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

N. Saha and L. Das
Biochemical Adaptation Laboratory, Department of Zoology, North-Eastern Hill University, Shillong - 793022, India (Fax: (+91) 364-250076)

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Abstract

The pattern of changes of activity of the urea cycle enzymes and the rate of urea-N excretion were studied in the perfused liver of an Indian air-breathing ureogenic walking catfish, *Clarias batrachus*. The liver was perfused with different concentrations of NH_4Cl for a period of 60 min to determine the role of ammonia for stimulation of hepatic ureogenesis and the threshold level of ammonia loading needed to cause such stimulation. Both the urea-N excretion and the ammonia uptake by the perfused liver were found to be a saturable process. Ammonia accumulated significantly in the liver infused with 1.25 μmoles g liver $^{-1}$ min^{-1} of NH_4Cl, followed by a maximum accumulation of about 28.5 μmoles g wet wt^{-1} with the infusion of 5.08 μmoles g liver^{-1} min^{-1}. The Vmax of the urea-N excretion (0.47 μmol g liver^{-1} min^{-1}) was obtained with the addition of 5.08 μmoles g liver^{-1} min^{-1} of NH_4Cl. Both the tissue and the specific activity of the urea cycle enzymes, except ornithine transcarbamylase and arginase, were stimulated significantly with the infusion of either 1.25 or 5.08 μmoles g liver^{-1} min^{-1} of NH_4Cl. Maximum stimulation of tissue activity of carbamoyl phosphate synthetase (about 120%) was seen with the infusion of 5.08 μmol g liver^{-1} min^{-1}, and for argininosuccinate synthetase (about 135%), and argininosuccinate lyase (about 50%) with the infusion of 10.81 μmol g liver^{-1} min^{-1} of NH_4Cl. Higher accumulation of ammonia of about 10–15 μmol g wet wt^{-1} from the physiological level in the perfused liver while infusing with NH_4Cl was suggested to be one of the major causes of stimulation of ureogenesis. The presence of such physiological adaptive strategy is probably necessary in this unique group of air-breathing walking catfish to survive under hyper-ammonia stress in their normal habitat or while living outside water or while burrowing inside mud.

Introduction

Nitrogen metabolism is considered as one of the most sensitive physiological systems showing adaptive changes in response to environmental variations. Accordingly, the nature of nitrogen excretory products in animals has changed with the evolution of vertebrates from water to the land habitat (Campell 1991; Wright 1995). Most teleostean fishes excrete ammonia as the major nitrogenous end product in response to their aquatic habitat (Randall and Wright 1987; Campbell 1991; Wood 1993; Saha and Ratha 1998). However, in special circumstances such as high ambient ammonia or aerial exposure, fish can hardly excrete ammonia, and toxic ammonia is concentrated in blood and body tissues. Fishes are generally known to tolerate relatively higher accumulation of ammonia than mammals. Plasma total ammonia ($NH_3 + NH_4^+$) normally remains between 0.05 to 2 mM in most teleost fishes (Campbell and Anderson 1991; Wood 1993). In contrast, blood ammonia levels greater than 0.05 mM can be toxic to the central nervous system of most mammals (Meijer et al. 1990). It is, therefore, interesting to study the different mechanism(s) of management of accumulated ammonia especially in

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

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

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

Materials and methods

Animals

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

Liver perfusion technique

The fish were decapitated before operation for liver perfusion. Livers were perfused via the portal vein in a non-circulating manner with haemoglobin-free media as used by French et al. (1981) and with certain modifications made by Saha et al. (1995). The isotonic media (265 mOsmol l^{-1}, determined by freezing point depression method) contained 119 mM NaCl, 5 mM KCl, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4$ and 1.25 mM $CaCl_2$ as a basic solution. The medium also contained 5 mM L-glucose and 2 mM L-ornithine. Different concentrations of NH_4Cl (0.05–2 mM) were infused along with the perfusion media to study the effect of ammonia on the urea cycle enzymes activity, its accumulation rate and also its conversion to urea in the perfused liver. The medium was gassed with O_2/CO_2 (99:1, v/v) before infusing into the liver at a flow rate of 4–5 ml g liver^{-1} min^{-1}. The pH of the medium was always maintained at 7.5 after gassing. The temperature of the medium was 30 °C. The effluent coming out of the perfused liver was collected through a cannula inserted at the superior vena cava at 2 min intervals for analysis of ammonia and urea-N.

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

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

Estimations

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

Enzyme assay

A ten percent homogenate (w/v) of the liver tissue was prepared in 0.1% cetyltrimethyl ammonium bromide (CTB) for assaying the activities of the urea cycle enzymes (Brown and Cohen 1959). The homogenate was centrifuged at 600× g at 0 ± 2 °C for 15 min. The supernatant was used for enzyme assays. The five enzymes of the urea cycle such as carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) were assayed following the method mentioned earlier in detail (Saha et al. 1995). This time, however, an extra 1 mM of uridine-5′-triphosphate (UTP) was added in the reaction mixture for the assay of urea synthesis-related CPS activity mainly to inhibit the pyrimidine synthesis-related CPS II activity which might interfere in the assay method. It should be noted that the assay method used here for CPS activity does not distinguish between the two different forms of urea synthesis-related enzymes namely CPS I (ammonia- and N-acetyl-L-glutamate-dependent, mitochondrial) and CPS III (glutamine- and N-acetyl-L-glutamate-dependent, mitochondrial). The reaction for all the enzymes was stopped by adding 10% PCA in 1:0.5 ratio after a specific time of reaction, and centrifuged to precipitate out the protein. Citrulline formed in the case of CPS and OTC, citrulline used in the case of ASS, and urea formed in the case of ASL and ARG were measured colorimetrically in the supernatant (Moore and Kauffman 1970) and expressed as enzyme activity. All the enzyme assays were carried out at 30 °C. One unit of enzyme activity was defined as that amount that catalyzed 1 μmol of product formed or substrate used per h at 30 °C. The enzyme activity was expressed as both tissue (U g wet wt of tissue^{-1}) and specific (U mg protein^{-1}) activity. Protein was estimated following the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Data collected from three to four replicates were statistically analysed and presented as mean ± SEM. Comparison of the paired mean values was made using Student's *t*-test (Croxton et al. 1982) and $P > 0.05$ was taken as non-significant.

Chemicals

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

Results

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

The changes in ammonia level in the liver perfused with different concentrations of NH_4Cl are shown in Table 1. No significant increase of ammonia level in the perfused liver was observed until increased the addition of 1.25 μmol g liver^{-1} min^{-1} of NH_4Cl. The ammonia level in the liver raised from 7.64 to about 28.5 μmol g wet wt^{-1} of liver with the infusion of 5.08 μmol g liver^{-1} min^{-1} of NH_4Cl, followed by no further increase at a higher rate of infusion.

Formation of urea-N in the perfused liver

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

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

Ammonia infusion rate (μmol g liver^{-1} min^{-1})	Ammonia level in the perfused liver (μmol g wet wt^{-1})	p
0 (control)	7.64 $\pm$ 0.75	
0.4 $\pm$ 0.04	8.84 $\pm$ 0.66 (16)	N.S.
0.85 $\pm$ 0.09	10.45 $\pm$ 0.82 (37)	N.S.
1.25 $\pm$ 0.11	14.50 $\pm$ 1.02 (90)	<0.05
2.32 $\pm$ 0.26	20.05 $\pm$ 1.85 (162)	<0.001
5.08 $\pm$ 0.38	28.45 $\pm$ 2.24 (272)	<0.001
10.81 $\pm$ 0.81	28.65 $\pm$ 2.55 (275)	<0.001

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

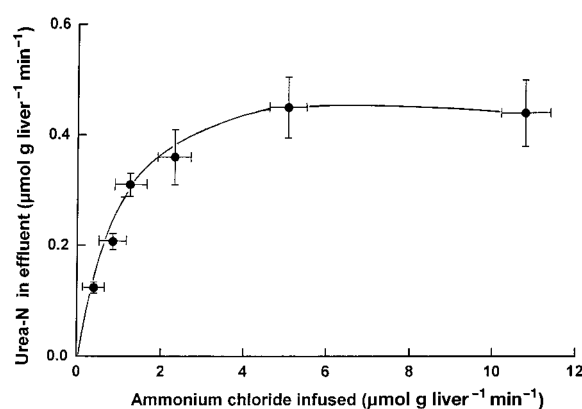

Figure 1. Urea-N excretion (μmol g liver^{-1} min^{-1}) in the effluent by the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl. Values were obtained between 50 and 60 min of infusion when the urea-N excretion already reached a steady state and are plotted as mean $\pm$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

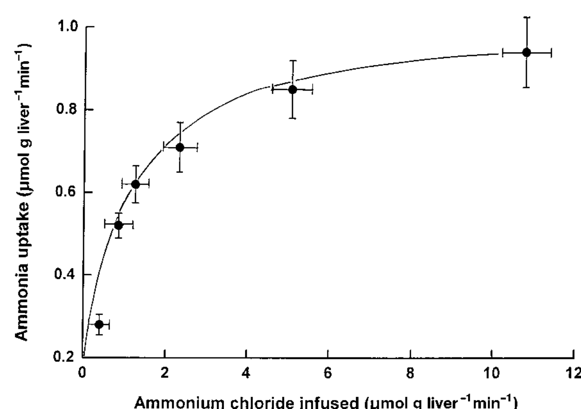

Figure 2. Ammonia uptake (μmol g liver^{-1} min^{-1}) by the perfused liver of *C. batrachus* infused with different concentrations of NH_4Cl. Values were obtained between 50 and 60 min of infusion when the ammoina uptake already reached a steady state and are plotted as mean $\pm$ SEM (n = 3–4). NH_4Cl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

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

Uptake of ammonia by the perfused liver

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

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

Ammonia infusion rate	CPS		OTC		ASS		ASL		ARG	
(μmol g liver^{-1} min^{-1})	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific	Tissue	Specific
0 (control)	3.92 ± 0.31	0.048 ± 0.005	190.4 ± 11.1	2.33 ± 0.45	65.8 ± 6.2	0.8 ± 0.1	100.3 ± 7.2	1.23 ± 0.14	4035 ± 212	49.4 ± 5.4
0.85 ± 0.09	4.29 ± 0.44	0.05 ± 0.004	187.6 ± 15.2	2.16 ± 0.32	74.3 ± 4.5	0.8 ± 0.08	97.3 ± 6.5	1.12 ± 0.08	4132 ± 224	47.7 ± 4.2
1.25 ± 0.11	5.68 ± 0.56	0.067 ± 0.004	218.2 ± 12.2	2.57 ± 0.28	130.3 ± 6.1^a	1.5 ± 0.7^a	122.4 ± 11.4	1.44 ± 0.1	3945 ± 191	46.4 ± 5.7
2.32 ± 0.26	6.45 ± 0.55^a	0.08 ± 0.006^a	212.6 ± 14.5	2.54 ± 0.44	152.4 ± 7.2^b	1.9 ± 0.1^b	147.6 ± 8.2^a	1.85 ± 0.1^a	4345 ± 191	54.1 ± 6.1
5.08 ± 0.38	8.68 ± 0.71^b	0.11 ± 0.02^b	212.6 ± 14.5	2.78 ± 0.34	148.5 ± 8.5^b	1.9 ± 0.1^c	140.8 ± 6.2^a	1.84 ± 0.1^a	4402 ± 224	57.5 ± 4.8
10.81 ± 0.81	8.59 ± 0.6^b	0.11 ± 0.6^b	219.3 ± 11.2	2.70 ± 0.22	153.6 ± 6.4^c	1.9 ± 0.1^c	151.3 ± 6.4^a	1.88 ± 0.1^a	4378 ± 237	54.5 ± 4.2

1 unit of enzyme activity was defined as the amount that catalyzed 1 μmol of product formed or substrate used per h at 30 °C; ^{a}, ^{b} and ^{c}: p values significant at <0.05, <0.01 and <0.005, respectively; CPS – carbamoyl phosphate synthetase, OTC – ornithine transcarbamylase, ASS – argininosuccinate synthetase, ASL – argininosuccinate lyase and ARG – arginase.

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

Pattern of changes of both the tissue and the specific activity of the urea cycle enzymes in the liver infused with different concentrations of NH_4Cl are shown in Table 2. At lower rates of ammonium chloride infusion, that is, up to 1.25 μmol g liver^{-1} min^{-1}, no significant increase of the tissue activity of any of the urea cycle enzymes were observed except for ASS, the activity of which increased significantly ($p < 0.05$) even at 1.25 μmol g liver^{-1} min^{-1} of NH_4Cl infusion. Significant stimulation of tissue activities of other urea cycle enzymes were seen from 2.32 μmol g liver^{-1} min^{-1} of infusion. The activity of CPS was stimulated maximally from 3.92 $\pm$ 0.31 to 8.68 $\pm$ 0.71 U g wet wt^{-1} with the addition of 5.08 μmol g liver^{-1} min^{-1} of NH_4Cl, and for ASS and ASL, from 65.8 $\pm$ 6.2 to 153.6 $\pm$ 6.4 and from 100.3 $\pm$ 7.2 to 151.3 $\pm$ 6.9 U g wet wt^{-1}, respectively, with the addition of 10.81 μmol g liver^{-1} min^{-1} of NH_4Cl. The activities of CPS, ASS and ASL increased maximally by 121%, 133% and 51%, respectively. However, the tissue activities of OTC and ARG did not show any significant change in all the concentrations of NH_4Cl infused.

Significant stimulation of the specific activity was also observed for ASS from 1.25 μmol g liver^{-1} min^{-1} of NH_4Cl addition, and for CPS and ASL, it was from 2.32 μmol g liver^{-1} min^{-1} onwards (Table 2). The specific activities rose maximally from 0.048 $\pm$ 0.005 to 0.11 $\pm$ 0.02 for CPS, 0.81 $\pm$ 0.1 to 1.94 $\pm$ 0.12 for ASS and from 1.23 $\pm$ 0.14 to 1.88 $\pm$ 0.09 U mg protein^{-1} for ASL. The specific activities of CPS, ASS and ASL increased maximally by 129%, 140% and 53%, respectively. Like the tissue activity, the specific activities of OTC and ARG also did not change in any of the concentrations of NH_4Cl infused.

Discussion

The liver of *C. batrachus* has been reported to be the major ureogenic tissue for conversion of ammonia to urea (Saha and Ratha 1989; Saha et al. 1999a) as in other ureotelic mammals and amphibia. Thus, instead of exposing the whole fish in higher ambient ammonia, the liver of this walking catfish was directly loaded with different concentrations of NH_4Cl for 60 min by the perfusion technique to find out its role in the detoxification of ammonia. The results obtained in the present study clearly indicated that the higher accumulation of ammonia could be one of the major factors for the stimulation of ureogenesis at least in the liver of this catfish. No significant increase in the ammonia level was observed in the perfused liver at a lower rate of NH_4Cl infusion (up to 0.85 μmol g liver^{-1} min^{-1}) for 60 min (Table 1). The excretion of ammonia into the effluent while infusing NH_4Cl at a lower rate was also found to be less (about 30% out of the total infused), indicating that most of the ammonia infused into the liver was either converted to urea-N and/or to some non-essential amino acids (not determined in the present study). Further, out of the total ammonia taken up by the perfused liver at a lower rate (up to 1.25 μmol g liver^{-1} min^{-1}) only about 40 to 50% was converted to urea-N causing any significant change of activity of the urea cycle enzymes except for ASS (Table 2). The normal physiological level of activity of the urea cycle enzymes in the liver of this ureogenic teleost, possibly along with other detoxification pathways, might be sufficient to detoxify most of the ammonia that was taken up by the perfused liver causing any change of ammonia level *in vivo*. Recently, the conversion of some part of accumulated ammonia to various non-essential amino acids under high ammonia load in the perfused liver has also been reported in this walking catfish (Saha et al. 1999b). The maximum accumulation of ammonia of about 28 μmol g wet wt^{-1} of tissue was observed with 5.08 μmol g liver^{-1} min^{-1} of NH_4Cl infusion with no further increase in ammonia level in the liver at a higher rate of ammonia addition (Table 1). It is, therefore, evident from this perfusion experiment that the accumulation of ammonia in the liver is a saturable process.

Both the urea-N excretion rate (Figure 1) and the rate of uptake of ammonia (Figure 2) by the perfused liver were also found to be a saturable process. The Vmax of urea-N excretion (0.47 μmol g liver^{-1} min^{-1}) was obtained with the addition of 5.08 μmol g liver^{-1} min^{-1} of NH_4Cl. At a still higher rate of ammonia addition the urea-N excretion rate became independent of ammonia (substrate) load, while the Vmax of ammonia uptake (1.34 μmol g liver^{-1} min^{-1}) was obtained at a still higher rate of NH_4Cl addition (10.81 μmol g liver^{-1} min^{-1}). It may so happen that initially at a lower ammonia load, it is the induced urea cycle which is mainly responsible for the detoxification of ammonia, whereas, at a higher ammonia load other detoxification pathways are also actively involved in the detoxification process in addi-

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

Significant stimulation of activity (both tissue and specific) of the urea cycle enzymes except for OTC and ARG was seen with the addition of NH_4Cl at the rate of 2.32 μmol g liver^{-1} min^{-1}, which was accompanied by the rise of tissue ammonia level from 7.64 ± 0.75 to 20.05 ± 1.85 μmol g wet wt^{-1} within 60 min of infusion (Tables 1 and 2). Although the level of ammonia in the liver increased further at a still higher rate of NH_4Cl infusion, it did not cause any change of activity of the urea cycle enzymes except for CPS, whose activity increased till the addition of 5.08 μmol g liver^{-1} min^{-1} of NH_4Cl (Table 2). Therefore, these results indicated that an increase of 10–15 μmol g wet wt^{-1} of hepatic ammonia from its physiological level is possibly necessary to achieve any significant and/or maximum stimulation of activity of the urea cycle enzymes in this walking catfish. Whereas, in the case of singhi catfish an increase of 3–5 μmol g wet wt^{-1} in the hepatic tissue was sufficient to cause any significant change of activity of the urea cycle enzymes (Saha et al. 1995). In the case of ureotelic alkaline-lake Magadi tilapia, a 3 fold increase of urea excretion was reported on exposure to 0.5 mM NH_4Cl, without causing any significant induction of the urea cycle and some other ammonia detoxifying enzymes (Wood et al. 1989; Walsh et al. 1993). In this case, possibly the accumulation of ammonia *in vivo* might not have reached the threshold level to cause any significant induction of the urea cycle enzymes activity, as has been noticed in the liver of *C. batrachus* infused with a lower rate of NH_4Cl. The increase in the rate of urea-N by the perfused liver of the walking catfish at a lower rate of NH_4Cl infusion and by the tilapia (whole fish) exposed to NH_4Cl were mainly because of high physiological activity of the urea cycle enzymes. In contrast, when a purely ammoniotelic teleost, the largemouth bass (*Micropterus salmoides*), where the levels of activity of various urea cycle enzymes are very low, was exposed to 0.25 and 1 mM of NH_4Cl, neither the change of urea-N excretion nor any change of urea cycle enzymes activity was observed (Kong et al. 1998).

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

Increase of pH in the external water was reported to stimulate the ureogenesis in Lahontan cutthroat, *Onchorhynchus clarki henshawi* (Wilkie et al. 1993), in tilapia, *Oreochromis nilotica* (Wright 1993), and in rainbow trout, *Onchorhynchus mykiss* (Wilkie and Wood 1991). However, in the present study, while perfusing the liver of walking catfish, the pH of the media containing different concentrations of NH_4Cl were kept constant (pH 7.5). Therefore, the stimulation of ureogenesis under higher ammonia load noticed herein cannot be considered the effect of pH change of the media. But, the intracellular pHi rising because of intracellular ammonia penetration was not taken into account in the present study and this might be another or an additional cause of induction of ureogenesis. In ureogenic toadfish, *O. beta* stimulation of ureogenesis was also reported while exposing to higher ambient ammonia and also to exposure to air for extended periods of time (Walsh et al. 1994), and the reason of

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

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