chapter IV).

The present study deals with the effect of hyper-osmotic stress on the nitrogen metabolism in **H. fossilis**. The fish

were exposed to different concentrations of mannitol. Mannitol remains in un-ionized form in the water and dehydrates the animal by exosmosis without penetrating into the body.

The pattern of excretion and accumulation in the body of ammonia and urea, and the activity of o-u cycle enzymes were studied in different tissues of **H. fossilis** treated with 250 mOsm mannitol for different periods upto 28 days.

Plan of work:

(1) **H. fossilis** was exposed to different concentrations of mannitol to determine its tolerance limits. A long term experiment was conducted by treating **H. fossilis** with 250 mOsm mannitol solution for 28 days and studying the following.

(2) The excretion pattern of ammonia and urea by **H. fossilis** under hyper-osmotic stress were studied at 48 hrs interval upto 28 days.

(3) Water content of the muscle was estimated at 7 days interval for 28 days.

(4) Ammonia and urea concentration in different tissues such as liver, kidney, muscle, brain and blood plasma of **H. fossilis** under hyper-osmotic stress were estimated at seven days interval upto 28 days.

(5) The activity of o-u cycle enzymes such as CPS, OTC, ARSS and ARG in the liver and kidney of **H. fossilis** under hyper-osmotic stress was assayed at seven days interval upto 28 days.

MATERIALS AND METHODS

Animal: **Heteropneustes fossilis** weighing 25-35 g acclimatised to laboratory conditions as described in Chapter I were used for experimentation.

Experimental set up: The experiment was conducted at the same light and temperature conditions to which the fishes were acclimatised. Fishes of similar size were used a day after last feeding. No food was given to the fishes during the experiment. They were treated in groups of five in plastic buckets containing different concentrations of mannitol (100, 200, 300, 400 and 500 mOsm) in 2 l bacteria free filtered tapwater with streptopenicillin (20 mg/l) to block microbial growth in the medium. The behaviour and rate of mortality of the treated fish were recorded. All the fishes in 400 and 500 mOsm solution of mannitol died within 4-5 days. Therefore, the experiment was conducted only at 250 mOsm mannitol. The treatment continued for 28 days. Groups of five fishes were kept in separate buckets containing 2 l of bacteria free filtered tapwater with streptopenicillin (20 mg/l) as control. The medium was replaced in each bucket at every 48 hrs after collecting samples for estimation of ammonia and urea excreted by the fish.

Concentration of water, ammonia and urea in different tissues and in the blood plasma were estimated at 7 days interval in both treated and control fishes. The activities of o-u cycle enzymes were assayed in liver and kidney simultaneously

with the estimation of tissue ammonia and urea level.

Blood sampling: Blood was collected from the caudal vein with a heparinized syringe and were processed for estimation of ammonia and urea level in the same way as mentioned in Chapter I.

Tissue preparation: Fishes were sacrificed by decapitation immediately after collecting the blood at 12 noon. Tissues such as liver, kidney, muscle and brain were quickly removed blotted dry and deep frozen at -20°C until used for estimations. All estimations were completed within two days of sampling.

Tissues were processed for estimation of their ammonia and urea level as described in Chapter I.

Estimation procedure: Estimation of ammonia and urea in the sample was done following the methods described in Chapter-I.

Enzyme assay: The activity of the enzymes of ornithine-urea cycle such as carbamyl phosphate synthetase (ammonia dependent) (CPS), ornithine transcarbamylase (OTC) and arginase (ARG) were assayed following the method described in Chapter II. Arginine synthetase system (ARSS) activity was assayed following the method described in Chapter III.

Protein: Protein concentration of the tissues were estimated following the method of Lowry **et al** (1951).

Estimation of water content: Water content of the muscle was estimated as mentioned in Chapter IV.

Expression of results: The rate of excretion or absorption of ammonia and urea during the experimental period were expressed as μmoles per g body weight per 48 hrs. The concentration of ammonia and urea in tissues were expressed as μmoles per g wet wt and in blood plasma as μmoles per ml. The enzyme activity were expressed both as total activity - units per g wet wt of tissue and as specific activity - units per mg protein.

Data were analysed statistically and expressed as mean ± S.D.. The levels of significance between two sets of data were calculated by student 't' test and 'p' value above 0.05 were taken as non-significant (N.S.).

Chemicals: All the enzymes, coenzymes and substrates used were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.. Other chemicals were of analytical grade and obtained indigenously. Deionized double glass distilled ammonia free water was used in all preparations.

RESULTS

Survival of **H. fossilis** in mannitol solution: **H. fossilis** were exposed to different concentrations of mannitol ranging from 100 to 500 mOsm. The fishes did not show much behavioural changes at 100 mOsm mannitol. They became hyper-excitable at higher concentrations which increased with increasing concentration. Any disturbance in the experimental bucket resulted in violent disoriented escape attempts. All the fishes died at 400 and 500 mOsm mannitol. Hence, the long term experiments for 28 days were carried out at 250 mOsm mannitol treatment.

Excretion of ammonia and urea:

Ammonia: (Table 34; Figs. 22 & 23) Ammonia excretion in **H. fossilis** significantly decreased immediately after exposure to 250 mOsm mannitol solution which continued to about 42% less than the control level by 10th day. The rate of ammonia excretion, thereafter, was maintained almost at that level till the end of the experimental period.

Urea: (Table 35; Figs. 22 & 23) There was a significant suppression of urea excretion in **H. fossilis** immediately after exposure to 250 mOsm mannitol solution. This gradually recovered to normal level by 10th day. The urea excretion significantly enhanced after 10th day, to 60% above the control level by 16th day of treatment. It was maintained thereafter, in that elevated level for the rest of the experimental period.

Water content of muscle: (Table 36) Treatment with 250 mOsm

mannitol reduced the water content of muscle of **H. fossilis** by about 2% as estimated at 7 days interval. This decrease, although appears negligible, was statistically significant althrough the period of experimentation.

Tissue ammonia and urea level:

Ammonia: (Table 37; Figs. 24 & 25). There were different tissue specific trends for alterations in ammonia level estimated at 7 days interval. Liver ammonia level remained lower than control althrough the treatment but was not statistically significant except on 28th day. On the contrary, kidney ammonia level remained significantly higher in the fish exposed to 250 mOsm mannitol than the control. Brain ammonia level significantly increased to its highest level (27%) within the first 7 days of treatment. This gradually decreased to the control level by the end of 4th week. There was no significant effect in the ammonia concentration in muscle and blood plasma.

Urea: (Table 38; Figs. 26 & 27) There was significant accumulation of urea in different tissues of **H. fossilis** treated with 250 mOsm mannitol. The accumulation was very fast upto 14th day in all the tissues studied after which the concentration was generally maintained. The maximum urea accumulation was found in liver (27.12) followed by kidney (19.77), brain (6.31) and muscle (3.22 μmoles/g wet wt). In plasma it was 4.98 μmoles/ml. However, the percentage increase in tissue urea level with mannitol treatment was maximum in plasma (568.5%) followed by liver (181.8%), muscle (119.3%), kidney (117.2%) and brain (112.4%).

The ratio of ammonia:urea concentration decreased significantly in all the tissues of mannitol exposed **H. fossilis** (Table 39). In most of the tissues the ratio decreased also in the control fish. However, the decrease in ratio was more prominent in the fish exposed to hyper-osmolar ambient medium.

Activity (total and specific) of o-u cycle enzymes in liver and kidney: (Tables 40-43; Figs. 28-31) There was significant induction in the activities of all the enzymes of o-u cycle except ARG in the liver and kidney of **H. fossilis** treated with 250 mOsm mannitol. The rate of induction gradually increased with the period of treatment with highest level usually on 28th day. The induction of enzyme activity was highest for ARSS followed by CPS and OTC in liver. Unlike liver, OTC activity was induced maximally in kidney followed by ARSS and CPS. ARG activity which was usually very high among the o-u cycle enzymes in liver and kidney, did not alter in liver under hyper-osmolar treatment. The specific activity of ARG showed significant induction (15-20%) in kidney of treated fish. In all other cases the total and specific activity showed similar pattern of induction. The induction rate of the total activity was higher in liver, while the induction of specific activity was higher in kidney.

TABLE 34. Alterations in the rate (μmoles/g body wt /48 hrs) of excretion of ammonia by **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	2	4	6	8	10	12	14	16	18	20	22	24	26	28
H_2O (control)	13.74 ±0.96	12.83 ±0.58	12.14 ±0.52	12.05 ±0.49	10.75 ±0.67	11.34 ±0.93	10.46 ±0.86	10.32 ±0.67	9.98 ±0.95	10.14 ±1.05	10.24	9.78	9.82	9.76
Mannitol (250 mOsm)	13.00 ±0.71	10.44 ±1.53	8.77 ±0.91	8.20 ±1.31	6.00 ±0.60	6.58 ±1.13	6.25 ±0.66	6.33 ±0.54	6.12 ±0.49	5.89 ±0.69	5.92	6.10	5.95	5.81
p	N.S. (-5.4)	<0.025 (-18.6)	<0.001 (-27.8)	<0.005 (-32.0)	<0.001 (-44.2)	<0.001 (-42.0)	<0.001 (-40.2)	<0.001 (-38.7)	<0.001 (-38.7)	<0.001 (-41.9)	(-42.2)	(-37.6)	(-39.4)	(-40.5)

% change compared to control are given in parentheses.

TABLE 35. Alterations in the rate (μmoles/g body wt /48 hrs) of excretion of urea by **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	2	4	6	8	10	12	14	16	18	20	22	24	26	28
H_2O (control)	1.58 ±0.30	1.39 ±0.14	1.23 ±0.19	1.33 ±0.08	1.33 ±0.18	1.20 ±0.17	1.28 ±0.12	1.31 ±0.13	1.36 ±0.10	1.25 ±0.20	1.22	1.26	1.29	1.25
Mannitol (250 mOsm)	0.80 ±0.22	0.73 ±0.30	0.71 ±0.12	1.10 ±0.10	1.56 ±0.31	1.58 ±0.32	1.95 ±0.38	2.06 ±0.35	2.02 ±0.38	2.01 ±0.42	1.95	2.18	1.99	2.00
p	<0.01	<0.01	<0.02	N.S.	N.S.	<0.02	<0.02	<0.01	<0.02	<0.02				
	(-49.4)	(-47.5)	(-42.3)	(-17.3)	(+17.3)	(+17.3)	(+52.3)	(+57.3)	(+48.5)	(+60.8)	(+59.8)	(+73.0)	(+54.3)	(+60.0)

% change compared to control are given in parentheses.

TABLE 36. Alterations in the water content (% wet wt) in the muscle of **H. fossilis** under hyper-osmotic (250 mOsm) condition (Mean ± S.D.)

Days after treatment	H_2O(control)	Mannitol (250 mOsm)	p
7	81.00 ± 0.82	79.5 ± 0.55 (-1.85%)	<0.02
14	82.00 ± 0.12	80.5 ± 0.60 (-1.83%)	<0.005
21	81.98 ± 0.20	80.52 ± 0.69 (-1.78%)	<0.005
28	82.13 ± 1.16	80.56 ± 0.32 (-1.91%)	<0.05

TABLE 37. Alterations in the concentration of ammonia in different tissues (μmoles/g wet wt.) and in blood plasma (μmoles/ml) of **H. fossilis** under hyper-osmatic (250 mOsm) condition. (Mean ± S.D.).

Tissue	Medium		Days after treatment				
			0	7	14	21	28
LIVER	H_2O (control)		14.56 ± 1.15	13.12 ± 1.32	12.72 ± 1.16	12.08 ± 1.17	12.28 ± 1.35
	Mannitol			12.48 ± 1.04	12.80 ± 1.55	11.63 ± 1.02	10.41 ± 1.05
		p		N.S. (-4.9)	N.S. (+0.6)	N.S. (-3.7)	<0.05 (-15.2)
KIDNEY	H_2O (control)		13.90 ± 1.22	13.12 ± 2.85	12.78 ± 2.24	12.68 ± 2.00	12.76 ± 1.76
	Mannitol			15.25 ± 3.01	17.27 ± 1.59	16.27 ± 1.47	15.32 ± 1.25
		p		N.S. (+16.2)	<0.02 (+35.1)	<0.025 (+28.3)	<0.05 (+20.1)
MUSCLE	H_2O (control)		12.43 ± 1.09	11.62 ± 0.91	10.42 ± 0.86	9.46 ± 0.77	9.24 ± 0.68
	Mannitol			11.99 ± 0.89	9.72 ± 0.80	9.13 ± 0.45	8.92 ± 0.42
		p		N.S. (+3.2)	N.S. (-6.7)	N.S. (-3.5)	N.S. (-3.5)
BRAIN	H_2O (control)		6.74 ± 0.81	5.82 ± 0.68	5.51 ± 0.55	5.40 ± 0.32	5.45 ± 0.52
	Mannitol			7.39 ± 0.54	6.62 ± 0.80	5.61 ± 0.81	5.46 ± 0.63
		p		<0.01 (+27.0)	<0.05 (+20.2)	N.S. (+3.9)	N.S. (+0.2)
PLASMA	H_2O (control)		0.473 ± 0.035	0.466 ± 0.04	0.461 ± 0.037	0.455 ± 0.05	0.438 ± 0.052
	Mannitol			0.491 ± 0.05	0.432 ± 0.045	0.415 ± 0.04	0.418 ± 0.043
		p		N.S. (+5.4)	N.S. (-6.3)	N.S. (-8.8)	N.S. (-4.6)

% change compared to control are given in parentheses.

TABLE 38. Alterations in the concentration of urea in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** under hyper-osmatic (250 mOsm) condition. (Mean ± S.D.).

Tissue	Medium	Days after treatment				
		0	7	14	21	28
LIVER	H_2O (control)	6.23 ± 0.61	7.81 ± 0.66	9.48 ± 0.72	11.88 ± 0.86	12.92 ± 1.25
	Mannitol		19.46 ± 1.68	26.71 ± 2.19	26.55 ± 2.68	27.12 ± 3.11
	p		<0.001 (+149.2)	<0.001 (+181.8)	<0.001 (+123.5)	<0.001 (+110.0)
KIDNEY	H_2O (control)	5.53 ± 0.97	6.98 ± 0.78	8.39 ± 1.10	10.22 ± 1.21	11.55 ± 0.92
	Mannitol		16.38 ± 1.57	18.22 ± 2.13	17.34 ± 1.94	19.77 ± 2.33
	p		<0.001 (+80.3)	<0.001 (+119.3)	<0.001 (+98.8)	<0.001 (+99.4)
MUSCLE	H_2O (control)	1.31 ± 0.32	1.42 ± 0.22	1.45 ± 0.38	1.62 ± 0.31	1.73 ± 0.39
	Mannitol		2.56 ± 0.35	3.18 ± 0.48	3.22 ± 0.51	3.45 ± 0.42
	p		<0.005 (+80.3)	<0.005 (+119.3)	<0.005 (+98.8)	<0.005 (+99.4)
BRAIN	H_2O (control)	2.49 ± 0.37	2.82 ± 0.21	2.91 ± 0.37	3.14 ± 0.42	3.22 ± 0.48
	Mannitol		4.85 ± 0.62	6.18 ± 0.72	6.31 ± 0.58	6.22 ± 0.66
	p		<0.001 (+72.0)	<0.001 (+112.4)	<0.001 (+101.0)	<0.001 (+93.2)
PLASMA	H_2O (control)	0.755 ± 0.081	0.742 ± 0.03	0.738 ± 0.04	0.742 ± 0.038	0.745 ± 0.029
	Mannitol		4.28 ± 0.62	4.92 ± 0.71	4.83 ± 0.42	4.98 ± 0.56
	p		<0.001 (+476.8)	<0.001 (+566.7)	<0.001 (+550.9)	<0.001 (+568.5)

% change compared to control are given in parentheses.

TABLE 39. Ratio of ammonia:urea concentration in different tissues and blood plasma of **H. fossilis** when treated with 250 mOsm mannitol.

Tissue	Medium	Days after treatment				
		0	7	14	21	28
LIVER	H_2O (control)	2.34	1.68	1.34	1.02	0.95
	Mannitol		0.64	0.48	0.44	0.38
KIDNEY	H_2O (control)	2.51	1.88	1.52	1.24	1.10
	Mannitol		0.93	0.95	0.94	0.77
MUSCLE	H_2O (control)	9.49	8.18	7.19	5.84	5.34
	Mannitol		4.68	3.06	2.83	2.59
BRAIN	H_2O (control)	2.71	2.06	1.89	1.72	1.69
	Mannitol		1.52	1.07	0.89	0.88
PLASMA	H_2O (control)	0.61	0.63	0.62	0.61	0.59
	Mannitol		0.11	0.09	0.09	0.09

TABLE 40. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver of **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	4.56 ±0.51		252.27 ±22.71		28.72 ±5.49		7699.49 ±933.48	
7	6.11 ±0.62 (+34.0)	<0.01	272.54 ±23.21 (+8.0)	N.S.	62.47 ±5.74 (+117.5)	<0.001	7828.32 ±456.38 (+1.67)	N.S.
14	6.54 ±0.71 (+43.4)	<0.005	339.42 ±30.45 (+34.6)	<0.005	78.29 ±8.23 (+172.6)	<0.001	7739.46 ±394.28 (+0.5)	N.S.
21	7.32 ±0.54 (+60.5)	<0.001	348.27 ±28.32 (+38.1)	<0.005	82.43 ±10.11 (+187.0)	<0.001	8045.27 ±561.62 (+4.5)	N.S.
28	7.96 ±0.69 (+74.6)	<0.001	358.74 ±27.68 (+42.2)	<0.005	91.66 ±9.68 (+219.2)	<0.001	7966.84 ±543.76 (+3.5)	N.S.

CPS - Carbamyl phosphate synthetase
OTC - Ornithine transcarbamylase
ARSS - Arginine synthetase system
ARG - Arginase
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 41. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver of **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.029 ±0.003		1.62 ±0.16		0.21 ±0.04		49.51 ±6.45	
7	0.04 ±0.004 (+37.9)	<0.005	1.76 ±0.14 (+8.6)	N.S.	0.40 ±0.08 (+90.5)	<0.005	50.74 ±4.74 (+2.5)	N.S.
14	0.043 ±0.005 (+48.3)	<0.005	2.21 ±0.18 (+36.4)	<0.005	0.51 ±0.10 (+142.9)	<0.005	50.40 ±5.94 (+1.8)	N.S.
21	0.048 ±0.003 (+65.5)	<0.001	2.28 ±0.17 (+40.7)	<0.005	0.54 ±0.10 (+157.1)	<0.001	52.58 ±6.21 (+6.2)	N.S.
28	0.052 ±0.004 (+79.3)	<0.001	2.35 ±0.21 (+45.1)	<0.005	0.60 ±0.11 (+185.7)	<0.001	52.16 ±6.55 (+5.4)	N.S.

Abbreviations are same as Table 40.
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 42. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	2.98 ±0.32		43.08 ±3.32		21.21 ±3.62		1585.60 ±103.54	
7	3.44 ±0.35 (+15.4)	N.S.	48.08 ±3.45 (+11.6)	N.S.	30.38 ±4.12 (+43.2)	<0.05	1621.62 ±117.21 (+2.3)	N.S.
14	3.56 ±0.29 (+19.5)	<0.05	66.39 ±5.68 (+54.1)	<0.001	34.42 ±4.62 (+62.3)	<0.005	1598.32 ±121.93 (+0.8)	N.S.
21	4.12 ±0.35 (+38.3)	<0.005	78.49 ±7.36 (+80.5)	<0.001	37.98 ±4.28 (+79.1)	<0.005	1632.47 ±132.49 (+3.0)	N.S.
28	4.28 ±0.37 (+43.6)	<0.005	84.35 ±9.12 (+95.8)	<0.001	39.27 ±4.95 (+85.2)	<0.005	1655.21 ±145.55 (+4.4)	N.S.

Abbreviations are same as Table 40
p value calculated compared to control
% change compared to control are given in parentheses.

TABLE 43. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition. (Mean ± S.D.)

Days after treatment	CPS	p	OTC	p	ARSS	p	ARG	p
0 (control)	0.02 ±0.003		0.28 ±0.024		0.14 ±0.021		9.43 ±0.74	
7	0.023 ±0.002 (+15.0)	N.S.	0.29 ±0.02 (+3.6)	N.S.	0.21 ±0.03 (+53.3)	<0.01	11.00 ±0.78 (+16.7)	<0.05
14	0.024 ±0.003 (+20.0)	N.S.	0.45 ±0.05 (+60.7)	<0.001	0.24 ±0.05 (+75.2)	<0.01	10.93 ±0.92 (+15.9)	<0.05
21	0.029 ±0.003 (+45.0)	<0.005	0.55 ±0.04 (+96.4)	<0.001	0.26 ±0.06 (+89.8)	<0.01	11.26 ±1.21 (+19.4)	<0.05
28	0.03 ±0.004 (+50.0)	<0.01	0.59 ±0.035 (+110.7)	<0.001	0.28 ±0.04 (+104.4)	<0.001	11.45 ±1.15 (+21.4)	<0.025

Abbreviations are same as Table 40.
p value calculated compared to control
% change compared to control are given in parentheses.

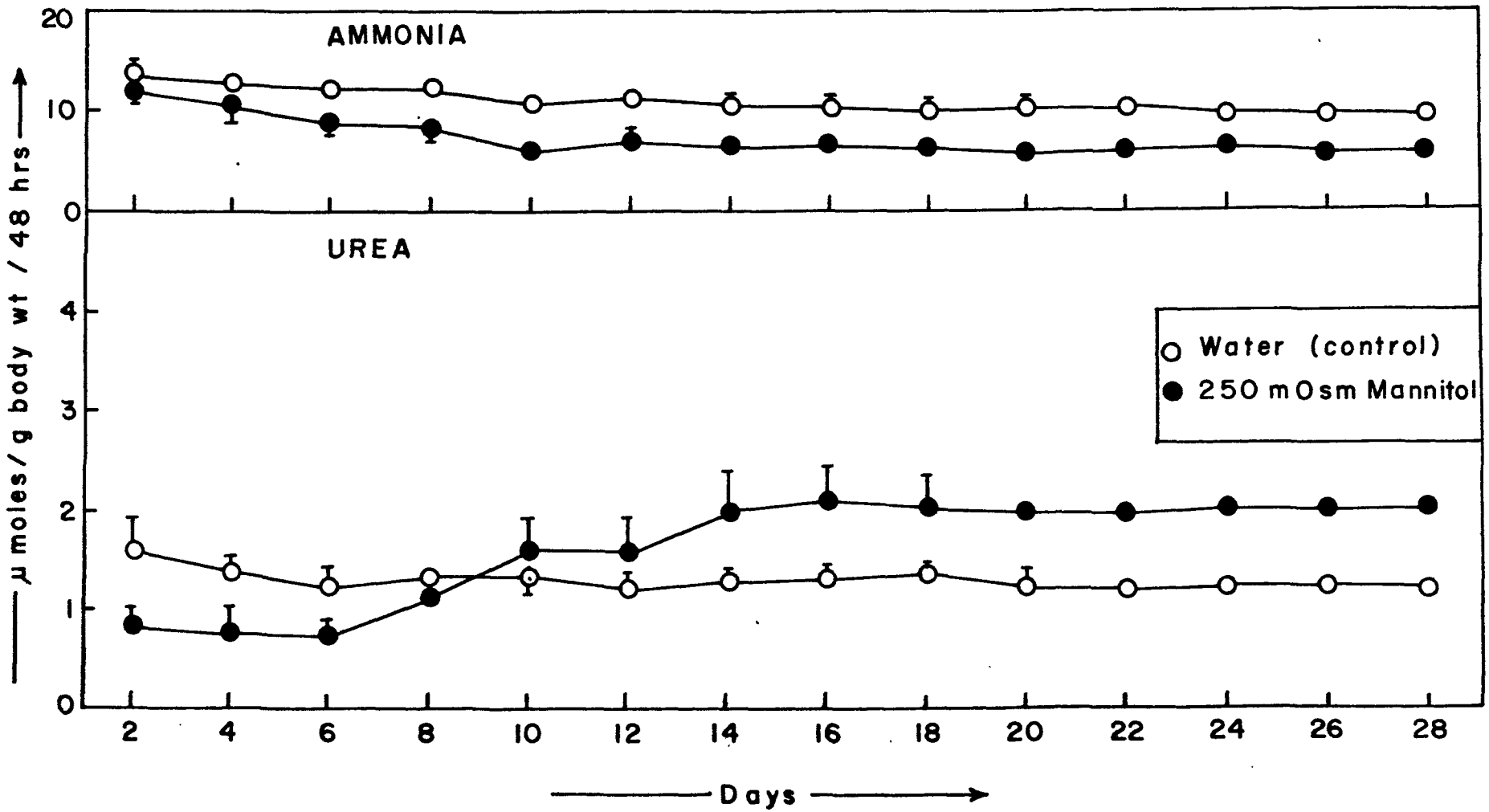


Fig. 22. Alterations in the rate of excretion (μmoles/g body wt./48 hrs) of ammonia and urea by **H. fossilis** under hyper-osmotic (250 mOsm) condition.

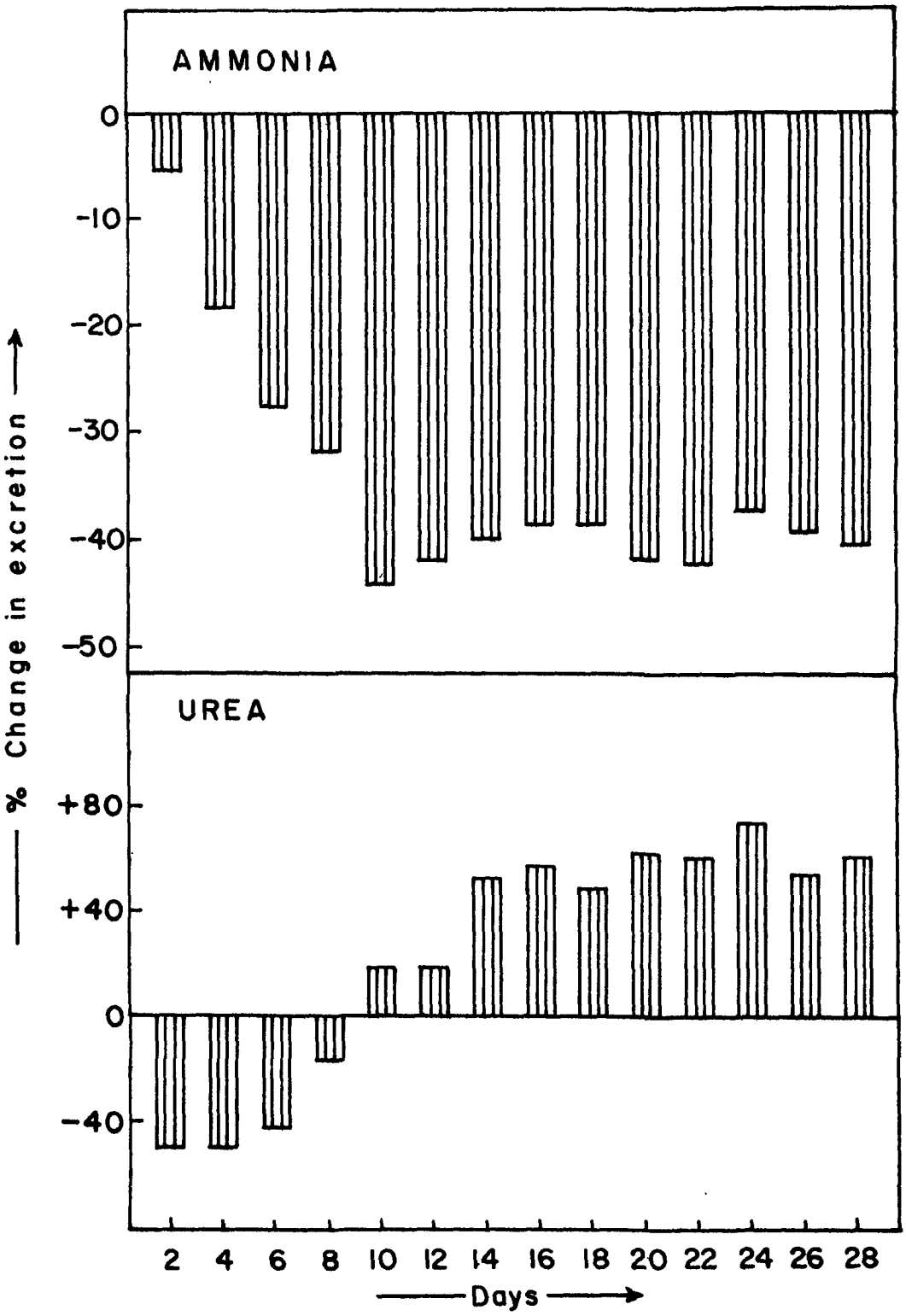


Fig. 23. % change in excretion of ammonia and urea by **H. fossilis** under hyper-osmotic (250 mOsm) condition.

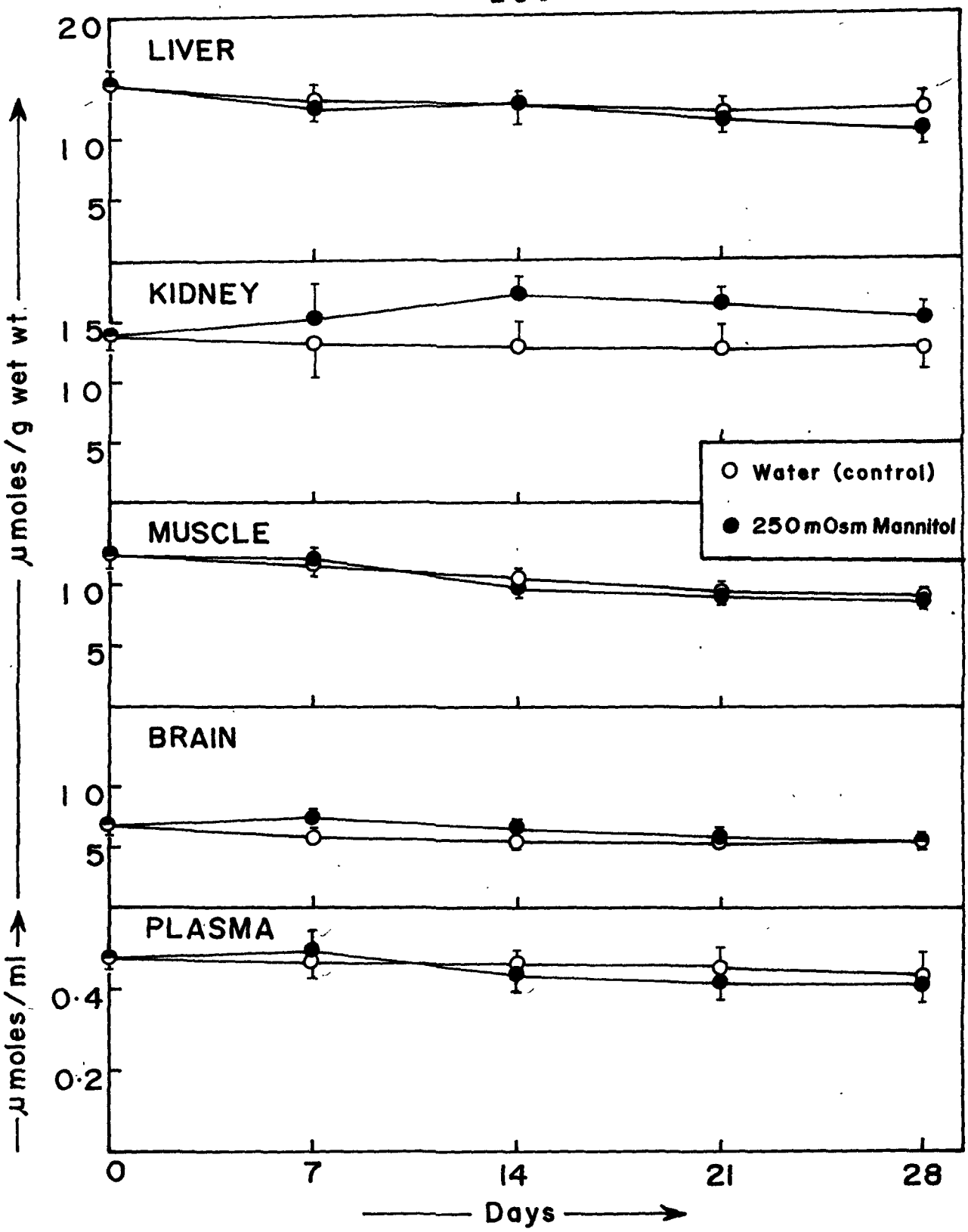


Fig. 24. Alterations in the concentration of ammonia in different tissues (µmoles/g wet wt) and in blood plasma (µmoles/ml) of **H. fossilis** under hyper-osmotic (250 mOsm) condition.

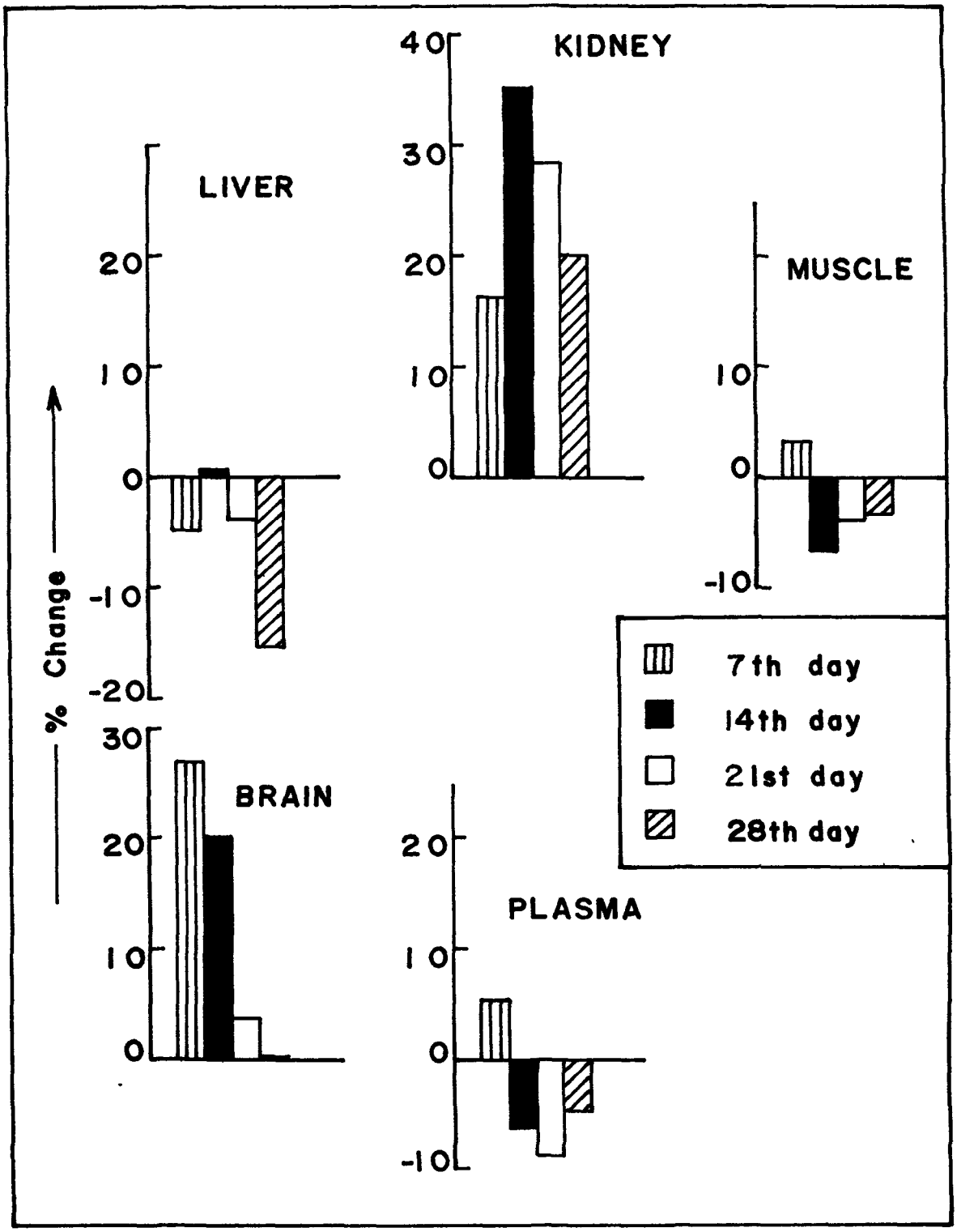


Fig. 25. % change in the concentration of ammonia in different tissues and in blood plasma of **H. fossilis** under hyper-osmotic (250 mOsm) condition

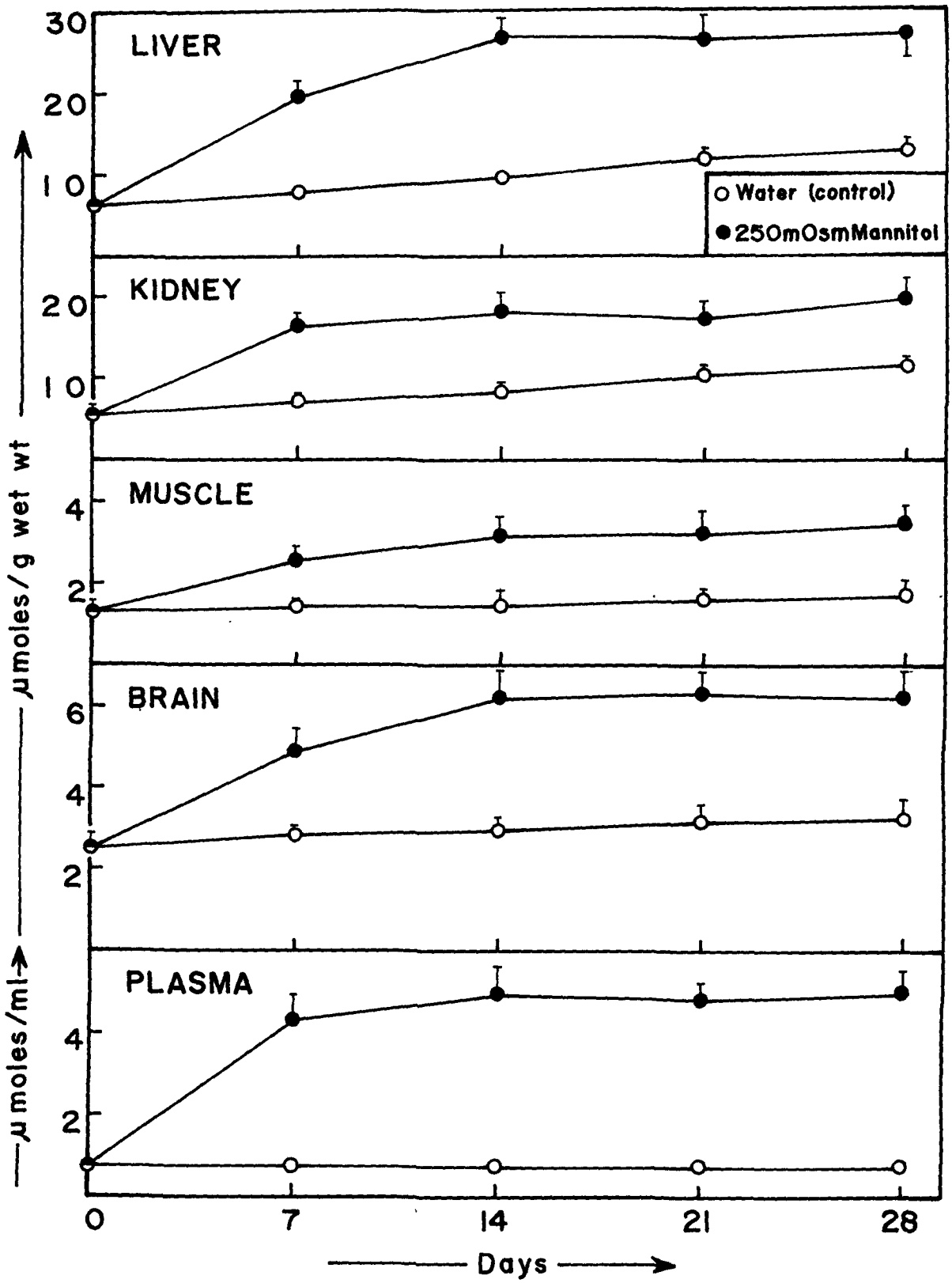


Fig. 26. Alterations in the concentration of urea in different tissues (μmoles/g wet wt) and in blood plasma (μmoles/ml) of **H. fossilis** under hyperosmotic (250 mOsm) condition.

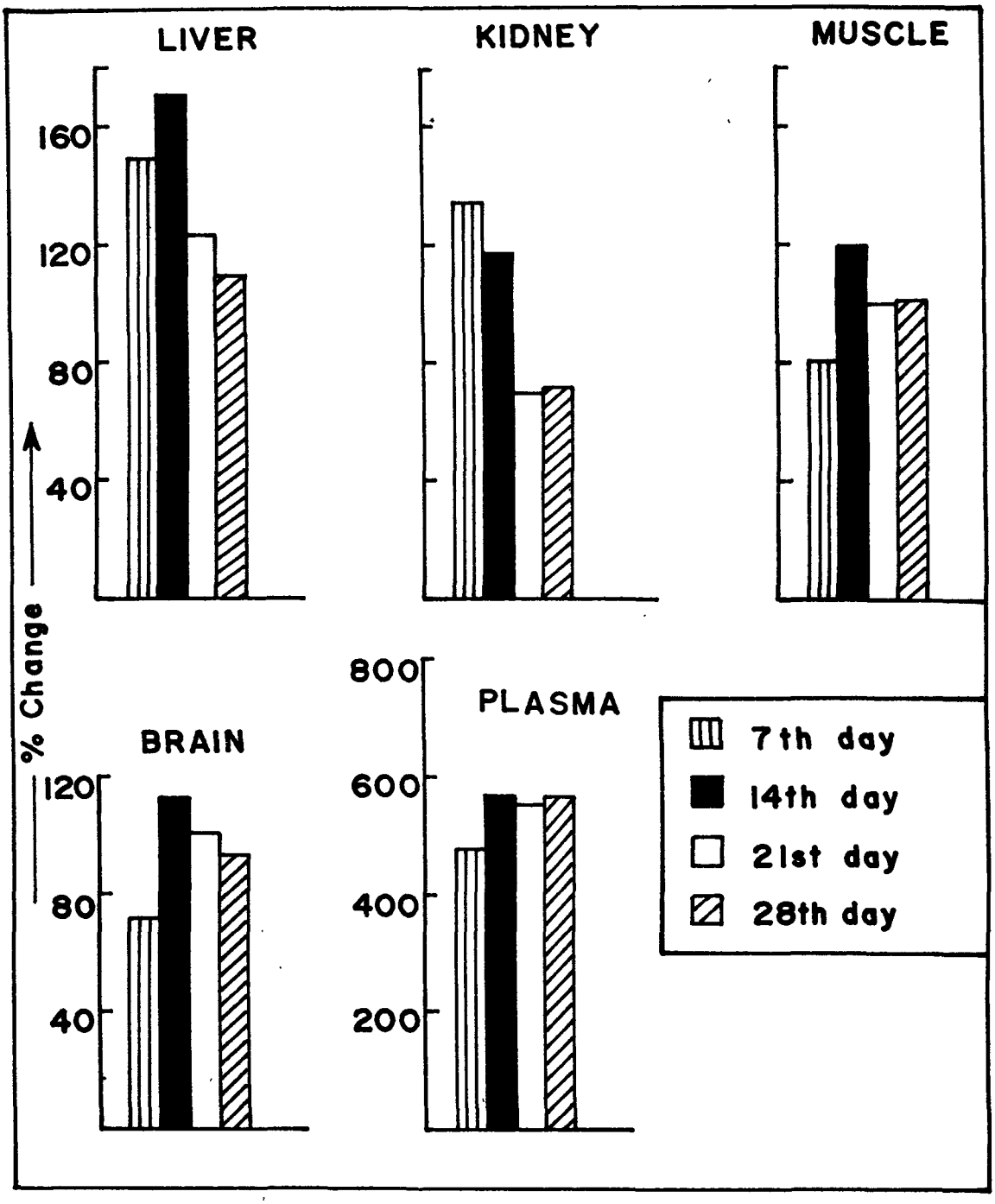


Fig. 27. % change in the concentration of urea in different tissues and in blood plasma under hyper-osmatic (250 mOsm) condition.

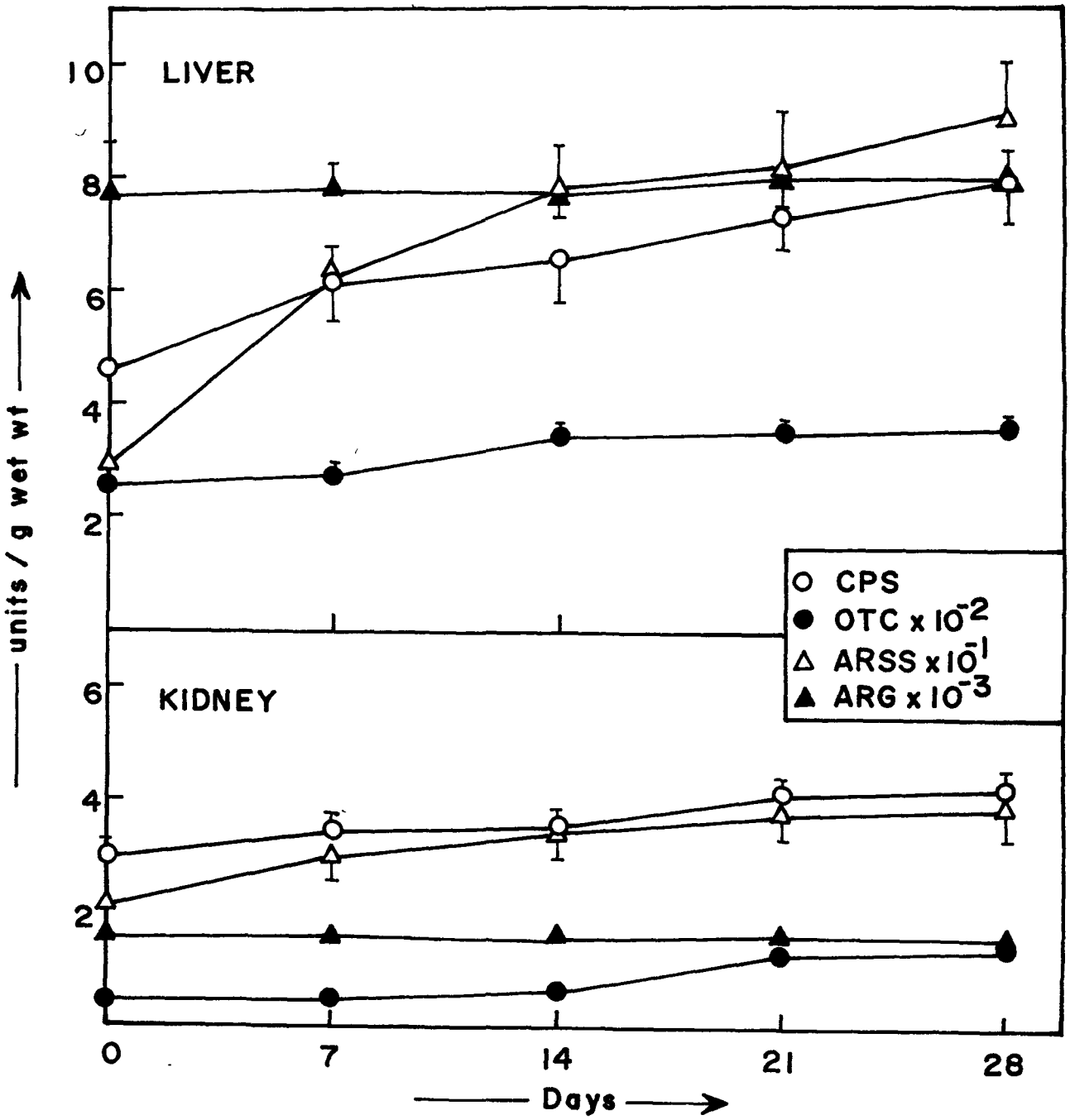


Fig. 28. Alterations in the total activity (units/g wet wt) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition.

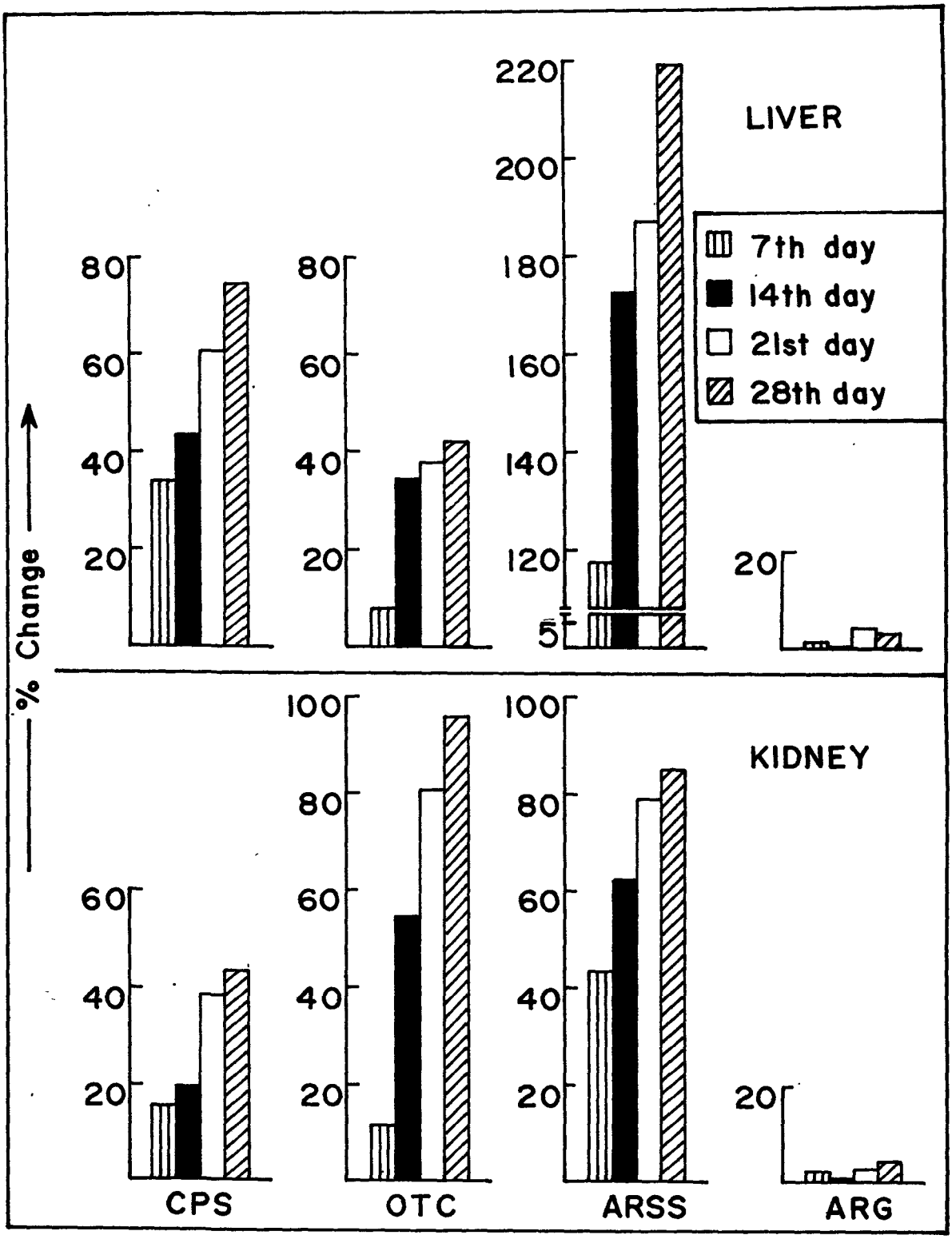


Fig. 29. % change in the total activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition.

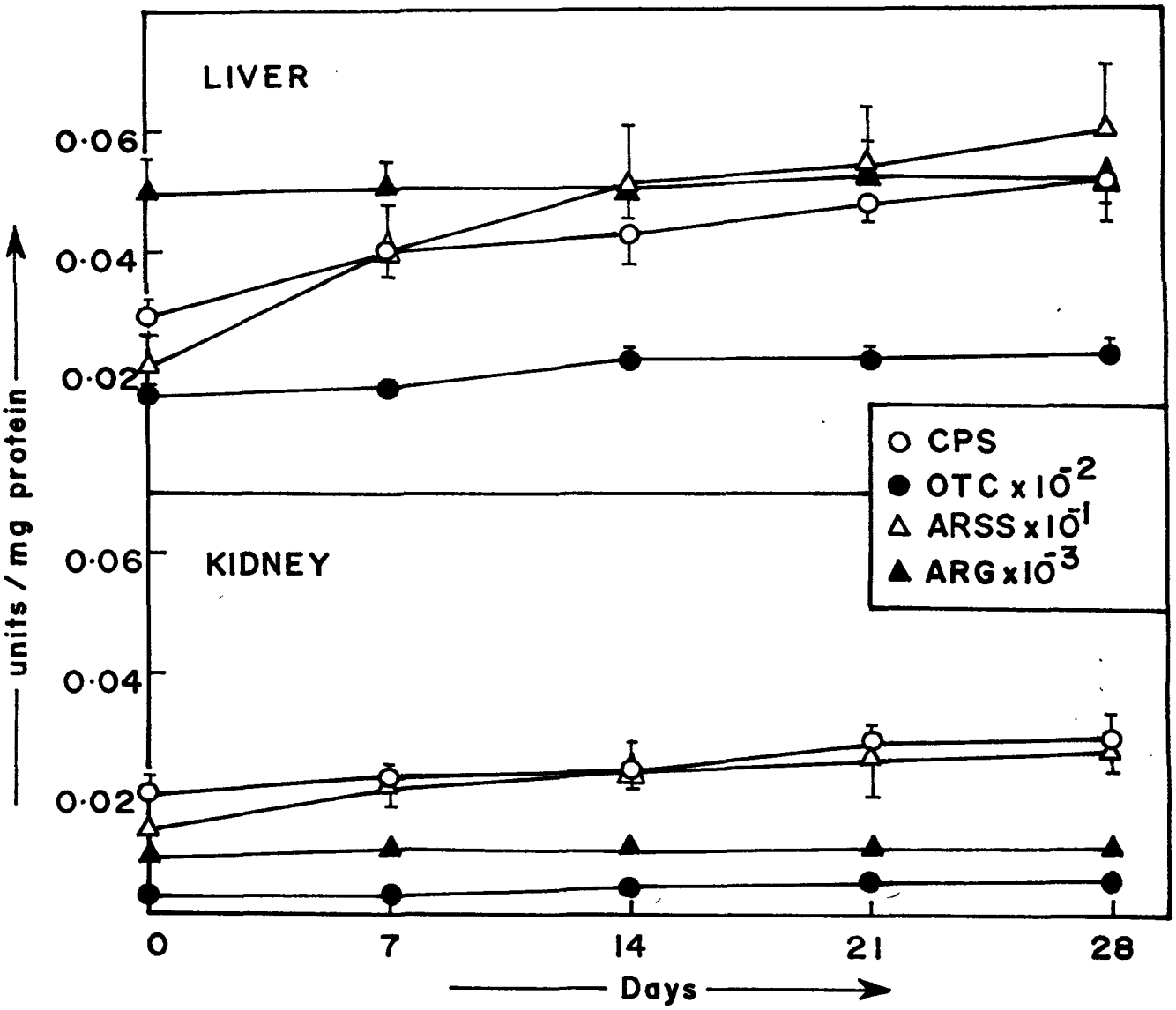


Fig. 30. Alterations in the specific activity (units/mg protein) of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition.

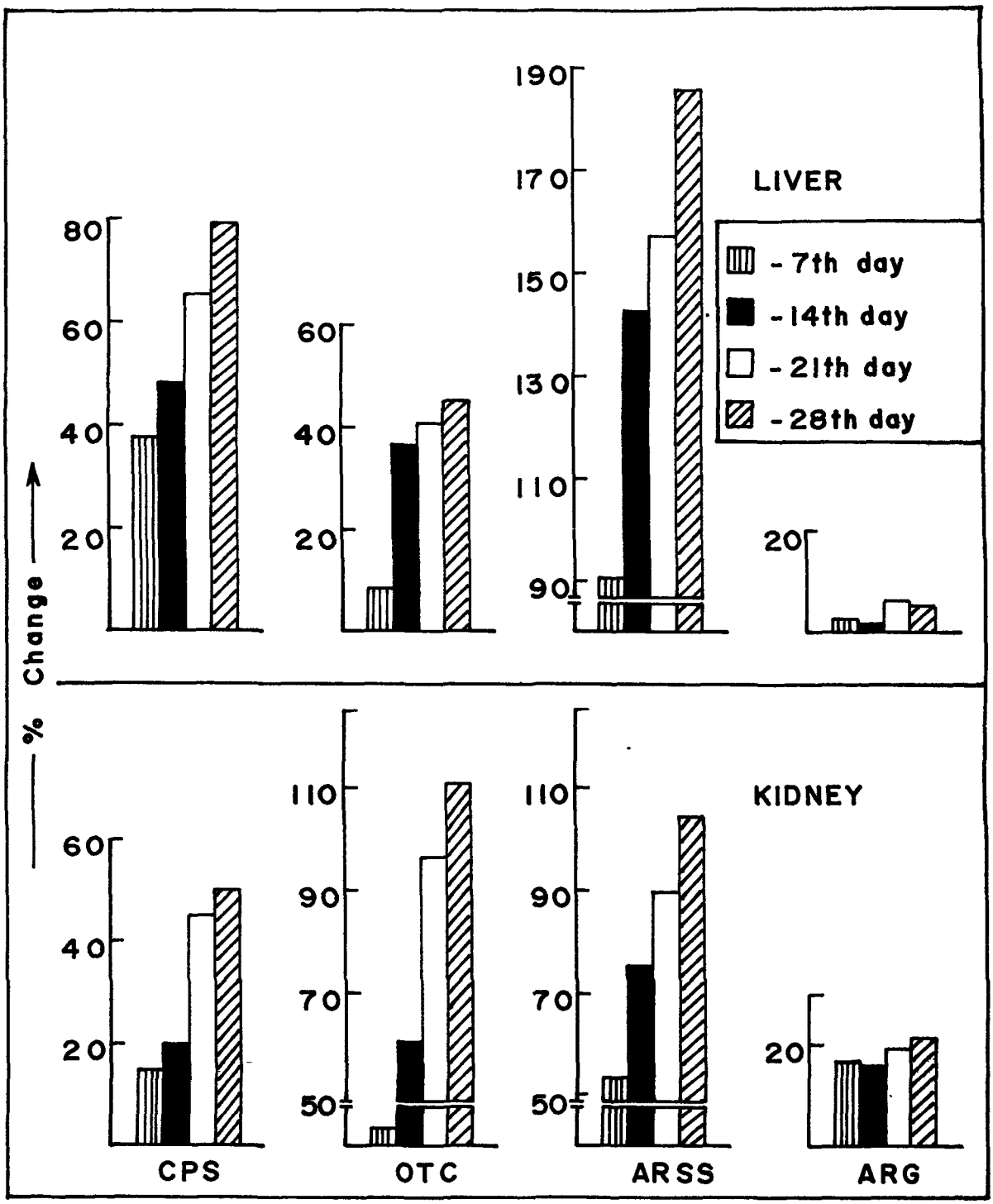


Fig. 31. % change in the specific activity of ornithine-urea cycle enzymes in the liver and kidney of **H. fossilis** under hyper-osmotic (250 mOsm) condition.

DISCUSSION

Heteropneustes fossilis tolerated a higher osmolar mannitol solution (300 mOsm) compared to other known fresh water teleosts. 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 hyper-osmolar stress. The present experiment clearly indicates that **H. fossilis** shares the ability to adapt to hyper-osmotic environment with some elasmobranchs and a number of amphibians (Gordon, 1965; Janssens & Cohen, 1968; McBean & Goldstein, 1970; Goldstein, 1972; Colley **et al**, 1972; Pang **et al**, 1977; Seiter **et al**, 1978; Schrock **et al**, 1980; Bruhman & Hanke, 1980) by possibly altering the levels of retained solutes, specially urea in its tissues. There was a marked reduction of both ammonia and urea excretion by **H. fossilis** immediately after being exposed to 250 mOsm mannitol solution. The decrease in urea excretion was noticed upto 8-10 days after which there was a marked increase of about 1-2 fold of urea excretion and continued till the end of the experiment. The suppression of ammonia excretion was between 40-50% and continued till the end of experiment. The immediate suppression in urea excretion recorded was gradually returned to normal level by 8-10 days. It then elevated to about 60% above control level between 10th to 16th day. This level was maintained thereafter. Seiter **et al** (1978) found the decrease in ammonia excretion in juveniles of **X. laevis** when treated with hyper-osmotic saline solution. The inhibition

was directly proportionate to the concentration of salt water. Treatment with iso-osmotic mannitol reduced ammonia excretion to zero after two days which continued for the following 26 days in **X. laevis**. Urea excretion in **X. laevis** also reduced to zero after 8 days. At a later stage it increased to one fifth of the control level (Funkhouser & Goldstein, 1973). However, when **X. laevis** was kept in hyper-osmotic saline solution the excretion of ammonia and urea (McBean & Goldstein, 1970) followed almost similar pattern as observed for **H. fossilis** exposed to hyper-osmotic mannitol for 28 days.

The initial decrease of urea excretion in **H. fossilis** was accompanied by the increase in tissue and plasma urea level. The peak of urea accumulation was observed on the 14th day of treatment in all tissues and blood plasma. The urea level in plasma increased by 6-7 fold. There was simultaneous decrease in ammonia excretion by the fish under hyper-osmotic stress. However, there was no accumulation of ammonia inside the body. Rather the ammonia level became lower than the control except in kidney where significant increase in ammonia level was noticed. Seiter **et al** (1978) reported decrease in ammonia content and increase in urea content of the blood of prometamorphic larvae of **X. laevis** treated with hyper-osmotic saline solution. In such situation where the availability of water was restricted, **H. fossilis** might have converted ammonia to urea and retained urea in their tissues. Thus, **H. fossilis** could avoid the toxic effects of accumulated ammonia and adjust the osmotic status with respect to the environment by

accumulating urea first by inhibiting its excretion and later by its synthesis from ammonia. Increase of urea level has been reported in plasma of carp, **Cyprinus carpio** (Gupta & Hanke, 1982a) and in anura, **Rana cancrivora** (Colley **et al**, 1972) treated with hyper- and hypo-osmotic mannitol. However, Gupta and Hanke (1982b) could not find any increase in plasma urea level in tilapia (**Sarotherodon mossambicus**) treated with 310 mOsm mannitol. The accumulation of urea in different tissues of *H. fossilis* might have maintained the osmotic equilibrium to reduce the loss of water and prevent the dehydration. This might have restricted the loss of water from muscle to only 2% which was reached by 7 days of exposure. In addition to the increase in urea, other compounds such as non-essential amino acids as reported in **X. laevis** (Schrock **et al**, 1980) and in **R. cancrivora** (Colley **et al**, 1972) might have also played some role in this fish for water retention and maintenance of osmotic equilibrium. Balinsky **et al** (1967) observed minimal excretion of nitrogenous end product due to reduction of renal function to a lower level during aestivation of **X. laevis**. During this period ammonia did not accumulate in the body fluids but urea increased to 15 times than those found in aquatic toads. Lowered rate of urine flow under hyper-osmotic condition has been suggested in **R. cancrivora** (Schmidt-Nielsen & Lee, 1962), **X. laevis** (McBean & Goldstein, 1970; Funkhouser & Goldstein, 1973) and **Scaphiopus cauchi** (McClanahan, 1972) to be one of the contributing factors for increase in tissue urea level besides its increased synthesis. A similar regulation might

have caused the decrease in renal excretion immediately after the exposure of **H. fossilis** to hyper-osmolar mannitol to help in accumulation of urea in the body. This might have been supplemented by the fresh synthesis of urea during the later periods of experimentation.

Increased excretion and tissue urea level were accompanied by the induction of the activity of the three enzymes of o-u cycle such as CPS, OTC and ARSS both in liver and kidney of **H. fossilis** under hyper-osmotic stress. Arginase activity was already very high. Therefore, the induction of the activity of other rate limiting enzymes of o-u cycle by about 2 fold must have enhanced urea synthesis from ammonia under hyper-osmolar stress by about 2 times of its normal rate of synthesis. Janssens & 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 four fold increase in liver CPS activity in **X. laevis** treated with hyper-osmotic saline solution. However, **X. laevis** kept in iso-osmotic mannitol solution induced CPS activity immediately by two fold in the liver which returned to normal on 28th day (Funkhouser & Goldstein, 1973). Increase in the activity of all the enzymes of o-u cycle was reported in the liver of **R. cancrivora** when the frog was preadapted to low concentration and then transferred to high concentration of saline solution (Colley **et al**, 1972). They suggested that increase of Na^+ion was essential for the induction of o-u cycle enzymes. However, Funkhouser and Goldstein (1973) could not find the correlation between the increase in Na^+ion concentration and

increasing activity of o-u cycle enzymes in **X. laevis.**

The decrease in ammonia excretion and accumulation observed in **H. fossilis** under osmotic stress indicated increased urea synthesis from ammonia during the treatment. McBean and Goldstein (1970) showed much more rapid initial increase in urea production (in 24 hrs) than the increase in enzymic activity (in 4 days) in **X. laevis** exposed to hyper-osmolar solution. Using immunological methods, they showed that the increased CPS activity probably resulted from an increase in the concentration of enzyme protein, and not from a conversion of pre-existing inactive, but immunologically similar protein into a catalyticatically active form. Another suggestion was made that the initial increase of urea synthesis in **X. laevis** in hyper-osmotic saline solution was probably due to the increase in ammonia level in tissues which was noticed after one day of treatment. In the case of **H. fossilis** we found significant increase in ammonia level only in kidney and to some extent in brain but not in liver and other tissues studied after 7 days of treatment. However, we did not check the level of ammonia within first 24 or 48 hrs. Ammonia concentration in different tissues of **H. fossilis** might have enhanced within 24 hrs as was reported in **X. laevis**, immediately after treatment with hyper-osmolar solution, and then that might have returned to normal after 2-3 days. The increase in ammonia level in the kidney of mannitol treated **H. fossilis** was observed till the end of the experiment. During higher ambient ammonia condition (Chapter III) and when the fish was kept outside water

(Chapter IV), higher increase in ammonia level along with induction of o-u cycle enzymes activity were also seen in kidney than liver. However, under hyper-osmotic condition the increase in ammonia level which was noticed in kidney was not as much as observed during hyper-ammonia and dehydration stress. The relative increase in o-u cycle enzymes activity in kidney was lower than liver. The increase in o-u cycle enzymes activity in the kidney might be correlated with the increase in ammonia level in the tissue rather than the water restriction. However, in liver both increase in ammonia level and water restriction might have induced the activity of o-u cycle enzymes. It is difficult to conclude definitely at present with the available data about whether ammonia, Na^+ or water restriction was responsible for the induction of o-u cycle enzymes in liver and kidney of **H. fossilis** subjected to hyper-osmolar stress.

The capacity of increased urea synthesis and accumulation during osmotic stress observed in **H. fossilis**, a fresh water air-breathing teleost, was uncommon among freshwater 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 have retained the "ureosmotic" character to synthesise and retain urea inside the body for osmotic balance.

GENERAL DISCUSSION

GENERAL DISCUSSION

Ammonia was found to be the major excretory product in all the five species of air-breathing teleosts studied (**Amphipnous cuchia, Clarias batrachus, Heteropneustes fossilis, Anabas testudineus** and **Channa punctatus**) in their aquatic medium like other freshwater teleosts.The major part of both ammonia and urea were also excreted through extra-renal sources as studied in **H. fossilis**. However, excretion and tissue level of urea observed in all the species (except in **C. punctatus**) were higher than in the freshwater teleosts reported earlier (Chapter I). Active synthesis of urea was evident due to the existence of functional o-u cycle in four out of the five species of air-breathing teleosts studied (Chapter II). The uricolytic pathway, which has been suggested as the major source of urea formation in freshwater teleosts (Goldstein & Forster, 1965; Cvancara, 1969a; Vellas & Serfaty, 1974) was also active in **H. fossilis**. However, formation of urea was more through o-u cycle than uricolytic pathway calculated from the relative activity of the enzymes (Chapter II; Saha & Ratha, 1986). Thus the 'deletion' hypothesis (Brown & Cohen, 1960; Cohen & Brown, 1960) suggested for freshwater teleosts, in general, will not be applicable at least in the case of air-breathing freshwater teleosts due to the existence of functional o-u cycle.

The o-u cycle in air-breathing teleosts was found to be regulated by various environmental factors such as higher ambient ammonia, aerial exposure and osmotic stress. These environmental conditions resulted in accumulation of ammonia and enhancement of urea synthesis by inducing the activity of o-u cycle enzymes in **H. fossilis.** These results support the view of Watts and Watts (1966) that the same enzyme system could serve different functions depending upon the need of the fish to adjust suitably to various environmental conditions. The o-u cycle in **H. fossilis** was induced at one hand to detoxify the accumulated ammonia under hyper-ammonia stress giving the fish a better tolerance for higher ambient ammonia and during aerial exposure when ammonia accumulated due to lack of its diffusion. At other hand excess synthesis of urea through induction of o-u cycle was also observed during hyper-osmolar treatment. Urea was accumulated in the tissues probably for osmoregulation in **H. fossilis** under hyper-osmolar stress. The presence of functionally active o-u cycle and its regulation under different environmental conditions in at least one species of air-breathing teleost (**H. fossilis**) is a unique observation for the fresh-water teleosts.

A shift towards ureotelism under hyper-ammonia stress (Chapter III) was found along with the induction of o-u cycle enzymes both in liver and kidney of **H. fossilis** probably to reduce ammonia toxicity. Ammonia level significantly increased in different tissues immediately after treatment

with higher concentration of NH_4Cl upto certain level followed by increase in both tissue level and excretion of urea. Accumulation of excess urea in the tissues might be for the osmotic balance as the ambient ionic concentration was quite high. Such adaptive modifications under ionic stress has been reported in some teleosts and aquatic amphibia, **Xenopus laevis** (Dêpêche & Schoffeniels, 1975; Gupta & Hanke, 1982 a,b; McBean & Goldstein, 1967, 1970; Janssens, 1972). Ammonia detoxification by some other pathways such as glutamate ⟶ glutamine synthesis through GDH (NADH dependent) and glutamine synthetase activity and uricolytic pathway cannot be ruled out at this stage without studying them under similar conditions. However, it is definite that a major part of accumulated ammonia were converted to urea via o-u cycle under higher ambient ammonia when all the key enzymes of the cycle got induced. Probably due to the presence of this detoxifying mechanism (might be along with other detoxifying mechanism(s)) **H. fossilis** could tolerate such a high ambient ammonia without any deleterious affect.

The shift towards ureotelism along with induction of o-u cycle which occurred during aerial exposure in freshwater air-breathing teleost, **H. fossilis** (present study) and probable shift to ureotelism reported in **Anabas scandens** and **Channa gachua** (Ramaswamy & Reddy, 1983) are significant findings relevant in discussion of the possible evolutionary adaptive value of ureotelism in the lower vertebrates. Such

shift to ureotelism have also been reported in other amphibious fishes such as **Periophthalmus sobrinus** (Gordon **et al**, 1969), **Sicyases sanguineus** (Gordon **et al**, 1970), **Periophthalmus cantonensis** (Gordon **et al**, 1978) and in amphibians (Balinsky **et al**, 1967; Janssens & Cohen, 1968; Balinsky, 1981). Thomson (1971) stated that ureotelism was increased in lungfishes whenever they lowered ventilation rates of their gills and increased aerial respiration.The cause of such shifts might be either the initiation of aestivation or lower aquatic oxygen or higher aquatic carbondioxide tension. He attributed the shift in nitrogen metabolism to the avoidance of possible toxaemia, since most ammonia excretion by fishes occurred through the gills. There was an immediate suppression of ammonia excretion by 75% which reduced further to 85% when **H. fossilis** was emersed. This resulted in immediate increase in tissue ammonia level followed by the induction of o-u cycle enzymes and increase in tissue urea level. Increased ammonia level in different tissues might have induced the activity of o-u cycle enzymes in liver and kidney. Thus a shift towards ureotelism with enhanced accumulation and excretion of urea and no or very less accumulation of ammonia at later stages of aerial exposure was noticed in **H. fossilis.** The occurrence of the shift towards ureotelism probably exist in all the amphibious fishes. If it is so, this mechanism has been phylogenetically widely distributed among living fishes and might have been one of the pre-existing physiological properties which played an important role in migration of

animals from water to land.

The functioning of o-u cycle and accumulation of urea also played a significant role for maintaining the osmotic balance in **H. fossilis** treated with hyper-osmotic mannitol (Chapter V). Urea has been known to play a significant role in osmoregulation (Alexander **et al**, 1968; Colley **et al**, 1972; Goldstein, 1972; Pang **et al**, 1977; Balinsky, 1981) as it can be uniformly distributed throughout the body water due to its uncharged hydrophilic nature. Possibly, therefore, urea excretion in **H. fossilis** was suppressed and accumulation increased **in vivo** immediately after transfer to hyper-osmotic medium. All the key enzymes of o-u cycle also got induced under osmotic stress both in the liver and kidney of **H. fossilis.** Reappearance of urea excretion at a higher rate from 10th day onwards of treatment might be due to more synthesis of urea via o-u cycle. Excretion rate of ammonia remained at a lower level than the control level althrough the period of treatment. In addition to the accumulated urea some amino acids and ions might have contributed to maintain the osmotic balance in **H. fossilis.** However, this needs further investigation.

Both in ambient hyper-ammonia (Chapter III) and dehydration stress (Chapter IV) accumulation of ammonia took place in all the tissues studied resulting in the induction of o-u cycle enzymes in liver and kidney of **H. fossilis.** The same effect cannot be ruled out under hyper-osmotic stress

for the induction of o-u cycle enzymes although no increase of ammonia was noticed in any tissues studied only after 7 days of treatment except in kidney. However, there might be an initial increase in ammonia level immediately after treatment inducing the activity of o-u cycle enzymes and thus reducing the ammonia level.These observations strongly suggest that increase in tissue ammonia level was one of the major reasons for induction of o-u cycle enzymes resulting in the shift towards ureotelism. In addition to this, the loss of water from the tissues, though very less but significant, might have contributed to this induction process. A diagrammatic model has been proposed (Fig. 32) to illustrate the mechanism of induction of o-u cycle in freshwater air-breathing teleosts under the three environmental conditions studied.

The observations discussed above suggest that freshwater air-breathing teleosts are unique in their adaptive nature of ammonia metabolism. This has given the capacity to these fish to tolerate at least higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Thus, freshwater air-breathing teleosts can be put into all the three classes i.e. ureogenic - for having the full complement of o-u cycle enzymes, ureotelic - for having the capacity to synthesise through o-u cycle and excrete sufficient urea and ureosmotic - for having the capacity to produce and accumulate urea for maintaining osmotic balance as proposed by Huggins **et al** (1969)

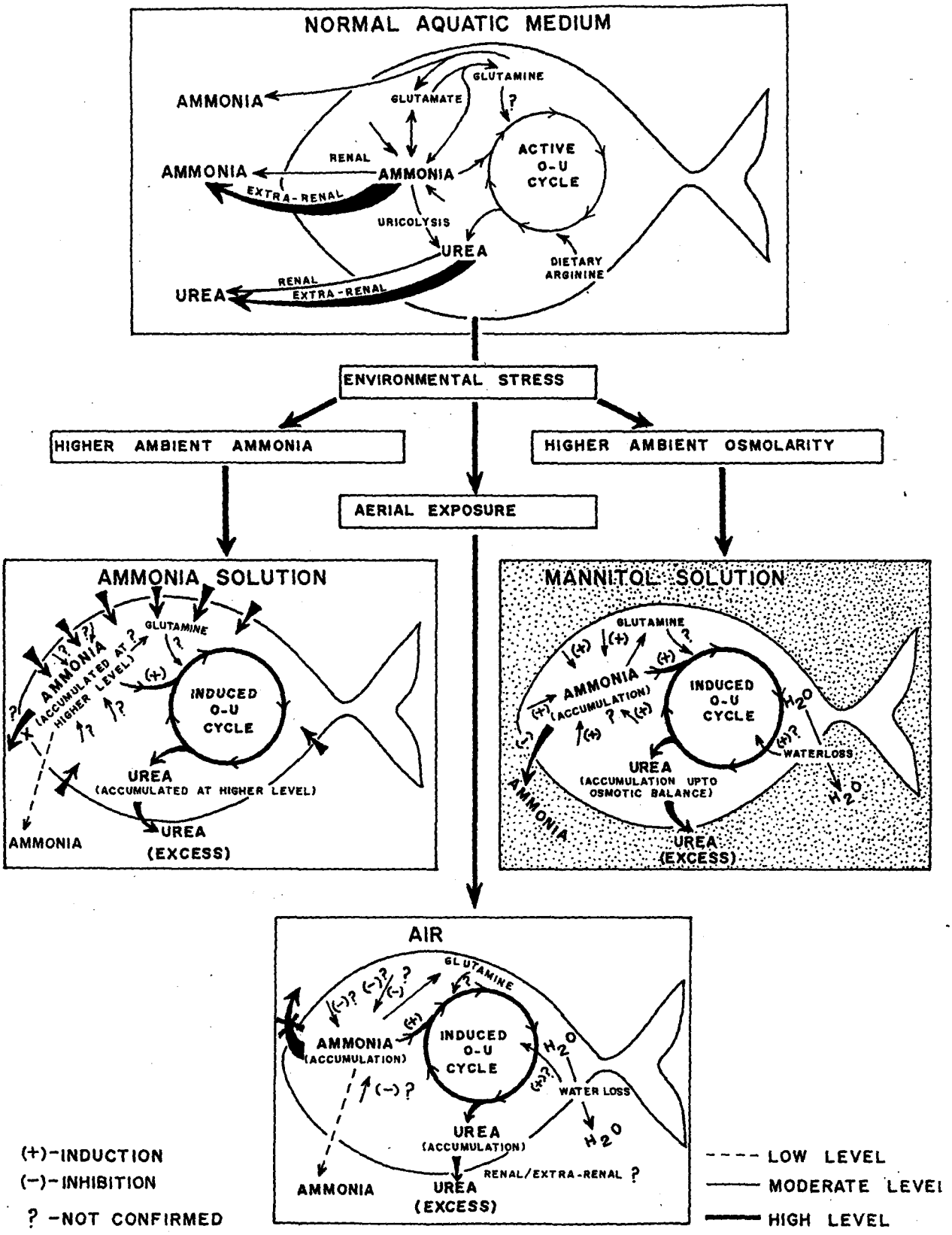


Fig.32. A diagramatic model for regulation of ureogenesis under environmental stress in *freshwater air-breathing teleosts.*

The presence of a functional and regulatory o-u cycle indicates that this important pathway still continues to persist in some of the freshwater teleosts necessitating an amendment to the 'deletion' hypothesis (Brown & Cohen, 1960; Cohen & Brown, 1960). We tend to agree with the suggestion of Watts and Watts (1966) that there is a need for some more sophisticated approach to the problem of evolution of the o-u cycle enzymes with relation to the evolution of terrestriality in vertebrates.

The present findings further suggest that the freshwater air-breathing teleosts might be either relatively primitive to the present day freshwater teleosts and the genes for o-u cycle enzymes might have been repressed at at a later stage of evolution. Alternatively it could be possible that these genes might have been derepressed as a secondary modification in these fish for their ability to adapt to higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Therefore, freshwater air-breathing teleosts have a separate physiological status and should be reclassified as an independent group among the freshwater teleosts. Studies on these fish might connect many missing links in the evolutionary story of regulation of nitrogen metabolism during teleostean evolution.

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