EXCRETORY NITROGEN METABOLISM IN THE CHINESE SOFT-SHELLED TURTLE, *PELODISCUS SINENSIS*

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SUMMARY

This study aimed to determine effects of four experimental conditions, namely feeding, salinity stress, emersion and acute ammonia toxicity, on nitrogen metabolism and excretion in the Chinese soft-shelled turtle *Pelodiscus sinensis*. *Pelodiscus sinensis* is ureogenic and primarily ureotelic in freshwater. Results reveal for the first time that a major portion of the urea was excreted through the buccopharyngeal epithelium. Approximately 72 h was required for *P. sinensis* to completely digest a meal of prawn meat. After feeding, ammonia contents in various tissues remained unchanged, but the tissue urea contents increased significantly. By hour 48, 68% of the assimilated nitrogen (N) from the feed was excreted, 54% of which was excreted as urea-N. The rate of urea synthesis apparently increased 7-fold during the initial 24 h after feeding. Increased urea synthesis effectively prevented postprandial surges in ammonia contents in the plasma and other tissues. In addition, postprandial ammonia toxicity was apparently ameliorated by increased transamination and synthesis of certain amino acids in the liver and muscle. For turtles exposed to a progressive increase in salinity from 1‰ to 15‰ through a 6-day period, there were significant increases in plasma osmolality, $[Na^+]$ and $[Cl^-]$ in 15‰ water on day 6. Free amino acids (FAAs) and urea were accumulated in various tissues for cell volume regulation. There were increases in proteolysis, which supplied FAAs as osmolytes, and catabolism of certain amino acids, which released ammonia for subsequent urea synthesis. Consequently, the rate of urea synthesis increased 1.4-fold. *Pelodiscus sinensis* was able to maintain its haematocrit and plasma osmolality, $[Na^+]$ and $[Cl^-]$ during 6 days of emersion. It reduced water loss through a reduction in urine output, resulting in a significant decrease in daily excretion of nitrogenous waste. There was a drastic decrease in the urea excretion rate due to a lack of water to flush the

buccopharyngeal lining, resulting in a shift from ureotely to ammonotely. Urea accumulated in various tissues, but it could only account for 13-22% of the deficit in urea excretion, indicating the occurrence of a decrease in the rate of urea synthesis. Indeed, there were significant decreases in activities of certain ornithine-urea cycle (OUC) enzymes from the liver. Because a decrease in urea synthesis occurred without accumulations of ammonia, total FAA (TFAA) or total essential FAA (TEFAA), it can be deduced that ammonia production through amino acid catabolism was suppressed with a proportional reduction in proteolysis. The ammonia content in the brain of *P. sinensis* increased transiently to 16 μmol g^{-1} brain 1 h after the injection with a sub-lethal dose of NH_4Cl, indicating that the brain of *P. sinensis* had high tolerance of ammonia at cellular and sub-cellular levels. Turtles which succumbed to a lethal dose of NH_4Cl had brain ammonia and glutamine contents of 21 μmol g^{-1} and 4.4 μmol g^{-1}, respectively. Because the brain glutamine content increased transiently to 8 μmol g^{-1} in turtles injected with a sub-lethal dose of NH_4Cl, astrocyte swelling resulted from glutamine accumulation could not be the major cause of death. Indeed, L-methionine S-sulfoximine (MSO), a glutamine synthetase (GS) inhibitor, had no effect on the mortality rate. In contrast, MK801, an N-methyl-D-aspartate (NMDA) receptor antagonist, reduced the 24 h mortality of turtles injected with a lethal dose of NH_4Cl by 50%, indicating that ammonia toxicity involved the activation of NMDA receptors.

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INTRODUCTION

The Chinese soft-shelled turtle

The Chinese soft-shelled turtle, *Pelodiscus sinensis* (Wiegmann, 1835) was known previously as *Trionyx sinensis* and belongs to the Family Trionychidae. It inhabits central and southern China, Vietnam, Korea and the islands of Hainan and Taiwan (Ernst and Barbour, 1989; Iverson, 1992). The natural habitat of *P. sinensis* includes standing or slow-flowing bodies of water, such as ponds, lakes, reservoirs, canals, marshes, creeks and rivers. Unlike other testudines, the flattened carapace of *P. sinensis* is covered with a leathery cutaneous surface instead of horny laminae, and hence the name "soft-shelled" turtle (Ernst and Barbour, 1989). It has a retractile, narrow, and elongate head, tipped with snorkel-snouted nostrils (Orenstein, 2001). *Pelodiscus sinensis* spend much of the time submerged in the water or buried in the mud of the bottom with only the tip of their snorkel poking occasionally above the surface. They are excellent, powerful swimmers and active hunters. These turtles are carnivorous, feeding on insects, worms, crustaceans, fishes, mollusks and frogs (Ernst and Barbour, 1989; Lim and Indraneil, 1999).

In spite of being primarily dependent on pulmonary respiration, *P. sinensis* is highly aquatic and can endure prolonged submersion (Ultsch and Wasser, 1990). It is able to satisfy much of its oxygen demand during diving or submergence through buccopharyngeal and cutaneous respiration. It performs rhythmic pharyngeal movements during forced submergence, and buccopharyngeal respiration accounted for 67% of the oxygen uptake, with the remaining 33% being accounted for through cutaneous uptake (Wang et al., 1989). *Pelodiscus sinensis* can survive well in brackish water and therefore can also be found in swamps and marshes (Lim and Indraneil, 1999). Under certain situations, it may be partially or completely exposed

to air; for example, emersion can occur when the ponds or creeks dry up during hot spells or when the turtle emerges from the waters to bask, but the turtle usually remains in contact with a moist or wet substratum in relatively high humidity.

Pelodiscus sinensis has been introduced into Malaysia and Thailand (Ernst and Barbour, 1989; Iverson, 1992), and is at present, the most commonly farmed turtle species in Asia (Orenstein, 2001). Turtles are grown in ponds and usually fed a high protein diet. Water in the pond may often have a high concentration of ammonia, as a result of fertilizer run-off from nearby farms. Thus, the ability of *P. sinensis* to flourish in such an environment hints at high ammonia tolerance.

Ammonia is produced mainly through amino acid catabolism in animals. Because ammonia is toxic (Cooper and Plum, 1987; Butterworth, 2002; Felipo and Butterworth, 2002; Ip et al., 2004a, b), it must be eliminated from the body or detoxified to another product. Ammonia can be detoxified to urea through the ornithine-urea cycle (OUC). A functional OUC is present in extant lungfishes, coelacanths, amphibians and the testudinid and rhynchocephalid reptiles. However, the OUC became dysfunctional in the reptilian line leading to the birds, and this may also have occurred in the reptilian line giving rise to Squamata and Crocodylia (Campbell, 1973). Thus, members of Reptilia exhibit the greatest plasticity among vertebrates with respect to the evolution of excretory function, because they exhibit all three major types of nitrogen metabolism, and undergo transitions between ammonotelism and ureotelism, ammonotelism and uricotelism, and ureotelism and uricotelism (Campbell, 1995).

Although Baze and Horne (1970) worked on a soft-shelled species, *Apalone mutica* (previously as *Trionyx muticus*), and presented results for hepatic ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase

(ASL) and arginase activities, they reported no results on carbamoyl phosphate synthetase I (CPS I), and this was cited later in the review by Campbell (1995). In view of the lack of information on CPS I from the liver of *Trionyx* (or *Pelodiscus*) spp., the first objective of this study was to determine the activities of various OUC enzymes and to estimate the CPS I capacity in the liver of *P. sinensis*.

Baze and Horne (1970) determined the activities of OUC enzymes in seven species of turtles (*Gopherus berlandieri*, *Terrapene ornata*, *Terrapene carolina*, *Clemmys insculpta*, *Trachemys scripta* (previously as *Pseudemys scripta*), *Chelydra serpentina* and *A. mutica* and reported that all of them were ureogenic and ureotelic except for *G. berlandieri*, which was uricotelic. To date, it is accepted that aquatic and semi-aquatic turtles are, in general, primarily ureotelic, although in some cases, ammonia is the predominant form of excretory-nitrogen (N) (Campbell, 1995; Dantzler, 1995). Thus, another objective of this study was to determine whether *P. sinensis* was ammonotelic or ureotelic when immersed in freshwater.

After establishing the baseline information on nitrogen metabolism and excretion in *P. sinensis* in freshwater (control), turtles were challenged with four different experimental conditions, namely feeding, salinity stress, emersion and acute ammonia toxicity. The objective was to study how *P. sinensis* responded to the perturbation of nitrogen homeostasis and how excretory nitrogen metabolism was modulated in response to the various types of environmental stresses.

Feeding

The normal dietary intake of protein by animals provides amino acids in excess of the amounts required for the synthesis of new protein to sustain protein turnover. After the consumption of a protein-containing meal, free amino acids (FAAs) produced by the actions of proteases in the alimentary tract and peptidases in

the intestinal mucosal cells (Matthews, 1975) enter the circulation. The majority of these amino acids, in excess of what is required for protein synthesis, are catabolized in the liver (Campbell, 1991), releasing ammonia and resulting in a momentary increase in ammonia level in the animal. Campbell (1995) suggested that aquatic and semi-aquatic turtles should be considered as facultative ammonoureoteles, and so it would be essential to examine conditions in which these turtles would detoxify ammonia to urea. Thus, this study was undertaken to examine whether the excess ammonia produced after feeding in *P. sinensis* would be excreted mainly as ammonia or detoxified to urea through the hepatic OUC. The hypothesis tested was that feeding would induce an increase in urea synthesis in this turtle, and a substantial portion of the ammonia released from the catabolism of excess amino acids was not excreted as such, despite *P. sinensis* being an aquatic and soft-shelled species. An attempt was also made to elucidate if the hepatic OUC capacity in this turtle would be up-regulated after feeding. To our knowledge, no such information is available for aquatic and semi-aquatic turtles at present.

It has been proposed that glycine and glutamine were synthesized from other amino acids consumed in excess of those required for protein synthesis in the liver of reptiles (Coulson and Hernandez, 1970). If indeed such a phenomenon occurred in *P. sinensis*, increased transamination and synthesis of certain amino acids could augment increased urea synthesis to defend against postprandial ammonia toxicity. Therefore, efforts were made in this study to determine the effects of feeding on contents of FAAs in the liver and muscle of this turtle.

Pelodiscus sinensis is of high commercial value and is therefore cultured in Malaysia, Vietnam, Indonesia and China for food consumption. They are usually fed a high protein diet (47 g protein per 100 g dried feed; Jia et al., 2005). For mammals,

it has been suggested that postprandial increases in the concentration of plasma ammonia and contents of brain glutamine and certain essential amino acids act as signals to decrease the intake of high protein diets (Peters and Harper, 1987; Semon et al., 1988). Therefore, we also made an effort to investigate whether feeding would lead to increases in FAAs, especially essential ones, in the brain of *P. sinensis*, with special emphasis on whether a postprandial increase in glutamine content would occur.

Salinity stress

Extant reptiles have not been particularly successful in adapting to the marine environment. Even the sea turtles, highly modified for a pelagic life, retain their dependence on land for purposes of egg-laying. One of the major difficulties encountered by reptiles living in waters of high salinity is the problem of ionic and osmotic regulation, because osmolalities of their body fluids, similar to those of other vertebrates, are about one-third that of seawater, and their kidneys cannot elaborate urine hyperosmotic to the plasma. The solution to this problem in marine reptiles is the development of the salt gland (Schmidt-Nielsen and Fänge, 1958; Dunson and Taub, 1967; Dunson, 1968, 1969a, b). For testudines, salt glands can be found in members of two families of true sea turtles: Cheloniidae and Dermochelyidae (Minnich, 1982). The only estuarine testudinid known to have a functional salt gland is the brackish water diamondback terrapin, *Malaclemys terrapin* (previously as *M. centrata*; Schmidt-Nielsen and Fänge, 1958). *Malaclemys terrapin* is restricted to estuarine, coastal waters of high salinity (Dunson, 1970), and its capacity of salt secretion through salt glands is lower than those of the true sea turtles. In spite of the development of salt glands (Dunson, 1970), tissue urea contents increase in *M. terrapin* acclimatized to various salinities. This increased osmolarity would help

decrease osmotic stress; osmolarity of the serum increases from 309 mmol l^{-1} in freshwater to 459 mmol l^{-1} in seawater. This increase was mainly due to an increased urea concentration from 22 mmol l^{-1} in freshwater to 115 mmol l^{-1} in seawater. Gilles-Baillien (1970), suggested that this was a result of the retention of urine in the bladder from where urea passed back to the blood unrelated to increased urea production.

The zone in which freshwater mixes with the sea is a biotope rich in food resources. *Pelodiscus sinensis* can survive well in brackish waters and can also be found in swamps and marshes (Obst, 1986; Lim and Indraneil, 1999). For a long time, the functional role of the integument of testudinid reptiles in ionic and osmotic regulation was assumed to be restricted. However, now it is well established that the skin of the soft-shelled turtle is not absolutely impermeable to water. Bentley and Schmidt-Nielsen (1970) compared the osmotic passage of water through the integument of the soft-shelled turtle *Apalone spinifera* (previously as *Trionyx spinifer*) and the pond slider *T. scripta*. The osmotic water transfer in either hypoosmotic or hyperosmotic solutions in *A. spinifera* was four times greater than *T. scripta*. Therefore, Bentley and Schmidt-Nielsen (1970) concluded that water uptake in *A. spinifera* approaches that seen in some aquatic amphibians, such as *Necturus* (Bentley and Heller, 1964), and that the leathery skins of soft-shelled turtles play a major role in water exchange in aqueous media. Thus, *P. sinensis* is likely to be confronted with greater osmotic stress than *M. terrapin* when exposed to waters of high salinity due to the absence of a horny carapace. However, to date, no information on adaptations of *P. sinensis* to a brackish environment is available.

This study was undertaken to determine effects of exposure to a progressive increase in salinity from 1‰ to 15‰ (half strength seawater) through a 6-day period

on nitrogen metabolism and excretion in *P. sinensis*, with a special emphasis on the roles of FAAs and urea in water retention. Because *P. sinensis* does not have a salt gland to facilitate iono- and osmo-regulation in brackish water, experiments were performed to test the hypotheses that its plasma osmolality and Na^+ and Cl^- concentrations would increase with increases in ambient salinity, and that its survival in brackish water would depend on the accumulation of osmolytes like FAAs and urea for cell volume regulation. Therefore, we also aimed to determine indirectly whether increased protein degradation would occur in *P. sinensis* during exposure to a progressive increase in ambient salinity, supplying FAAs and/or urea for osmoregulatory purposes. Because *P. sinensis* was subsequently found to be ureogenic and primarily ureotelic in freshwater, efforts were made to examine whether salinity stress would result in increases in the rate of urea synthesis and activities of OUC enzymes in the liver. If indeed FAAs and urea played a role in osmoregulation in *P. sinensis*, there could also be decreases in rates of ammonia and/or urea excretion. Therefore, we also determined the effects of 6 days of exposure to a progressive increase in salinity followed with 1 day of recovery in freshwater on rates of ammonia and urea excretion in *P. sinensis*.

Emersion

Pelodiscus sinensis may be partially or completely exposed to air under certain situations. Bentley and Schmidt-Nielsen (1970) reported that, in air, the total evaporative water loss through the integument of the soft-shelled turtle *A. spinifera* was three times greater than that of the pond slider *T. scripta*. Therefore, *P. sinensis* is likely to experience more severe dehydration stress than hard-shelled turtles during emersion due to the absence of a horny carapace. During emersion, turtles have to conserve water in order to prevent dehydration. Water conservation may result in the

impediment of nitrogenous excretion and require special adaptation to ameliorate ammonia toxicity. An effective strategy in this case, would be to increase the rate of urea synthesis. However, to date, no information is available on the effects of emersion on nitrogen metabolism and excretion in this turtle.

Severe water loss would lead to elevated levels of electrolytes, because *P. sinensis*, as mentioned above, is incapable of elaborating hyperosmotic urine (Shoemaker and Nagy, 1977) or a hyperosmotic salt gland secretion (members of Trionychidae are not known to possess salt glands; Shoemaker and Nagy, 1977; Minnich, 1979). Hence, this study aimed to determine whether 6 days of emersion would result in increases in plasma osmolality and Na^+ and Cl^- concentrations in *P. sinensis*. The hypothesis tested was that *P. sinensis* could effectively conserve water during this period. In order to achieve this, it would be essential for *P. sinensis* to reduce urine production during prolonged emersion, and this would impede the excretion of nitrogenous waste. Thus, another objective was to determine whether 6 days of emersion would result in decreases in ammonia and urea excretion in this turtle. During the course of the study, it was discovered unexpectedly that emersion had differential effects on ammonia and urea excretion, and therefore efforts were made to determine whether ammonia and urea excretion occurred through different routes (urine versus non-urine) in *P. sinensis*.

Traditionally, defense against ammonia toxicity in animals has been focused on the detoxification of ammonia to less toxic compounds like urea, uric acid and/or glutamine (Campbell, 1973, 1991, 1995). Since *P. sinensis* is ureogenic, and urea can act as an osmolyte to reduce evaporative water loss in certain animals (Horne, 1971; Campbell, 1973; Chew et al., 2004), this study also aimed to examine whether urea would be accumulated to high levels in various tissues and indirectly to elucidate

whether the rate of urea synthesis would be enhanced in *P. sinensis* during 6 days of emersion. In addition, efforts were made to determine whether 6 days of emersion would result in changes in activities of various OUC enzymes in the liver. In addition, it has been suggested recently that a reduction in ammonia production can be an important adaptation, which ameliorates ammonia toxicity in some tropical air-breathing fishes during emersion (Ip et al., 2001a, 2004a; Chew et al., 2005). This can be achieved through the suppression of ammonia production in general through a reduction in amino acid catabolism (Jow et al., 1999; Lim et al., 2004, Ip et al., 2001a; Chew et al., 2001, 2003a, 2004; Tay et al., 2003; Loong et al., 2005), or through the partial catabolism of certain amino acids leading to the formation of alanine (Ip et al., 2001a, b; Chew et al., 2001, 2003b). Such a phenomenon has not been reported in soft-shelled turtles kept out of water. Therefore, the final objective of this study was to examine changes in tissues ammonia and FAA contents in *P. sinensis* during 6 days of emersion and to evaluate indirectly whether a reduction in amino acid catabolism and/or protein degradation had occurred. We hypothesized that *P. sinensis* could effectively reduce ammonia production during emersion, which would manifest as a deficit between reduction in nitrogenous excretion and increase in nitrogenous accumulation (including urea) together with a decrease in the rate of urea synthesis during the 6-day experimental period.

Acute ammonium toxicity

Ammonia is toxic to animals for many reasons. At the molecular level, NH_4^+ can substitute for K^+ in Na^+, K^+-ATPase and in $Na^+/K^+/2Cl^-$ co-transport (see Wilkie, 1997, 2002 for reviews; Person-Le Ruyet et al., 1997), and for H^+ in Na^+/ H^+ exchanger (Randall et al., 1999). In neurons, NH_4^+ can substitute for K^+ and permeate through K^+ background channels, affecting the membrane potential (Binstock and

Lecar, 1969). Ammonia can interfere with energy metabolism through inhibition of certain glycolytic enzymes and impairment of the tricarboxylic acid cycle (TCA) (Campbell, 1973). In vertebrates, ammonia toxicity normally manifests as encephalopathy at the organismal level; the animal enters into a coma and succumbs to the deleterious effects of ammonia. In recent years, several theories, i.e. glutamatergic dysfunction, activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, and glutamine accumulation leading to astrocyte swelling, have been proposed as mechanisms involved in chronic and/or acute ammonia toxicity in mammalian brains (Felipo et al., 1994; Margulies et al., 1999; Hermenegildo et al., 2000; Desjardins et al., 2001; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002).

In patients suffering from acute liver failure and hepatic encephalopathy (HE), glutamatergic dysfunction (Hilgier et al., 1999; Michalak et al., 1996) resulted from high levels of brain ammonia (1-3 mmol l^{-1}; Kosenko et al., 1994) remains the leading candidate in the pathogenesis of HE. The concentration of extracellular glutamate increases (Michalak et al., 1996) due to the inhibition of glutamate uptake (Oppong et al., 1995) or increased glutamate release from neurons (Rose, 2002). Extracellular glutamate binds with and activates NMDA receptors (Marcaida et al., 1992; Hermenegildo et al., 1996), which are coupled with the nitric oxide-cyclic GMP signal transduction pathway (Hermenegildo et al., 2000), leading to extensive destruction of proteins in the neurons (Kosenko et al., 1993 1994, 1995, 1997, 1999, 2000). It has also been demonstrated in rats that an activation of NMDA receptors may precede the increase in extracellular glutamate (Hermenegildo et al., 2000), initiated probably by a depolarization of the neuronal membrane (Sugden and Newsholme, 1975; Fan and Szerb 1993). When (5R, 10S)-(+)-methyl-10, 11-

dihydro-5H-dibenzo[a, d]cyclohepten-5, 10-imine hydrogen maleate (MK801; a NMDA receptor antagonist) is injected into rats before the injection of CH_3COONH_4, it can delay or eliminate the fatal effects of acute ammonia toxicity (Marcaida et al. 1992; Hermenegildo et al. 1996), because it binds to NMDA receptors and prevents their activation by glutamate.

In mammals, glutamine synthesis via glutamine synthetase (GS) is activated in the brain to remove the excess ammonia present when there is an increase in ammonia level (Suárez et al., 2002). In patients with urea cycle disorders, hyperammonemic encephalopathy is a consequence of astrocyte swelling and dysfunction resulting from the osmotic effects of astrocyte glutamine synthesis (activated by ammonia) and accumulation (Brusilow, 2002) and the loss in expression of aquaporin 4 and the astrocytic/endothelial cell glucose transport protein GLUT-1 (Margulies et al., 1999; Desjardins et al., 2001). Cell swelling may be so severe as to cause raised intracranial pressure and, as a consequence, brain herniation, which is the major cause of mortality in patients with acute liver failure. Indeed, the administration of L-methionine S-sulfoximine (MSO), an inhibitor of GS, to rats delays or even eliminates the fatal effects of ammonia toxicity (Warren and Schenker, 1964; Takahashi et al., 1991; Willard-Mack et al., 1996; Brusilow, 2002), probably due to the inhibition of GS, or the inhibition of glutamate release which prevents the activation of NMDA receptors (Kosenko et al., 1994, 1999; Kosenko et al., 2003).

Despite recent advances in the understanding of mechanism of ammonia toxicity in mammals, there is a dearth of knowledge on ammonia toxicity on other vertebrates. Unlike mammals, some tropical air-breathing fishes can tolerate high levels of ammonia (see Ip et al., 2001a, 2004a, b, and Chew et al., 2005 for reviews), and/or synthesize and accumulate high levels of glutamine in their brains and extra-

cranial tissues (Peng et al., 1998; Anderson et al., 2002; Tsui et al., 2002; Tay et al., 2003; Ip et al., 2004a, b). Thus, the mechanisms of ammonia toxicity in the brains of fish species with high ammonia tolerance are likely to be different from those in mammalian brains (Ip et al., 2005; Veauvy et al., 2005). Indeed, Ip et al. (2005) demonstrated recently that, unlike patients suffering from hyperammonemia, glutamine synthesis and accumulation in the brain was not the major cause of death in two mudskippers, *Periophthalmodon schlosseri* and *Boleophthalmus boddarti*, confronted with acute ammonia toxicity. Although MSO was an inhibitor of GS activities from the brains of both mudskippers MSO, at a dosage (100 μg g^{-1} fish) protective for rats, it did not reduce the mortality in these two mudskippers injected with a lethal dose of CH_3COONH_4. In addition, MK801 (2 μg g^{-1} fish) had no protective effect on these two mudskippers either, indicating that activation of NMDA receptors was not the major cause of death during acute ammonia intoxication (Ip et al., 2005). Therefore, this study was undertaken to extend our investigation to mechanisms of ammonia toxicity to reptiles, and in particular *P. sinensis*.

In the first series of experiment, MK801 was injected into the peritoneal cavity of *P. sinensis* prior to the injection of a lethal dose of NH_4Cl. The objective was to determine whether acute ammonia toxicity in this turtle was mediated through NMDA receptor activation as in rats. Efforts were also made to evaluate if the administration of MSO prior to the injection of a lethal dose of NH_4Cl would exacerbate or ameliorate ammonia toxicity in *P. sinensis*. We aimed to test the hypothesis that the synthesis and accumulation of glutamine and the release of glutamate into extracellular compartments did not contribute significantly to ammonia toxicity in the brain of *P. sinensis* and, therefore, that MSO would not reduce the mortality of turtles confronted with acute ammonia toxicity.

Indeed, results obtained subsequently revealed that the mechanisms of ammonia toxicity in *P. sinensis* differed from those of mammals. Therefore, in the second series of experiments, a sub-lethal dose of NH_4Cl was injected intraperitoneally into *P. sinensis* with the aims of determining during the subsequent 24 h (1) how much ammonia would accumulate in the brain and other tissues, (2) whether ammonia would be detoxified to glutamine, leading to its accumulation, in the brain, (3) the urea contents in various tissues in order to elucidate if excess ammonia would be detoxified to urea, (4) the FAA contents in various tissues and activities of enzymes involved in the synthesis of glutamate and glutamine and (5) the rates of ammonia and urea excretion to confirm whether a major portion of the ammonia injected into the peritoneal cavity was excreted as ammonia per se. It is hoped that results obtained would shed light on adaptations for defense against ammonia toxicity in this soft-shelled turtle.

LITERATURE REVIEW

Amino acids as substrates for gluconeogenesis

Normal dietary intake of protein by animals provides amino acids in excess of the amounts required for the synthesis of new protein to sustain protein turnover (Baldwin, 1964; Campbell, 1973). As amino acids cannot be stored, they are degraded and a major portion, the carbon skeleton is converted to glucose, i.e. amino acid gluconeogenesis (Krebs, 1972). The liver is considered to be the "glucostat" of the body (Jungermann and Katz, 1986), and is therefore the main site of amino acid gluconeogenesis in mammals and most other vertebrates. Other tissues, especially the kidney (Friedman and Toretti, 1978), are also capable of gluconeogenesis.

The majority of amino acids are gluconeogenic in that they can either be degraded to pyruvate or TCA intermediates for eventual conversion to oxaloacetate (Leverve, 1995). In carnivorous species, they are a major source of energy. In trout, for example, 90% of the calories utilized during sustained swimming are from protein (Van den Thillart, 1986). Cats, whose natural diet also consists mainly of protein, illustrate some unique adaptations of mammals to such diets. They, for example, maintain essentially maximal rates of gluconeogenesis irrespective of protein intake (Silva and Mercer, 1986). This is unlike omnivorous animals in which increases or decreases in dietary protein intake cause corresponding changes in the rates of hepatic gluconeogenesis (Lardy and Hughes, 1984).

The liver also acts on amino acids formed by extrahepatic tissues. Alanine and glutamine are major products of muscle metabolism in mammals, accounting for 50% or more of the amino acids released by this tissue (Ruderman and Berger, 1974; Tischler and Goldberg, 1980). Alanine and glutamine are also released by adipose tissue (Tishler and Goldberg, 1980). These two amino acids are formed from the

catabolism of other amino acids, especially the branched chain amino acids (leucine, isoleucine and valine), which are rapidly transaminated in both cardiac and skeletal muscle (Goldberg and Chang, 1978; Harris et al., 1986). The resulting α-keto acids may be returned to the liver or may be oxidized directly by muscle as an alternate energy source (Goldberg and Odessey, 1972; Odessey and Goldberg, 1972).

The main fate of alanine formed in extrahepatic organs is conversion to glucose in the liver (Felig et al., 1970; Hall et al., 1977). This has been referred to as the "glucose-alanine cycle" (Felig, 1973). Glutamine can also serve as a gluconeogenic substrate for liver but its main fate is uptake by either intestine or kidney (Goldstein, 1976; Welbourne and Phromphetcharat, 1984; Windmueller, 1984). Glutamine is a major energy source for intestinal tissues. Alanine formed during glutamine catabolism in intestinal tissues may be released to be taken up by liver for glucose synthesis. In fact, as much as 50% of the alanine utilized by the liver may come from the intestine. In the kidney, glutamine serves as a source of ammonia for acid-base balance (Campbell, 1991).

Amino acid catabolism – Transdeamination

The main pathway for amino acid catabolism requires an initial transfer of the α-amino function to α-ketoglutarate by an aminotransferase to form glutamate and the corresponding α-ketoacid (Braunstein, 1985, Torchinsky, 1987). Aminotransferase reactions are at or near equilibrium so any increase or decrease in plasma concentrations of amino acids causes a corresponding increase or decrease in their rate of degradation (Krebs et al., 1972; Torchinsky, 1987). Glutamate is then taken up by mitochondria where it is oxidatively deaminated by glutamate dehydrogenase (GDH), resulting in the removal of the α-amino group as ammonium ion. The

coupled amino acid aminotransferase – GDH reactions are referred to as "transdeamination" (Fig. 1) (Braunstein, 1985, Torchinsky, 1987).

Alternate routes of amino acid catabolism

A second general mechanism for amino acid catabolism in specific mammalian extrahepatic tissue has been proposed (Lowenstein, 1972; Lowenstein and Tornheim, 1971). This mechanism requires coupling of transamination reactions with the purine nucleotide cycle, which is made up of the enzymes adenylosuccinate synthetase and lyase and AMP deaminase (Fig. 2). Ammonia is released by the purine nucleotide cycle in the cytosolic compartment.

In addition to mammalian muscle, in which it was first described, the cycle has been shown to be present in mammalian brain (Schultz and Lowenstein, 1976, 1978) and kidney tissues (Bogusky et al., 1976). These tissues are characterized by high aspartate aminotransferase and AMP deaminase activities and low glutamate dehydrogenase. In working muscle, these enzymes of the cycle function as a unit and are a major source of the ammonia formed by this tissue. The purine nucleotide cycle is also a major source of ammonia produced by brain tissue (Schultz and Lowenstein, 1976), but the extent to which it operates in mammalian kidney tissues is not agreed upon (Nissim et al., 1986; Strzelecki et al., 1983; Tornheim et al., 1986).

Some amino acids, such as asparagine, glycine, serine and threonine, may also undergo direct deamination in the cytosol, at least in mammals (Campbell, 1995). The L-amino acid oxidases catalyze the direct formation of ammonia from several amino acids. However, their activity is generally felt to be of minor importance in vertebrates (Campbell, 1991). A NAD^+-specific L-threonine dehydrogenase is present in both avian (Aoyama and Motokawa, 1981) and mammalian liver (Bird and Nunn, 1983; Ray and Ray, 1985). Glycine produced by this reaction may be

Fig. 1 Function of aminotransferases and glutamate dehydrogenase (GDH) in the release of the α-amino group of amino acids via transamination (modified from Campbell, 1973).

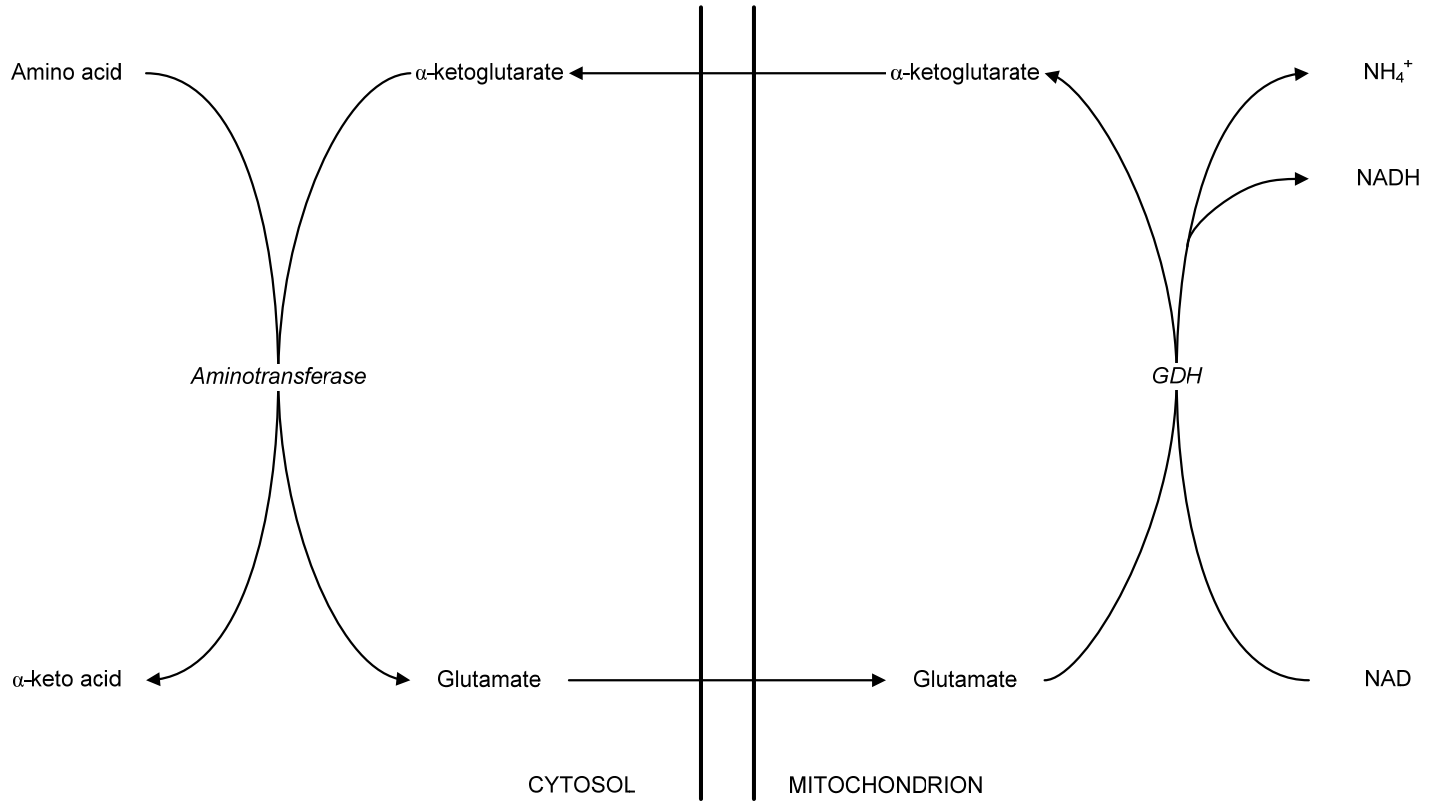
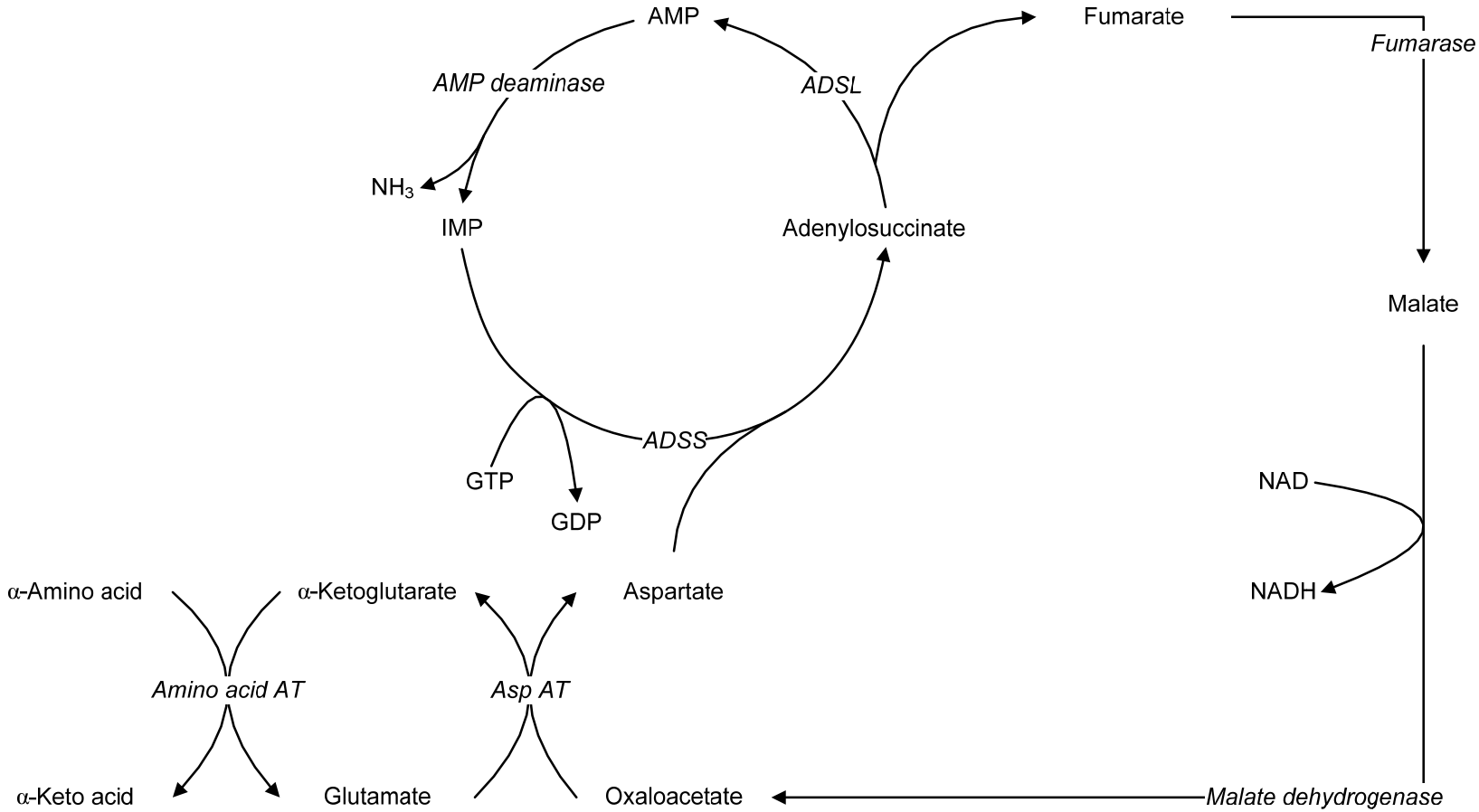


Fig. 2 The purine nucleotide cycle (From Campbell, 1991). The abbreviations are: ADSS, adenylosuccinate synthetase; ADSL, adenylosuccinate lyase; AT, aminotransferase



important in birds, which require a source of glycine for hepatic urate synthesis. Threonine and serine are generally not transaminated in the mammalian liver and are instead deaminated by serine dehydratase in the cytosol. However, this enzyme is low in non-mammalian vertebrates (Rowsell et al., 1979; Yoshida and Kikuchi, 1971).

In mammals, another major source of ammonia for hepatic urea synthesis is the amide function of glutamine. Glutamine is formed in extrahepatic organs, such as the brain, intestine and muscle in mammals and birds and presumably other ureotelic reptiles. Glutamine is deaminated by glutaminase in liver mitochondria releasing ammonia as shown below (Campbell, 1995).

$$\text{L-glutamine} + H_2O \xrightarrow{\text{Glutaminase}} \text{L-glutamate} + NH_3$$

Amino acid catabolism and gluconeogenesis in reptiles

There have been relatively few studies done on amino acid gluconeogenesis in reptiles. Lizards rapidly resynthesize glycogen from non-dietary sources after exercise, so they are clearly capable of gluconeogenesis (Gleeson, 1982). Thus, reptiles appear capable of converting amino acids to glucose. Reptiles also appear to be able to control rates of amino acid catabolism, as evidenced by an increased rate of ureogenesis in the tortoise, *G. berlandieri* after prolonged starvation (Horne and Findeisen, 1977).

Ammonia generated during amino acid catabolism is toxic

The mechanisms of ammonia toxicity in reptiles may be similar to those in mammals. In humans, ammonia is a major factor in the pathogenesis of a severe neuropsychiatric disorder, HE (Butterworth, 1999; Felipo and Butterworth, 2002; Katayama, 2004; Shawcross, 2005). In acute liver failure, HE is characterized by

rapid progression of symptoms starting with altered mental status progressing to stupor and coma within hours or days. Seizures are occasionally encountered and mortality rates are high. In contrast, chronic liver failure results in significant portal-systemic shunting of portal blood with more modest increases in arterial ammonia concentrations. HE in this case develops slowly and is often precipitated by ammononiagenic conditions such as ingestion of a protein load, constipation or a gastrointestinal bleed. Early symptoms include altered sleep patterns and personality changes, followed by shortened attention span and asterixis progressing through stupor to coma as the severity of liver disease progresses (Butterworth, 1999; Felipo and Butterworth, 2002).

Toxic effects of ammonia on cerebral metabolism – Astrocyte swelling

The mammalian brain relies on GS localized in astrocytes to detoxify ammonia (Cooper and Plum, 1987). In acute liver failure, glutamine accumulation is believed to cause swelling of astrocytes. This leads to raised intracranial pressure and as a consequence brain herniation. Brain herniation is the major cause of mortality in acute liver failure (Felipo and Butterworth, 2002; Jalan, 2005).

Ammonia also results in altered mRNA and protein expression profiles. In astrocytes, glial fibrillary acidic protein (GFAP) mRNA and protein were significantly reduced in frontal cortex of rats with acute hyperammonemia (Bélanger et al., 2002). GFAP is the major protein of intermediate filaments in differentiated astrocytes (Eng, 1985). Thus, it was suggested that decreased GFAP could exacerbate cell swelling and subsequent brain edema due to its role in maintaining the visco-elastic properties of the astrocyte (Bélanger et al., 2002).

Toxic effects of ammonia on neurotransmission

Glutamate is the principle excitatory neurotransmitter in the central nervous system (CNS) (Dingledine and McBain, 1999). Glutamate receptors fall into two classes, ionotropic (NMDA or AMPA-Kainate subtype) or metabotropic receptors. The activation of ionotropic receptors, which gate ion channels, lead to an influx of Na^+, K^+ and Ca^{2+} into the cell (Felipo and Butterworth, 2002). In contrast, metabotropic receptors are coupled to G proteins and activation leads to modulation of specific enzymes and ion channels, such as phospholipase C and adenylate cyclase (Felipo and Butterworth, 2002). Thus, it is obvious that dysregulation of glutamate levels will have far-reaching consequences.

Hyperammonemic disorders are associated with increased extracellular brain glutamate in rats and rabbits (Bosman et al., 1992; de Knegt et al., 1994; Michalak et al., 1996; Hilgier et al., 1999). These increased levels could come about due to decreased uptake by astrocytes or increased release from neurons or astrocytes (Butterworth, 2002; Rose et al., 2005; Rose, 2006). In rats, astrocytes remove neuronally-released glutamate from the synaptic cleft through a high affinity, energy-dependent glutamate transporters, GLT-1 and GLAST. Glutamate uptake is decreased in hyperammonemia due to a significant loss in expressions of GLT-1 (Chan and Butterworth, 1999; Knecht et al., 1997) and GLAST (Chan et al., 2000) mRNA and protein. Increased release of glutamate from astrocytes can occur through a Ca^{2+}-dependent vesicular release or due to cell swelling (Rose, 2006). Ca^{2+}-dependent vesicular release occurs in cultured astrocytes in response to acute application of ammonia (5 mmol l^{-1}), which causes a transient alkalinization and mobilizes Ca^{2+} from intracellular stores (Rose et al., 2005). Cell swelling in cultured cortical astrocytes exposed to hypoosmotic medium is thought to increase membrane

permeability allowing for the efflux through volume-regulated anion channels as well as other mechanisms (Kimelberg et al., 1990; Evanko et al., 2004)

In addition, NMDA receptor activation can be modulated by a few compounds. One of them is glycine and increased extracellular levels could result in increased glutamatergic transmission (Ascher and Johnson, 1994). In rats with acute liver failure from liver ischaemia, these increased levels are a consequence of a significant loss of expression of the astrocytic glycine transporter GLYT-1 in cerebral cortex (Zwingmann et al., 2001). Ammonia is also able to exert a direct effect on rat NMDA receptors. In acute hyperammonemia, ammonia is able to activate NMDA receptors, most likely through removal of the Mg^{2+} block on the NMDA receptor (Fan and Szerb, 1993).

The increased neuronal Ca^{2+} due to NMDA receptor stimulation leads to deleterious effects. Increased intracellular Ca^{2+} would activate Ca^{2+}-dependent enzymes including protein kinases, protein phosphatases and proteases. This would lead to decreased protein kinase C-mediated phosphorylation and concomitant activation of Na^{+}, K^{+}-ATPase (Felipo and Butterworth, 2002).

On the other hand, NMDA-induced currents can also be decreased in chronic hyperammonemia through impairment of NMDA receptor function and by inhibition of NMDA-receptor mediated signal transduction pathways (Felipo and Butterworth, 2002). In addition, the decarboxylation of glutamate leads to formation of γ-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the mammalian CNS (Olsen and DeLorey, 1999). In rats, extracellular GABA is increased due to decreased uptake and increased release leading to increased activation of the GABA-A receptor in acute ammonia exposure (Bender and Norenberg, 2000). In addition, neuroinhibition can also be increased due to a direct

interaction of ammonia with the GABA-A receptor complex in the presence of GABA as shown in cultured rat neurons (Takahashi et al., 1993). Neuroinhibition can also be increased by neurosteroids, which are potent positive allosteric modulators of the GABA-A receptor (Krueger and Papadopoulos, 1992). Neurosteroids syntheses may be stimulated in chronic moderate hyperammonemia as a consequence of increased expression of peripheral-type benzodiazepine receptor (PTBR) isoquinoline binding protein mRNA in rat astrocytes (Desjardins et al., 1997). Increased stimulation of GABA-A receptor could contribute to the neuroinhibition that is characteristic of HE (Hazell and Butterworth, 1999; Desjardins and Butterworth, 2002).

Finally, NH_4^+ can also directly affect membrane potential by substituting for K^+ and permeating through K^+ background channels in neurons (Binstock and Lecar, 1969).

Toxic effects of ammonia on cerebral energy metabolism

Hyperammonemia disrupts cerebral energy metabolism in several ways. In acute hyperammonemia, brain glucose concentration is increased possibly due to increased expression of the endothelial cell/astrocytic glucose transporter GLUT-1 (Desjardins et al., 2001). This may lead to increased glucose utilization in rats with brain ammonia concentrations in the 1.4-1.5 mmol l^{-1} range (Hawkins et al., 1973).

ATP depletion in rats has been suggested to occur via ammonia-induced activation of NMDA receptors. Activation starts a cascade leading to increased entry of Ca^{2+} and Na^+. ATP is consumed by Na^+, K^+, ATPase in the effort to maintain Na^+ homeostasis (Kosenko et al., 1994). Increased Ca^{2+} contents that result from NMDA stimulation also exerts effects on key mitochondrial enzymes involved in energy metabolism. In glycolysis, ammonia activates phosphofructokinase in brain extracts from a variety of invertebrates and vertebrates (Sugden and Newsholme, 1975). In

the TCA cycle, α-ketoglutarate dehydrogenase, a rate-limiting enzyme, is inhibited in rat brain mitochondrial preparations exposed to ammonia between 0.2 to 2 mmol l^{-1} (Lai and Cooper, 1986). In rats that had convulsions induced by injecting 7 mmol kg^{-1} of ammonium intraperitoneally, it was found that activities of the electron transport chain (ETC) enzyme, succinate dehydrogenase, was reduced significantly (Kosenko et al., 1996). These inhibitions may contribute to decreased brain ATP concentrations observed in experimental animals exposed to lethal doses of ammonium salts and to brain ammonia concentrations in excess of 3 mmol l^{-1} (McCandless and Schenker, 1981; Kosenko et al., 1994).

Hyperammonemia can also affect energy metabolism by interference with the malate-aspartate shuttle (Fig. 3). In order for glycolysis to proceed, NAD must be regenerated from NADH produced by glycolysis. Since NADH cannot cross the mitochondrial membrane easily, reoxidation of cytoplasmically generated NADH must occur through the transport of reduced equivalents across the inner mitochondrial membrane in lieu of NADH (Cooper and Plum, 1987). This shuttle's effectiveness can be reduced following decreases in brain glutamate seen in hyperammonemia (Hindfelt et al., 1977).

In summary, ammonia exerts its toxic effects over many cellular aspects and since there is cross-talk between many of the cascades, a complex picture of pathogenesis emerges for this toxicant. An overview of the toxic effects discussed so far is listed in Fig. 4. It becomes obvious that in order to avoid deleterious effects, ammonia and hence nitrogen metabolism and excretion in organisms must be precisely regulated.

Fig. 3 The malate-aspartate shuttle (From Cooper and Plum, 1987).

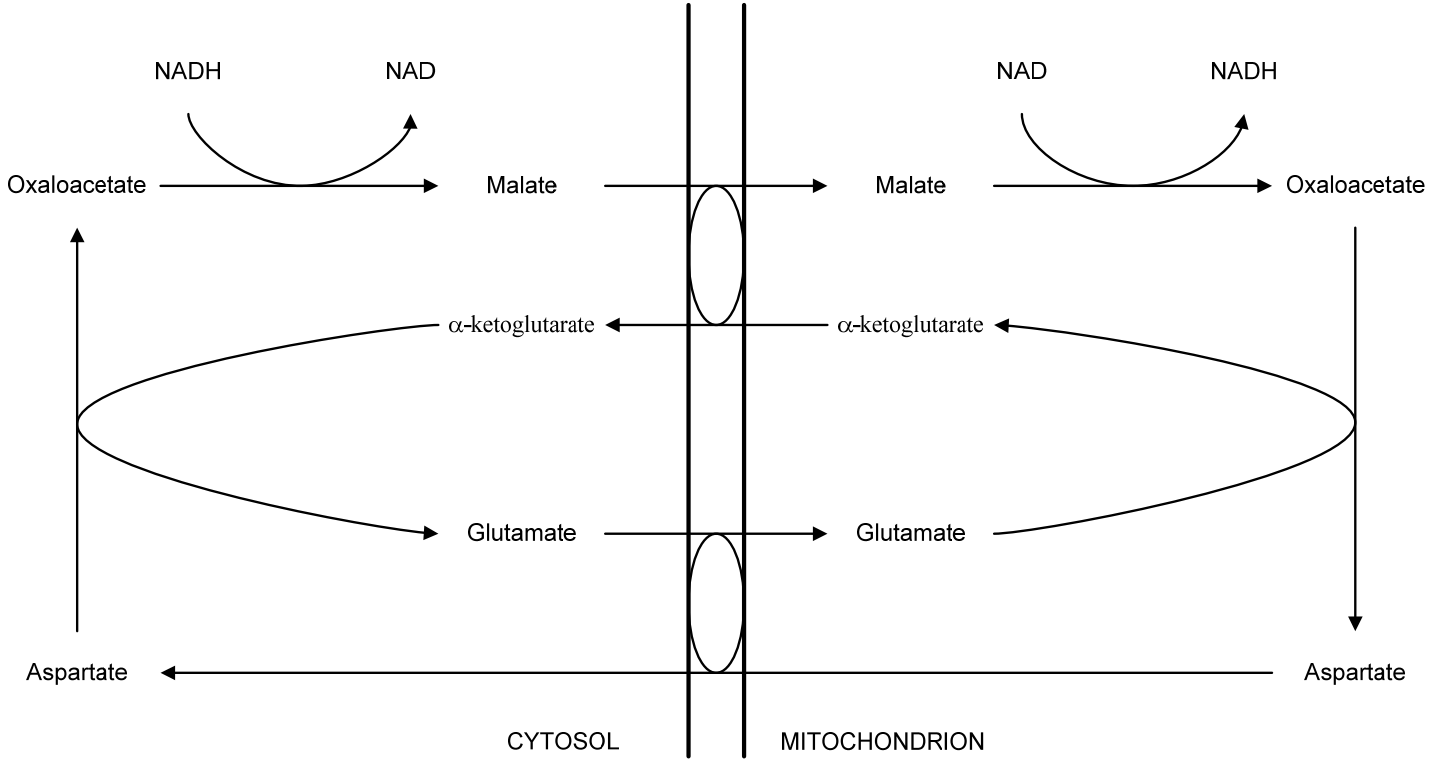
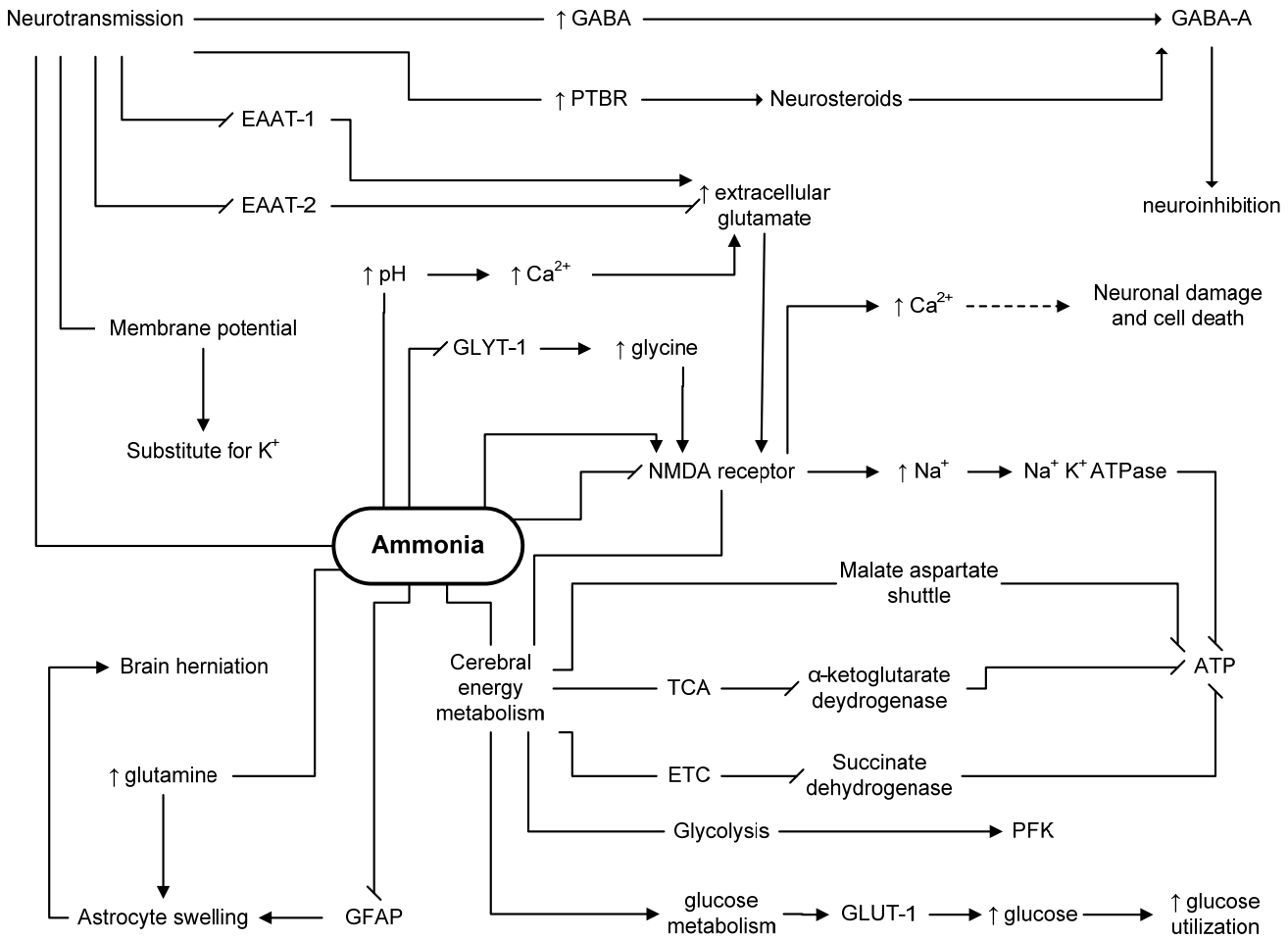


Fig. 4 An overview of ammonia toxicity



Defense against ammonia toxicity

Ammonia toxicity can be ameliorated by preventing ammonia accumulation in the body. This can be done at several levels; ammonia production can be decreased, ammonia can be converted to less toxic compounds for storage or excretion and ammonia excretion can be enhanced. As can be seen later, animals can make use of one or more of these strategies to avoid ammonia toxicity depending on the conditions that lead to ammonia intoxication.

Strategy 1--Reduction in ammonia production through reduced amino acid catabolism

Decreasing amino acid catabolism leads to a concurrent decrease in endogenous ammonia production. Exposure of the rainbow trout, *Oncorhynchus mykiss*, to pH 10 water results in a reduction of ammoniagenesis (Wilson et al., 1998). The Lahontan cutthroat trout, *Oncorhynchus clarki henshawi*, appears to permanently lower its rates of nitrogenous waste production immediately following transfer from its juvenile freshwater habitat (pH 8.4) to Pyramid Lake, Nevada (Wilkie et al., 1997). The giant mudskipper, *P. schlosseri*, reduces proteolysis and amino acid catabolism when exposed to terrestrial conditions under a dark:light regime (Lim et al., 2001). Boddart's goggle-eyed goby, *B. boddarti*, also undergoes a reduction in the rate of amino acid catabolism during aerial exposure (Lim et al., 2001). It can be seen that this strategy is especially important in situations where excretion is impeded, such as in alkaline waters or when access to water is limited.

Strategy 2--Partial amino acid catabolism leading to formation and storage of alanine

Most of the FAAs can be converted to alanine without releasing ammonia. The overall quantitative energetics for this conversion appears to be quite favorable.

The net conversion of glutamate to alanine would yield 10 ATP per mole of alanine formed (Ip et al., 2001a, b). This value would be even higher for the conversion of proline or arginine to alanine (Hochachka and Guppy, 1987).

Periophthalmodon schlosseri relies on this strategy when exposed to aerial conditions for 24 h (Ip et al., 2001b). The advantages of such a strategy include the high levels of ATP generated and also the formation of alanine itself. Alanine produced can serve as a carrier of amino acid carbon for further metabolism elsewhere. For example, it can be converted to pyruvate for further oxidation in the heart, liver and red muscle (Ip et al., 2001a) or it could serve as a glucose precursor in gluconeogenic tissues such as the kidney or liver (Hochachka and Guppy, 1987). Thus, the production of alanine reduces the dependence on carbohydrate metabolism and spares the glycogen stores. The swamp eel *Monopterus albus* and the small snakehead *Channa asiatica* also use partial amino acid catabolism as a strategy to avoid ammonia toxicity during aerial exposure (Ip et al., 2001a).

Thus, it can be seen that this strategy in addition to decreasing the endogenous production of ammonia, allows amino acids to be used as an energy source. This would be a strategy especially suited for animals that remain active during the adverse conditions that limit excretion.

Strategy 3--Ammonia detoxification and glutamine synthesis

In mammals, GS is an extramitochondrial enzyme in the brain and extrahepatic tissues, acting mainly for the detoxification of exogenous ammonia (Campbell, 1973). Glutamine is produced from glutamate and NH_4^+ as shown below.

$$\text{L-Glutamate} + NH_4^+ + \text{ATP} \xrightarrow{GS} \text{L-glutamine} + \text{ADP} + P_i$$

Glutamate may in turn be produced from α-ketoglutarate and NH_4^+ or α-ketoglutarate and other amino acids catalyzed by various transaminases (Ip et al., 2001a).

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \xrightarrow{GDH} \text{L-Glutamate} + NAD$$

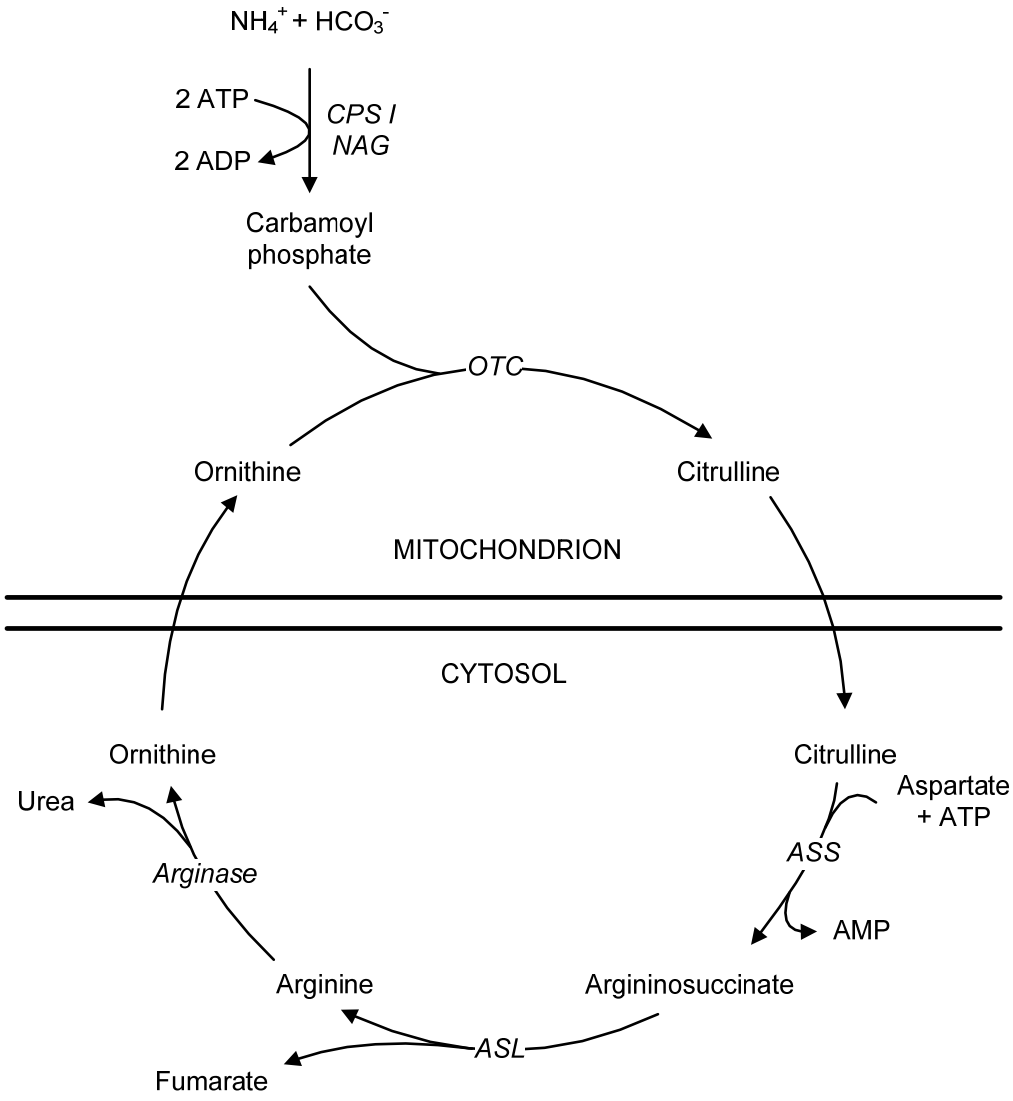
Thus, glutamine formation has the capacity to remove 2 mole of ammonia per mole of glutamine formed. After the ammonia insult, glutamine formed can be channeled towards biosynthesis of other nitrogenous compounds or transported to the liver and deaminated, releasing ammonia for urea synthesis and ultimately excreted (Campbell, 1973).

Strategy 4--Ammonia detoxification and ureogenesis

In ureotelic amphibians, turtles, and mammals, ammonia generated intramitochondrially is converted to urea via the OUC for excretion (Campbell, 1991). The pathway for urea biosynthesis is shown in Fig. 5. In addition to its role in detoxification of ammonia, the product formed, urea, can also serve as an osmotically active solute in water regulation in many vertebrates (King and Goldstein, 1985).

The primary ammonia-detoxifying enzyme here is CPS I, which is allosterically activated by N-acetylglutamate (NAG) (Lusty, 1981). Both CPS I (Clarke, 1976) and OTC (Raijman, 1974) are exclusively mitochondrial in mammalian liver. CPS I acts in concert with OTC to form citrulline, a neutral molecule that exits to the cytosol for conversion to urea for excretion. ASS and ASL are exclusively cytosolic in the ureotelic liver (Cornell et al., 1985). In mammalian livers, arginase is also predominantly cytosolic although a small fraction appears to be associated with the outer mitochondrial membrane (Cheung and Raijman, 1981). In the chicken (Grazi et al., 1975; Kadowaki et al., 1976) and elasmobranch (Casey and Anderson, 1983; King and Goldstein, 1983) liver and kidney tissues, arginase is

Fig. 5. The ornithine-urea cycle (OUC) (Modified from Campbell, 1995). The abbreviations are: NAG, N-acetylglutamate; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS I, carbamoyl phosphate synthetase I; OTC, ornithine transcarbamylase.



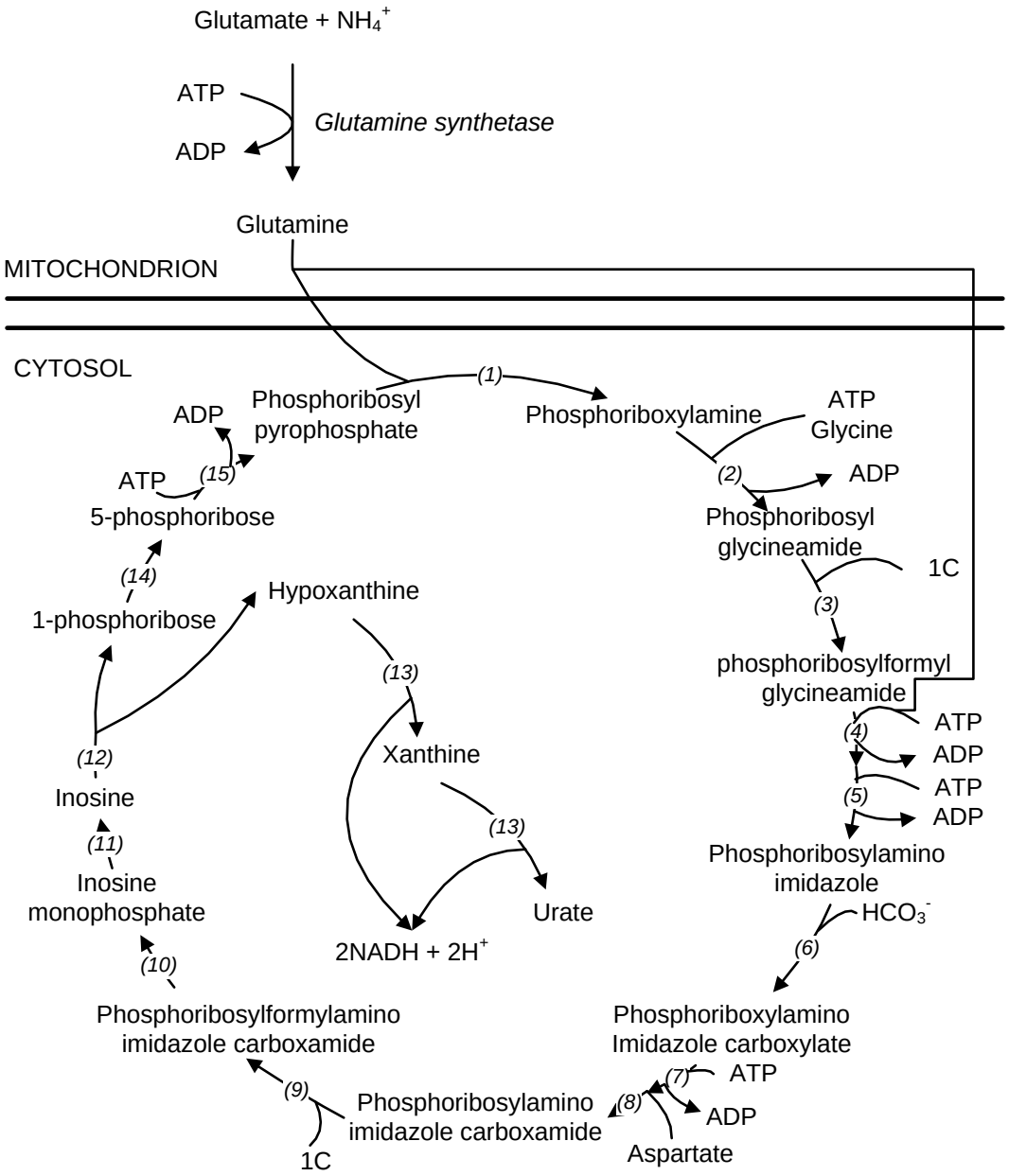
mainly mitochondrial. In the mammalian liver, biosynthesis of urea is energetically expensive and directly utilizes four pyrophosphate bonds from three ATPs (Campbell, 1995).

Strategy 5--Ammonia detoxification and uricogenesis

In birds, crocodilians and certain other reptiles, ammonia formed during amino acid catabolism is converted to uric acid for excretion (Campbell, 1991). The formation of the purine ring of uric acid in the cytosol requires the sequential additions of atoms from the precursors glutamine, glycine, HCO_3^- and of one-carbon units as 5, 10-methylene tetrahydrofolate (5, 10-MeFH4) (Campbell, 1995) (Fig. 6). In uric acid formation, the primary ammonia-detoxifying enzyme is GS, which is located in mitochondria of the uricogenic liver (Vorhaben and Campbell, 1972). The discovery that GS is localized in avian and reptilian liver mitochondria, in contrast with its cytosolic localization in mammalian liver (Vorhaben and Campbell, 1972), allowed for the formulation of the uricotelic system for hepatic ammonia detoxification (Campbell, 1991). The remaining enzymes of the purine biosynthetic pathway are localized in the cytosol, possibly as a loosely associated complex (Rowe et al., 1978) or multifunctional protein (Schrimsher et al., 1986).

There are some similarities between ureagenesis and uricogenesis. One of these similarities is that the amino acid exported to the cytosol (in this case, glutamine) is also a neutral molecule. Another similarity is that formation of urate is also energetically expensive and consumes six pyrophosphate bonds from five ATP. However, the exact energetic cost is not known due to the uncertainty with respect to the metabolism of ribose and P-riboses in the uricotelic liver (Mapes and Krebs, 1978).

Fig. 6. Biosynthesis of urate (Modified from Campbell, 1995). The enzymes are (1) glutamine phosphoribosyl pyrophosphate amidotransferase; (2) glycineamide ribonucleotide synthetase; (3) glycineamide ribonucleotide transformylase; (4) formylglycineamide ribonucleotide amidotransferase; (5) aminoimidazole ribonucleotide synthetase; (6) aminoimidazole ribonucleotide carboxylase; (7) N-succinylcarboxamide aminoimidazole ribonucleotide synthetase; (8) adenylosuccinate lyase; (9) aminoimidazole carboxamide ribonucleotide transformylase; (10) IMP cyclohydrase; (11) 5'-ribonucleotide phosphohydrolase; (12) nucleoside phosphohydrolase; (13) xanthine: NAD^+ oxidoreductase; (14) phosphopentomutase and (15) ATP: ribose-5-P pyrophosphotransferase.



Evolutionary divergence of ureotelic and uricotelic tetrapods

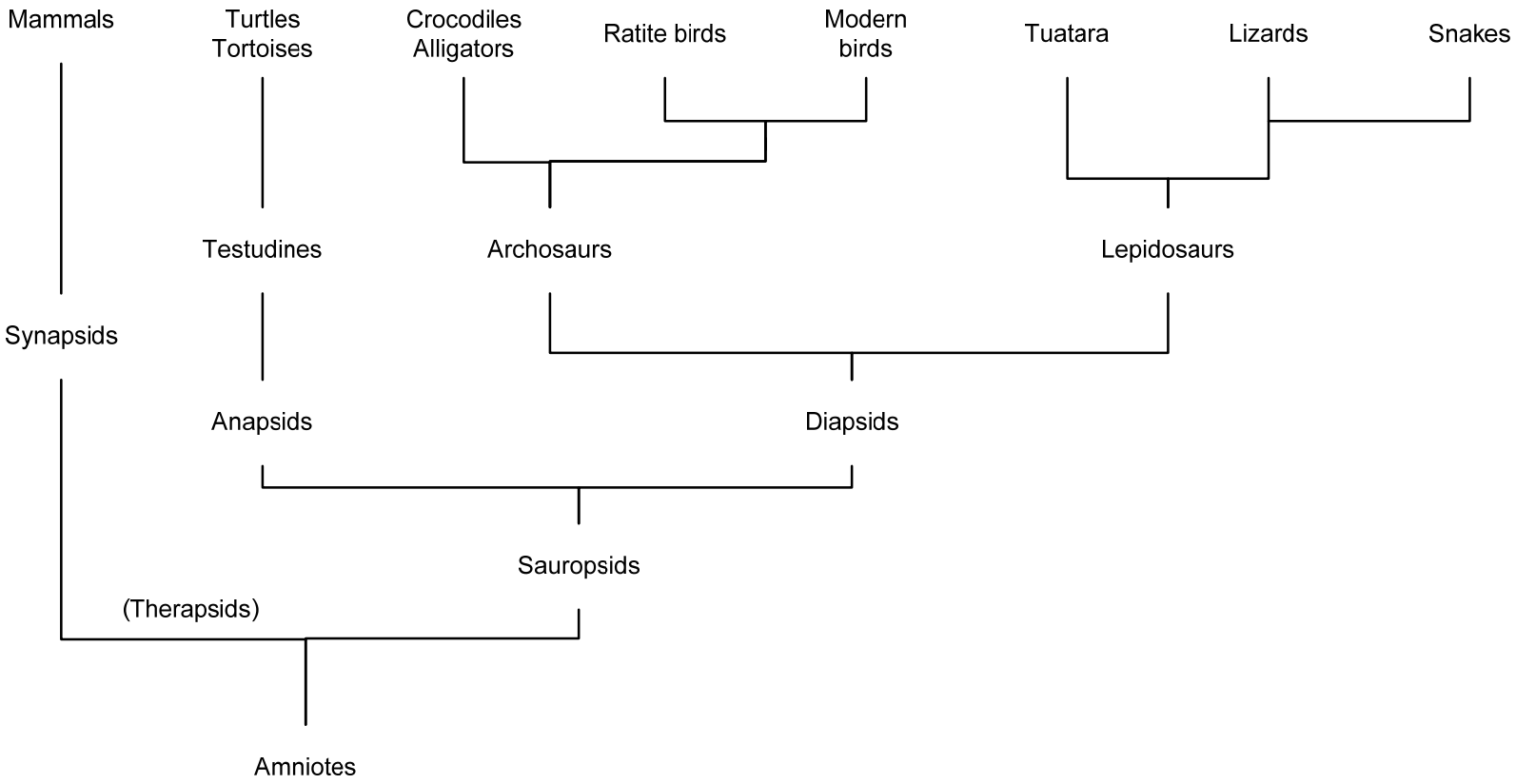
Reptiles have successfully invaded the vast majority of terrestrial, freshwater and even some marine environments of the temperate and tropical zones. The class Reptilia consist of orders Squamata (amphisbaenians, lizards and snakes), Crocodylia (crocodiles and alligators), Rhynchocephalia (tuataras) and Testudines (turtles and tortoises).

The amniote tetrapods may be divided into the mammalian and reptilian-avian lines (Fig. 7). These lines of descent rose from the cotylosauria and were represented primitively by the mammalian-like therapsid reptiles on the one hand and the sauropsid reptiles, on the other (Benton, 1990). The latter included the reptilian ancestors of the dinosaurs and their kin (archosaurs), the primitive turtles (testudines and the ancestors of modern reptiles (lepidosaurs). The mammalian and reptilian lines are generally thought to have diverged around 300 million years ago. The sauropsid line now contains the extant turtles and tortoises, lizards, snakes, the tuatara, alligators, crocodiles and birds (Campbell, 1995).

Terrestrial vertebrates are thought to have originated in the marine environment and used a freshwater route to land (Little, 1983). It is believed that the early amphibians gave rise to the first reptiles, which are considered the first truly terrestrial vertebrates (Little, 1983, 1990).

The main factors that allowed reptiles to colonize the driest of habitats, despite an inability to produce hyperosmotic urine, seem to be a combination of larger size and the development of a great reduction in the permeability of the skin to water (Little, 1990). Their low permeability is due to a barrier to water loss in the inner layer of the stratum corneum, which contains both keratin and phospholipid (Lillywhite and Maderson, 1982).

Fig. 7 Phylogeny of the amniote tetrapods (From Benton, 1990)



The transition to land, which necessitates conservation of water, limited the role of ammonia in nitrogenous excretion due to its high toxicity. Only aquatic species can take advantage of the energy savings of excreting ammonia by diluting their waste ammonia in large volumes of water (Walsh and Mommsen, 2001). In terrestrial animals, ammonia is detoxified to urea or uric acid depending on access to water. An added advantage of urea is that it can be used as an osmolyte to prevent water loss.

The ability of primitive fish to synthesize urea as an osmolyte and the presence of ureoteley in terrestrial amphibians suggests that this was the primitive mode of nitrogen excretion in early amniote tetrapods (Campbell, 1995). This capacity to synthesize urea preadapted the early vertebrates for their transition to land (Campbell, 1973). The synthesis of less toxic urea prevented endogenous ammonia from reaching harmful levels in a situation where availability of water for excretion is limited.

Subsequently, the changing water relationships may have spurred the transition to uricotelism (Campbell, 1973). During the late Triassic, it became increasingly arid and one of the factors that may have contributed to the marked radiation of the archosaurs at this time was the ability to cope with this aridity (Tucker and Benton, 1982). Most of the large continental mass of Pangaea was distributed along the equator during the Triassic, making for predominantly arid tropical or subtropical environments (Parrish et al., 1986). The development of uricoteley as a means of detoxifying ammonia, along with a cloaca with high postrenal reabsorptive capacity as a water-conserving mechanism, may have been a critical part of the physiological adaptation to these environments (Robinson, 1971). Excretion of uric acid only requires minimal amounts of water, compared to urea, because this

compound contributes little to urine osmolality (McNabb and Poulson, 1970). Whereas the concentrating ability of the archosaurian kidney is less than that of certain desert mammals (Dantzler, 1982), the postrenal reabsorption of water and electrolytes by the lower intestine and cloaca in reptiles, birds and crocodilians result in the final excretion of a semisolid paste of urate containing a minimal amount of water (Coulson and Hernandez, 1970; Schmidt-Nielsen, 1988; Skadhauge, 1981). In those tetrapod vertebrates capable of "facultative uricoteley", as defined by a shift toward increased percentage urate nitrogen excreted, this transition always occurs during conditions of water restriction. Examples include treefrogs (Loveridge, 1970; Drewes et al., 1977; Shoemaker and Bickler, 1979), tortoises (Khalil and Haggag, 1955), certain lizards (Dessauer, 1952; Perschmann, 1956), the tuatara (Schmidt-Nielsen and Schmidt, 1973) and crocodilians (Coulson and Hernandez, 1970). Thus, uricotelism represents a complete adaptation to the terrestrial environment.

Excretory nitrogen metabolism in reptiles

Animals can be classified on the basis of which compounds predominate in their excreta; those excreting mainly ammonium ion, urea or uric acid are ammonotelic, ureotelic or uricotelic respectively (Campbell, 1973). Members of the Reptilia exhibit the greatest plasticity among vertebrates with respect to excretory function, because different species exhibit the three major types of nitrogen metabolism, and some undergo transitions between ammonotelism and ureotelism, ammonotelism and uricotelism, and ureotelism and uricotelism (Campbell 1995). Although, there is great variation among the orders, some generalizations can be made about the major component of their excreta.

Squamata: Neither snakes nor lizards excrete a significant amount of excretory-N as urea nor do they, in general, excrete much ammonia-N. All attempts

to demonstrate a complete set of urea cycle enzymes in squamate reptiles have yielded negative results. CPS I activity is especially undetectable as is, in most cases, ASS activity (Rogers, 1966; Brown et al., 1960; Mora et al., 1965; Baby and Reddy, 1980). Low levels of OTC, ASS and arginase have been found in liver of the lizard *Ctenosaura pectinata* (Mora et al., 1965), but this may reflect the situation in isolated species of Squamata. Neither OTC nor ASS could be detected in other snakes and lizards and hepatic arginase levels are generally low, typical of uricoteles. A large number of squamate reptiles are adapted to very arid environments and the excretion of urate as the major nitrogenous end product reflects this adaptation. In their transition to uricotelism, the urea cycle appears to have been rendered nonfunctional by "silencing" the CPS I gene (Campbell, 1995). A hepatic GS is expressed in garter snakes of the genus *Thamnophis* in levels higher than those in the facultatively uricotelic tortoises but comparable to those in birds. This GS is localized in hepatic mitochondria (Vorhaben and Campbell, 1972) and presumably functions as the sole ammonia-detoxifying enzyme for uricogenesis. There is very little ammonia-N excreted by the squamate reptiles, in contrast to the uricotelic archosaurs.

Crocodylia: Crocodiles, alligators and the caiman also excrete very little urea-N and are either ammonotelic or uricotelic, depending on the water load. The American alligator, *Alligator mississippiensis*, excretes near equal parts of ammonia- and urate-N in its normal habitat. When forcibly hydrated, ammonia becomes the main end product; when dehydrated, urate excretion predominates (Coulson and Hernandez, 1970). CPS I is not detectable in liver of the American alligator (Smith and Campbell, 1987), which does contain relatively high levels of GS activity. GS is mitochondrial in alligators (Lemieux et al., 1984; Smith and Campbell, 1987) and is expressed in all hepatocytes (Smith and Campbell, 1987, 1988). Thus, although

crocodilians are predominantly ammonotelic when water loaded, they are constitutively uricotelic with respect to their hepatic ammonia metabolism.

Rhyncocephalia: The sole representative of the rhyncocephalia, the tuatara, is predominantly uricotelic (Schmidt-Nielsen and Schmidt, 1973). It also excretes a significant amount of urea-N. The percentage of urea-N appears to increase with dietary protein intake whereas the percentage urate-N decreases (Hill and Dawbin, 1969). Although a CPS activity has not been reported in the tuatara, the activities of other urea cycle enzymes are present in liver at levels comparable to those of strict ureoteles (Hill and Dawbin, 1969).

Effects of environmental stresses on excretory nitrogen metabolism and other relevant physiological processes in reptiles

Feeding: A strategy taken to prevent postprandial ammonia toxicity is to increase transamination and synthesis of certain non-essential amino acids. Crocodylia are capable of eating amounts greater than 20% of their body weight at a single meal (Coulson et al., 1950), which represents a substantial amount of excess N. In the caiman post-feeding, 80% of the increase in amino acids in both the extracellular and intracellular fluids was attributed to glutamine, glycine and alanine. This amino acid composition does not resemble that of the food eaten and hence substantial transamination must have occurred. These amino acids ultimately carry the excess N to the liver, where it can be incorporated into uric acid or to the kidney where it can be excreted as ammonia (Coulson and Hernandez, 1967). Similarly, in chameleons post-feeding, 74% of the increased levels of amino acids was due to glycine, alanine, glutamine and glutamate with glycine alone accounting for 44% of the total (Coulson and Hernandez, 1968). *Alligator mississipiensis* also exhibited

considerable increases in glutamine, glutamate, glycine and alanine post-feeding (Herbert and Coulson, 1976).

Another strategy taken post-feeding is to increase excretion of nitrogenous waste to prevent harmful accumulations in the body. The Burmese python, *Python molurus*, is a carnivore that consumes large animals at infrequent intervals (Secor and Diamond, 1998). Following a large protein meal, there are increases by 50-100% in the masses of the kidneys, heart and lungs (Secor and Diamond, 1997, 1998). This increase in mass is presumably related in part to the increased requirement for renal excretion following increased production of nitrogenous waste.

Salinity stress: The osmotic stress of increased salinity is counteracted in several ways. For the whole body, the major strategy taken by marine and many estuarine reptiles, which cannot elaborate hyperosmotic urine, is excretion through the salt glands. Several groups of marine and estuarine reptiles have independently evolved salt-secreting glands (Minnich, 1982), resulting in five non-homologous structures that function as salt glands; the nasal gland of marine and estuarine lizards (e.g. *Amblyrhynchus cristatus*, *Varanus semiremex*), the lachrymal gland of marine turtles (Cheloniidae and Dermochelyidae) and the emydid diamondback terrapin (*M. terrapin*), the posterior sublingual gland of sea snakes (Hydrophiidae) and the acrochordid little file snake (*Chersydrus granulatus*), the premaxillary gland of the marine homalopsid snake *Cerberus rhynchops*, and the lingual glands of *Crocodylus porosus*. The salt glands of marine reptiles excrete mainly Na and Cl.

Increased levels of plasma osmolarity, $[Na^+]$, $[Cl^-]$ and urea may help to decrease the gradient for osmotic water loss. Marine turtles, such as *Caretta caretta* (Dessauer, 1970; Minnich, 1982) and *Chelonia mydas* (Holmes and Mcbean, 1964; Minnich, 1982), have relatively high plasma osmotic concentrations (270-465

mosmol l^{-1}) compared to freshwater and terrestrial turtles. Much of this difference in *C. caretta* (Dessauer, 1970; Minnich, 1982) is due to high levels of [Na^+] (166 mM), [Cl^-] (110 mM) and urea (30 μmol ml^{-1} plasma). When the freshwater *Mauremys leprosa* and the estuarine *M. terrapin* are acclimated to seawater, the levels of plasma osmolarity, Na, Cl and urea increases to reach levels reminiscent of that in the marine turtles. For *M. leprosa* acclimated to seawater for 15 days, the plasma osmolarity increases from 362 to 534 mosmol l^{-1}, [Na^+] from 146 to 161 mmol l^{-1}, [Cl^-] from 88 to 129 mmol l^{-1} and urea from 4.7 to 26 μmol ml^{-1} plasma (Minnich, 1982). For *M. terrapin* acclimated to seawater, the plasma osmolarity increases from 300 to 459 mosmol l^{-1}, [Na^+] from 130 to 174 mmol l^{-1}, [Cl^-] from 90 to 140 mmol l^{-1} and urea from 22 to 115 μmol ml^{-1} plasma (Gilles-Baillien, 1970; Robinson and Dunson, 1976). However, it appears that the increased osmotic concentrations in *M. terrapin* that accompany acclimation to seawater apparently result mainly from water loss rather than a gain of NaCl (Robinson and Dunson, 1976).

Individual cells in various tissues also have to make metabolic and osmotic adjustments. The main substances accumulated as intracellular solutes are amino acids and urea (King and Goldstein, 1983). Accumulation of amino acids in this case can also serve as metabolic energy sources for oxidation to provide ATP for osmoregulation (Ballantyne and Chamberlin, 1988). *Malaclemys terrapin* when acclimated to seawater increases urea, taurine and amino acids substantially in its muscle (Gilles-Baillien, 1973).

Other strategies taken by reptiles include behavioral regulation. Estuarine snakes *Nerodia fasciata* in comparison to the freshwater species, do not drink seawater and can survive indefinitely in seawater. Other marine reptiles, such as sea snakes, marine turtle and crocodiles also do not appear to drink seawater (Dunson,

1969b). These species obtain most of their water from their food, which is osmotically less concentrated that seawater (Minnich, 1982). In addition, estuarine reptiles, such as *M. terrapin* may move into freshwater periodically to rehydrate. In captivity, it has been observed that *M. terrapin*, returned to freshwater after seawater acclimation, drinks up to 15% of its body weight to rehydrate and excrete excess salt (Cowan, 1981). This is in spite of it having a functional salt gland. The salt gland of *M. terrapin* tends to be stimulated by high salinity and has a lower capacity as that in marine turtles (Holmes and McBean, 1964; Dunson, 1969b). As such, its salt gland may not be able to indefinitely prevent the extracellular Na concentration from rising above a certain level without drinking (Dunson, 1970). Sometimes, the sheer size of the animal allows them to take advantage of saline environments. *Trionyx triunguis* are occasionally found in the eastern Mediterranean Sea. As they lack salt glands, only adults are able to survive in seawater (Minnich, 1982) and their survival may depend on an integument that has a low permeability to salt and water (Lillywhite and Maderson, 1982).

Emersion: For *T. scripta* prevented access to water for up to 40 h, mild dehydration decreases glomerular filtration rate and increases tubular reabsorption of water. Increases of only 20 mosmol kg^{-1} caused a complete cessation of renal function in this aquatic turtle and caused anuria (Dantzler and Schmidt-Nielsen, 1966). In contrast, the desert tortoise, *Gopherus agassizii* prevented access to water for 6 weeks, does not cease renal function as a result of moderate increase in plasma osmolality and only becomes anuric when blood osmolality is increased by more than 100 mosmol kg^{-1}. Being uricotelic, it is able to continue producing hypoosmotic urine, which is stored in the bladder and not voided. Within the bladder, urates precipitate out and urine equilibrates with the blood, allowing it to store an insoluble

nitrogenous waste and yet conserve water simultaneously (Dantzler and Schmidt-Nielsen, 1966). Similarly, the uricotelic gopher tortoise, *Gopherus polyphemus*, is able to greatly reduce urinary water loss (Ross, 1977).

The snake-necked turtle, *Chelodina longicollis*, left to dehydrate for 20 days, increases the percentage of ammonia and uric acid in the urine and at the same time, decreases the percentage of urea in the urine. However, it still remains ureotelic through the course of the experiment. During dehydration, the volume of urine in the bladder of *C. longicollis* decreased from a mean of 32 to 2.0 ml, showing that water was reabsorbed from the bladder (Rogers, 1966). Thus, in general, water loss can be limited by reduction of urine volume voided. The desert tortoises have an additional advantage in that they are uricotelic, which allows them in comparison to conserve the most water with the least toxic nitrogenous waste.

Another way to conserve water is to decrease pulmonary water loss by increasing oxygen extraction from air. This strategy has been observed in the chuckwalla, *Sauromalus obesus*, the royal snake, *Spalerosophis cliffordii* and the asp *Cerastes cerastes* (Bentley, 1976). Osmotic stress during drought is also alleviated in some terrestrial lizards by secretion of excess salt from the salt glands (Minnich, 1982).

Excretory nitrogen metabolism in testudines

In general, tortoises excrete approximately equal amounts of urea-N and urate-N. However, the urea to urate ratio exhibits large variations. For example, the Berlandier's tortoise, *G. berlandieri* can be primarily ureotelic or uricotelic in its excretion pattern. Desert tortoises, *G. agassizii* and *G. berlandieri* express CPS I and GS in the liver mitochondria (Campbell et al., 1985). Hepatic mitochondria from both species form and release both citrulline and glutamine to the medium *in vitro*. Both

the ureotelic and uricotelic hepatic ammonia-detoxifying systems are therefore present and functional in tortoise liver tissue. The main physiological stimulus for a switch to increased urate-N excretion by tortoises appears to be water restriction or dehydration (Dantzler and Schmidt-Nielsen, 1966). Thus, tortoises are classified as facultative ureouricoteles on the basis not only of their partitioning of excretory-N, but also for the duality in their capacity to detoxify ammonia in liver tissue (Campbell, 1995).

Given that reptiles have terrestrial origins, the subsequent evolution of the Order Testudines has seen a remarkable proliferation of aquatic taxa. And it is believed that the marine turtles have recolonized the marine environment via a freshwater route (Pritchard, 1997).

In contrast to terrestrial tortoises, aquatic turtles in general, are primarily ureotelic in terms of their partition of excretory-N. Many species examined so far have a complete complement of hepatic urea cycle enzymes (Brown et al., 1960; Mora et al., 1965; Baze and Horne, 1970). However, at present the levels of CPS I activity is not known for soft-shelled species. No GS activity can be detected in the liver of either aquatic or semi-aquatic turtles (Wu, 1963, Campbell, 1995), so CPS I appears to be the sole ammonia-detoxifying enzyme in these species. The levels of most urea cycle enzymes in turtle liver are comparable to those in rat liver, especially if assay temperature differences and overall metabolic rate differences between poikilotherms and homeotherms are taken into account (Campbell, 1995). Turtles also excrete a significant amount of ammonia-N, which in some cases, is the predominant form of excretory-N. Aquatic turtles should therefore be considered to be facultative ammonoureoteles (Campbell, 1995).

The great variation in nitrogen metabolism within Reptilia is believed to come about due to the OUC becoming dysfunctional in the reptilian line leading to the birds, and this may also have occurred in the reptilian line giving rise to Squamata and Crocodylia. A functional OUC remains present only in extant lungfishes, coelacanths, amphibians, and the testudinid and rhynchocephalid reptiles (Campbell 1973).

Pelodiscus sinensis – *delving into unknown territory*

Generally speaking, the study of reptiles has been neglected in comparison to other species, such as those in the class Mammalia. The studies done on excretory nitrogen metabolism in soft-shelled turtles remains small in number. One study found activities of 4 enzymes of the OUC (OTC, ASS, ASL and arginase) in the liver of *A. mutica* (Baze and Horne, 1970). However, CPS I activity has not been measured in soft-shelled species and remains unknown. Another study found that when *A. spinifera* was acclimated to 0.7% seawater for 11 weeks, the plasma osmolality increases from 252 to 281 mosmol l^{-1}, $[Na^+]$ from 113 to 126 mmol l^{-1} and urea from 2.0 to 3.5 μmol ml^{-1} plasma (Seidel, 1975). There is little known about soft-shelled species and in particular, very little research has been done on the Chinese soft-shelled turtle, *P. sinensis*, and none on its excretory nitrogen metabolism. Thus far, research on *P. sinensis* has focused on a few limited aspects. Many of the studies carried out involve its importance as a farmed species. They focused on the composition of feed for *P. sinensis* (Huang and Lin, 2002; Zhou et al., 2003, 2004; Huang et al., 2005; Lei, 2006), diseases affecting it (Shortridge, 1975; Chen et al., 1999; Zhou et al., 2002; Orós et al., 2003; Hsieh et al., 2006), incubation of its eggs (Du and Ji, 2003) and the benefits of *P. sinensis* as a food item (Yao and Li, 2005; Yin et al., 2005; Tan et al., 2006). Another linked aspect is the cloning, sequencing

and expression of genes of interest (Zhang et al., 2000; Chien et al., 2005, 2006), such as those for growth hormone receptor (Zhang et al., 2000) and follicle stimulating hormone (Chien et al., 2005). Another area of interest is the buccopharyngeal region (Yokosuka et al., 2000a, b) and its capacity for salt uptake (Yokosuka et al., 2000a; Yoshie et al., 2000). The last three popular aspects focus on its aquatic respiration (Wang et al., 1989; Hua and Wang, 1993; Yokosuka, 2000a), phylogeny (Mao and Chen, 1982; Liao et al., 2001; Matsuda et al., 2005) and development (Kuraku et al., 2005; Ohya et al., 2005).

A summary of the evolution of the Reptilia first involved the colonization of land, followed by a return to freshwater and finally invasion of the marine environment (Little, 1983, 1990; Pritchard; 1997). This transition to land would first require strategies to deal with acute ammonia toxicity in lieu of a dilute watery environment in which to excrete ammonia. In addition, the carnivorous nature of early inhabitants would levy additional stress on animals, as they would have to deal with a postprandial surge of ammonia (Walsh and Mommsen, 2001). Secondly, this transition would require adaptations to retain and/or prevent water loss. Similarly, when the marine environment was invaded, osmotic stress remains a problem due to seawater being hypertonic and hyperionic to the animals' contents. Together, these constituted the main thrust for the author to undertake this project on the excretory nitrogen metabolism in *P. sinensis*. *Pelodiscus sinensis* is an appropriate specimen for this study because it can survive on land for an extended period and acclimate to brackish water in spite of being a soft-shelled aquatic freshwater turtle. In addition, preliminary studies in the author's laboratory revealed that, unlike other animals, there was no postprandial ammonia surge in *P. sinensis* which could be related to its

being ureogenic, although confirmation on the presence of a functional OUC in its liver is lacking.

CHAPTER 1. FEEDING

MATERIALS AND METHODS

Procurement and maintenance of animals

Specimens of *P. sinensis* were purchased from a turtle farm in Johore Bahru, Malaysia and transferred to the National University of Singapore. Animals were maintained individually in plastic aquaria (L30 cm x W30 cm x H25 cm) containing 8 l of freshwater at 25^{o}C in the laboratory, and water was changed daily. No attempt was made to separate the sexes. The turtles were acclimated to laboratory conditions for at least 1 week. During the adaptation period, they were fed with meat from the tiger prawn, *Panaeus monodon* until satiated. Food was withdrawn prior to experiments. All experiments were performed under a 12h:12h dark:light regime.

Analysis of N and carbon (C) contents in feed

The wet masses of prawn meat were obtained to the nearest milligram. Samples of prawn meat were then freeze-dried and the dry masses recorded. Subsequently, they were analyzed for N and C by a Eurovector EA3011 Elemental Analyzer (Milan, Italy) equipped with the Callidus software. BBOT ($C_{26}H_{26}N_2O_2S$) standard obtained from Eurovector was used as a standard for comparison. In addition some samples were extracted in 15 volumes (w/v) of 70% ethanol for 24 h to remove non-protein N-compounds (MacInnis, 1970) before being freeze-dried for N and C analyses. The difference between samples obtained with and without 70% ethanol extraction gives an indication to the combined contribution of ammonia, urea, FAAs, purines and pyrimidines to the N and C contents of the prawn meat.

Determination of ammonia, urea, FAAs and protein amino acids (PAAs) in feed

In order to determine contents of ammonia, urea, FAAs and PAAs in prawn meat, samples of prawn meat were weighed, ground to a powder in liquid nitrogen,

and homogenized three times (20 s each with 10 s intervals) in 5 volumes (w/v) of 6% trichloroacetic acid at 24,000 rpm using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 10,000 *g* and 4°C for 15 min to obtain the supernatant and precipitated proteins. The supernatant obtained was kept at -25°C until analysis.

An aliquot of the supernatant was used for ammonia and urea assay after the pH was adjusted to 5.5-6.0 with 2 mol l^{-1} $KHCO_3$. Total ammonia was analyzed using the enzymatic method of Bergmeyer and Beutler (1985). The change in absorbance was monitored at 340 nm using a Shimadzu UV-1601 spectrophotometer with CPS controller. Freshly prepared NH_4Cl solution was used as a standard for comparison. Urea was determined according to the method of Jow et al. (1999). The difference in absorbance obtained from the samples with and without urease treatment was used for the estimation of the urea concentration in the sample. A urea standard was processed through the same procedure for comparison.

Another aliquot of the supernatant was used for FAAs analysis; the supernatant obtained was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). FAAs were analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column.

For PAAs, the precipitated proteins were hydrolyzed with 4 mol l^{-1} methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce, USA) under vacuum in Pierce hydrolysis tubes at 115°C for 22 h according to the method of Simpson et al. (1976). The hydrolysate was centrifuged, adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2) to be analyzed by the Shimadzu LC-6A amino acid analysis system.

Although complete FAA and PAA analyses were performed on the prawn meat, only the contents (μmol g^{-1} wet mass) of free and protein-bound arginine (which would produce urea after argininolysis), total FAA (TFAA) and total PAA were presented in this thesis.

Feeding the animals

Pelodiscus sinensis ranging between 264 to 507 g body mass were used in this series of experiments. Food was withdrawn 7 days prior to experiments, which gave sufficient time for the gut to be emptied and a high probability that feeding would occur.

Turtles were divided into two groups. The first group of control turtles was not fed while the second group of experimental turtles was allowed to feed on 2.0% body mass of prawn meat *ad libitum*. Time 0 h for the experiment was considered to commence when the turtle stopped feeding upon satiation. The fed and control (72 h) turtles were gently transferred to individual tanks containing 2 l of freshwater. The actual mass of food consumed by the turtle was then calculated by subtracting the mass of any left over food from the initial mass of food given to the turtle. During the course of the experiment, turtles were maintained at 25°C in the laboratory, under a 12h:12h dark:light regime.

Collection of water samples for analyses

Water samples (3 ml) for unfed (*N*=5) and fed (*N*=5) turtles were collected at hours 0, 12, 24, 36, 48, 60 and 72 during the subsequent 3-day post-feeding, and acidified with 70 μl of 1 mol l^{-1} HCl. These samples were spun at 5,000 *g* for 5 min before being kept at 4°C until analysis. Randomly selected water samples were collected in duplicate left at 25°C for 24 h and then acidified and stored at 4°C until analysis. These served as a control for microbial activity, and results obtained

confirmed that the ammonia and urea concentration remained relatively unchanged after 24 h of incubation at 25^{o}C.

Determination of ammonia and urea concentrations in water samples

Ammonia and urea concentrations in water samples were determined as described by Jow et al. (1999). Ammonia was determined colorimetrically using freshly prepared NH_4Cl solution as the standard. Urea was determined colorimetrically according to the method of Jow et al. (1999) using a prepared urea standard for comparison. The rates of ammonia or urea excreted were expressed as μmol N h^{-1} g^{-1} mass of the turtle.

Collection of tissue samples for analyses

The control (0 h or 72 h unfed; *N*=5 each) and experimental turtles (12, 24, 36, 48 or 72 h post feeding; *N*=5 each) were killed by a strong blow to the head. Blood samples were collected by cardiac puncture into heparinized syringes, and centrifuged at 5,000 *g* and 4^{o}C for 5 min to obtain the plasma. The plasma was deproteinized by the addition of an equal volume (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10,000 *g* and 4^{o}C for 15 min. The resulting supernatant was kept at -25^{o}C until analysis. The muscle, liver, stomach, intestine and brain were quickly excised. The stomach and intestine was removed, flushed well with water, and divided into 2 halves longitudinally. The excised tissues and organs (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs (Faupel et al. 1972). Frozen samples were kept at -80^{o}C until analysis.

Determination of ammonia, urea and FAAs in tissue samples

Ammonia, urea and FAA contents in tissue samples were determined as described above on page 47. The TFAA content was calculated by the summation of all FAAs, while total essential (TEFAA) content was calculated as the sum of

histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine contents. Results were expressed as μmol g^{-1} tissue or μmol ml^{-1} plasma.

Determination of OUC enzyme and GS activities

Preliminary results indicated that OUC enzymes were present only in the liver of *P. sinensis*. The liver of fed and unfed *P. sinensis* were excised quickly and homogenized three times (20 s each with 10 s intervals) in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT and 0.5 mmol l^{-1} PMSF using an Ultra-Turrax homogenizer. The homogenate was sonicated (110 W, 20 kHz; Misonix Incorporated Farmingdale, NY, USA) three times for 20 s each, with a 10 s break between each sonication. The sonicated sample was centrifuged at 10,000 *g* and 4°C for 15 min. After centrifugation, the supernatant was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with the extraction buffer without EDTA and PMSF. The filtrate obtained was used directly for enzyme assays.

CPS I (E.C. 2.7.2.5) activity was determined with NH_4^+ as a substrate in the presence of NAG according to the method of Anderson and Walsh (1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). Enzyme activity was expressed as μmol [^{14}C]urea formed min^{-1} g^{-1} tissue.

OTC (E.C. 2.1.3.3) activity was determined by combining the methods of Anderson and Walsh (1995) and Xiong and Anderson (1989). Absorbance was measured at 466 nm using a Shimadzu 160 UV VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan). Enzyme activity was expressed as μmol citrulline formed min^{-1} g^{-1} tissue.

ASS (E.C. 6.3.4.5) and ASL (E.C. 4.3.2.1) activities were determined together (ASS + ASL) assuming that both were present, by measuring the formation of [^{14}C]fumarate from [^{14}C]aspartate using the method of Cao et al. (1991). Radioactivity was measured using a Wallac 1414 liquid scintillation counter. ASS + ASL activity was expressed as μmol [^{14}C]fumarate formed min^{-1} g^{-1} tissue.

Arginase (E.C. 3.5.3.1) was assayed as described by Felskie et al. (1998). Urea was determined as described above on page 47. Arginase activity was expressed as μmol urea formed min^{-1} g^{-1} tissue.

GS (E.C. 6.3.1.2) activity was determined colorimetrically according to the method of Shankar and Anderson (1985). GS activity was expressed as μmol γ-glutamyl hydroxamate formed min^{-1} g^{-1} tissue. Freshly prepared glutamic acid monohydroxamate solution was used as a standard for comparison.

Determination of plasma volume and wet masses of various tissues and organs

Turtles (*N=4*) were weighed with a Shimadzu animal balance (Shimadzu Co., Kyoto, Japan) and the mass recorded to the nearest 0.1 g. The blood volume was estimated according to the method of Thorson (1968). A known quantity of T-1824 (Evans blue dye) was injected into the bloodstream through cardiac puncture. Over a period of 20-40 min, which permitted complete circulation of the dye, three or four blood samples were drawn to make colorimetric comparison of the diluted dye with standard solutions prepared with plasma for calculation of plasma volume. The liver, stomach, intestine, and brain were excised and their wet mass obtained to the nearest 0.1 g. Then, muscle was removed as much as possible from the skeleton and carapace to estimate the total muscle wet mass.

Statistical analyses

Results are presented as means ± standard error of the mean (S.E.M). Data in Fig. 8 was analyzed using 2-way repeated-measures ANOVA followed by least-square means (LSMEANS) to evaluate differences between means. Data in tables were assessed using one-way analysis of variance followed by Bonferroni's multiple range test to evaluate differences between means. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Feed analysis

For every 1 g wet mass of prawn meat (*N*=3), there was 0.25 g of freeze-dried materials, of which 12.4 ± 0.15% and 42.5 ± 1.5% of the dry mass were N and C, respectively. After ethanol extraction, the percentages of dry mass being represented by N and C were 11.1 ± 0.28 and 39.8 ± 2.5, respectively, indicating that proteins were the major contributor of N. The ammonia and urea contents (μmol g^{-1} wet mass) of prawn meat were 7.6 ± 0.2 and 0.94 ± 0.09, respectively. The free and protein-bound arginine contents were 36 ± 1 and 111 ± 1, respectively. The total PAA content (1843 ± 25 μmol g^{-1} wet mass) was 11-fold greater than the TFAA content (162 ± 2 μmol g^{-1} wet mass).

Food intake of experimental animals

The averaged body mass of *P. sinensis* (*N*=25) and the wet mass of prawn meat ingested were 316 ± 7 g and 5.4 ± 0.2 g, respectively. So the food ration represented 1.73 ± 0.08% of the body mass.

Activities of OUC enzymes and GS

A full complement of OUC enzymes was present in the liver of *P. sinensis* (Table 1). CPS I activity was detected because it utilized ammonia as a substrate in the presence of NAG (Table 1). Judging by activities, both CPS I and ASS + ASL could be rate-limiting in the hepatic OUC in this turtle. The GS activity present in the liver of *P. sinensis* was low. There were no significant changes in activities of various hepatic OUC enzymes and GS after 24 h of feeding (Table 1), when there were peaks in urea excretion and tissues urea contents.

Table 1 Activities (μmol min^{-1} g^{-1} liver) of carbamoyl phosphate synthetase I (CPS I), ornithine transcarbamylase (OTC), argininosuccinate synthetase + lyase (ASS+ASL), arginase, and glutamine synthetase (GS) in the liver of *Pelodiscus sinensis* without food (unfed control) or 24 h post-feeding.

Enzymes	Activities (μmol min^{-1} g^{-1} liver)	
	Unfed	24 h post-feeding
CPS I (with NAG)	1.2±0.2	0.93±0.08
CPS I (without NAG)	n.d.	n.d.
OTC	66±4	60±7
ASS + ASL	0.36±0.03	0.36±0.04
Arginase	234±25	235±47
GS	1.8±0.3	1.1±0.3

Values represent means ± S.E.M, *N*=5.

NAG, N-acetyl-L-glutamate; n.d., not detectable (detection limit = 0.001 μmol min^{-1} g^{-1} liver).

Effects of feeding on tissue ammonia and urea contents

There were significant increases in ammonia contents in the stomach at hour 24 and in the intestine between hours 12 and 36 (Table 2); but, ammonia contents in the liver, muscle, brain and plasma remained unchanged throughout the 72 h post-feeding (Table 2). In contrast, urea contents in the stomach, intestine, liver, muscle, brain and plasma increased significantly by 2.9-, 3.5-, 2.6-, 2.9-, 3.4 and 3.0-fold, respectively, at hour 24 (Table 3).

Effects of feeding on contents of FAAs in the brain, liver and muscle

At hour 24 post-feeding, there was a significant increase (1.8-fold) in the brain glutamine content in *P. sinensis* (Table 4). In spite of significant changes in contents of arginine, glycine, histidine isoleucine, leucine, phenylalanine, serine and valine, there were no significant changes in the contents of TFAA and TEFAA in the brain during the 72-h period post-feeding (Table 4). In contrast, there were significant increases in the TFAA content at hour 24 and the TEFAA content at hours 12 and 24 in the liver (Table 5). This was mainly due to increases in contents of glutamate and alanine (Table 5), with minor but significant increases in contents of aspartate, glycine, isoleucine, leucine, phenylalanine, proline, serine, threonine and valine. Histidine and tryptophan, which were below the detection limit (0.001 μmol g^{-1} tissue) in the liver of the unfed control, became detectable at the level of 0.38 ± 0.03 and 0.07 ± 0.01 μmol g^{-1}, respectively, 24 h after feeding. As for the muscle, feeding led to increases in contents of alanine, glutamine, glycine (Table 6) and several essential amino acids (isoleucine, leucine, lysine, phenylalanine, threonine and valine). Overall, the muscle TFAA content remained relatively constant after feeding, but a significant increase in the muscle TEFAA content occurred at hours 12, 24 and 36 (Table 6).

Table 2 Ammonia contents (μmol g^{-1} tissue) in various tissues of *Pelodiscus sinensis* during the 72-h period post-feeding.

	Ammonia contents (μmol g^{-1} tissue)						
	Unfed	Fed					Unfed
Tissue	0 h	12 h	24 h	36 h	48 h	72 h	72 h
Brain	1.3±0.1	1.6±0.1	1.3±0.2	1.9±0.9	1.4±0.2	1.5±0.3	1.3±0.2
Intestine	1.2±0.1^{ab}	2.3±0.2^{cd}	2.7±0.2^{d}	2.1±0.2^{cd}	1.9±0.2^{bc}	1.0±0.1^{a}	1.2±0.1^{ab}
Liver	3.1±0.4	4.1±0.7	3.9±0.6	2.6±0.4	2.0±0.2	2.3±0.3	3.1±0.3
Muscle	0.74±0.03	0.68±0.06	0.67±0.08	0.66±0.13	0.55±0.07	0.69±0.11	0.70±0.09
Plasma	0.43±0.02	0.39±0.05	0.43±0.06	0.47±0.04	0.57±0.10	0.63±0.05	0.50±0.09
Stomach	0.74±0.03^{ab}	0.88±0.09^{abc}	1.3±0.2^{c}	0.84±0.04^{abc}	1.1±0.1^{bc}	0.79±0.07^{ab}	0.54±0.03^{a}

Values represent means± S.E.M, N=5, except for the 72 h unfed muscle value (N=4).

Means of contents not sharing the same letter are significantly different, P<0.05.

Table 3 Urea contents (μmol g^{-1} tissue) in various tissues of *Pelodiscus sinensis* during the 72-h period post-feeding.

	Urea contents (μmol g^{-1} tissue)						
	Unfed	Fed					Unfed
Tissue	0 h	12 h	24 h	36 h	48 h	72 h	72 h
Brain	0.72±0.17^{a}	1.1±0.2^{a}	2.5±0.5^{b}	1.3±0.2^{a}	0.71±0.16^{a}	0.44±0.11^{a}	0.17±0.02^{a}
Intestine	0.60±0.12^{ab}	1.2±0.2^{b}	2.1±0.4^{c}	1.0±0.1^{ab}	0.68±0.20^{ab}	0.18±0.03^{a}	0.42±0.10^{ab}
Liver	0.86±0.29^{a}	1.4±0.3^{ab}	2.2±0.4^{b}	1.0±0.2^{ab}	0.96±0.40^{ab}	0.53±0.16^{a}	0.19±0.05^{a}
Muscle	0.79± 0.14^{ab}	1.4±0.2^{bc}	2.3±0.4^{c}	1.3±0.2^{ab}	0.91±0.26^{ab}	0.58±0.16^{ab}	0.23±0.03^{a}
Plasma	0.96±0.13^{ab}	1.8±0.3^{bc}	2.9±0.6^{c}	1.5±0.2^{ab}	1.2±0.3^{ab}	0.61±0.18^{ab}	0.41±0.06^{a}
Stomach	0.66±0.11^{ab}	1.1±0.2^{b}	1.9±0.3^{c}	1.2±0.2^{bc}	0.75±0.18^{ab}	0.48±0.10^{ab}	0.23±0.03^{a}

Values represent means±S.E.M., N=5, except for the 72 h unfed brain value, N=4.

Means of contents not sharing the same letter are significantly different, P<0.05.

Table 4 Contents (μmol g^{-1} brain) of various free amino acids (FAA), total FAA (TFAA) and total essential FAA (TEFAA) in the brain of *Pelodiscus sinensis* during the 72-h period post-feeding.

	FAA contents (μmol g^{-1} brain)						
	Unfed	Fed					Unfed
FAA	0h	12h	24h	36h	48h	72h	72h
Alanine	0.37±0.05	0.35±0.05	0.28±0.01	0.22±0.03	0.31±0.04	0.35±0.04	0.32±0.04
β-alanine	0.060±0.006	0.053±0.003	0.050±0.005	0.072±0.017	0.063±0.007	0.046±0.006	0.047±0.001
Arginine	0.19±0.01^{b}	0.19±0.01^{b}	0.10±0.01^{a}	0.15±0.01^{ab}	0.18±0.01^{b}	0.20±0.01^{b}	0.17±0.01^{b}
Aspartate	0.78±0.06^{ab}	0.81±0.08^{ab}	1.0±0.1^{b}	0.73±0.06^{ab}	0.75±0.15^{ab}	0.68±0.04^{ab}	0.61±0.05^{a}
Glutamate	6.4±0.1	7.3±0.3	6.0±0.5	6.0±0.4	6.4±0.3	6.6±0.2	6.5±0.1
Glutamine	2.1±0.2^{a}	3.0±0.2^{ab}	3.7±0.4^{b}	2.4±0.2^{a}	2.4±0.2^{a}	2.5±0.2^{a}	2.1±0.1^{a}
Glycine	0.20±0.01^{a}	0.21±0.01^{ab}	0.28±0.02^{b}	0.23±0.02^{ab}	0.23±0.02^{ab}	0.23±0.02^{ab}	0.24±0.01^{ab}
Histidine	0.082±0.005^{a}	0.061±0.010^{a}	0.15±0.02^{b}	0.046±0.005^{a}	0.056±0.019^{a}	0.066±0.012^{a}	0.067±0.007^{a}
Isoleucine	0.034±0.002^{ab}	0.072±0.010^{b}	0.024±0.003^{a}	0.063±0.015^{ab}	0.047±0.006^{ab}	0.044±0.009^{ab}	0.058±0.005^{ab}
Leucine	0.067±0.006^{a}	0.17±0.03^{b}	0.073±0.010^{a}	0.14±0.03^{ab}	0.11±0.02^{ab}	0.092±0.018^{ab}	0.14±0.01^{ab}
Lysine	1.0±0.1^{b}	1.1±0.1^{b}	0.16±0.03^{a}	0.97±0.01^{b}	0.95±0.08^{b}	1.0±0.1^{b}	0.85±0.04^{b}
Phenylalanine	0.059±0.006^{a}	0.058±0.004^{a}	0.14±0.01^{b}	0.038±0.004^{a}	0.036±0.007^{a}	0.049±0.011^{a}	0.030±0.001^{a}
Proline	0.056±0.003	0.086±0.013	0.087±0.008	0.067±0.014	0.055±0.004	0.057±0.003	0.066±0.004
Serine	0.24±0.02^{bc}	0.26±0.03^{bc}	0.20±0.02^{abc}	0.21±0.02^{abc}	0.13±0.02^{a}	0.18±0.01^{ab}	0.27±0.02^{c}
Taurine	2.0±0.1	2.1±0.1	2.3±0.2	2.0±0.1	2.2±0.1	2.0±0.1	2.0±0.1
Threonine	0.073±0.003	0.12±0.01	0.079±0.009	0.10±0.03	0.074±0.013	0.086±0.013	0.069±0.006
Tyrosine	0.050±0.007	0.035±0.003	0.040±0.003	0.032±0.006	0.029±0.004	0.043±0.012	0.025±0.004
Valine	0.10±0.01^{ab}	0.14±0.02^{b}	0.054±0.006^{a}	0.15±0.02^{b}	0.12±0.01^{b}	0.12±0.02^{b}	0.14±0.01^{b}
TFAA	13±1	16±1	15±1	13±1	13±1	14±1	13±1
TEFAA	0.96±0.12^{ab}	1.1±0.1^{b}	0.68±0.03^{a}	0.95±0.09^{ab}	0.77±0.05^{ab}	0.84±0.05^{ab}	0.94±0.04^{ab}

Values represent means±S.E.M., N=4.
Means not sharing the same letter are significant different, P<0.05.

Table 5 Contents (μmol g^{-1} liver) of various free amino acids (FAA), total FAA (TFAA) and total essential FAA (TEFAA) in the liver of *Pelodiscus sinensis* during the 72-h period post-feeding.

	FAA contents (μmol g^{-1} liver)						
	Unfed	Fed					Unfed
FAA	0h	12h	24h	36h	48h	72h	72h
Alanine	0.076±0.019^{ab}	0.26±0.17^{ab}	0.57±0.21^{b}	0.056±0.010^{a}	0.034±0.004^{a}	0.074±0.023^{a}	0.058±0.008^{a}
β-alanine	0.028±0.002	0.033±0.007	0.028±0.005	0.026±0.001	0.028±0.004	0.036±0.006	0.032±0.003
Arginine	0.14±0.01	0.16±0.03	0.081±0.033	0.085±0.009	0.067±0.003	0.089±0.010	0.081±0.011
Aspartate	0.10±0.04^{a}	0.12±0.03^{a}	0.64±0.12^{b}	0.064±0.006^{a}	0.043±0.003^{a}	0.062±0.008^{a}	0.082±0.022^{a}
Glutamate	0.96±0.11^{ab}	0.84±0.18^{ab}	1.9±0.1^{c}	0.62±0.07^{ab}	0.47±0.03^{a}	1.2±0.2^{b}	0.56±0.05^{a}
Glutamine	0.24±0.13	0.21±0.10	0.68±0.33	0.035± 0.011	0.032±0.004	0.22±0.09	0.15±0.04
Glycine	0.25±0.03^{a}	0.32±0.03^{ab}	0.83±0.27^{b}	0.15±0.01^{a}	0.15±0.01^{a}	0.23±0.04^{a}	0.19±0.03^{a}
Isoleucine	0.062±0.001^{b}	0.24±0.03^{b}	0.14±0.03^{a}	0.16±0.01^{b}	0.12±0.01^{a}	0.11±0.03^{a}	0.13±0.01^{a}
Leucine	0.12±0.01^{a}	0.56±0.06^{b}	0.27±0.06^{a}	0.37±0.01^{ab}	0.28±0.04^{a}	0.22±0.05^{a}	0.31±0.02^{a}
Lysine	0.48±0.10	0.85±0.07	0.48±0.13	0.73±0.30	0.94±0.08	0.98±0.07	1.1±0.1
Phenylalanine	0.11±0.01^{a}	0.18±0.02^{a}	0.33±0.06^{b}	0.13±0.01^{a}	0.093±0.008^{a}	0.10±0.01^{a}	0.090±0.004^{a}
Proline	0.064±0.005^{a}	0.42±0.12^{ab}	0.87±0.26^{b}	0.12±0.04^{a}	0.058±0.003^{a}	0.076±0.009^{a}	0.093±0.014^{a}
Serine	0.40±0.09^{ab}	1.5±0.2^{bc}	1.7±0.6^{c}	0.23±0.05^{ab}	0.11±0.02^{a}	0.32±0.14^{ab}	0.17±0.01^{a}
Taurine	6.3±0.5	5.7±0.6	6.9±1.0	5.3±0.5	6.9±1.0	6.1±0.5	5.6±0.1
Threonine	0.15±0.04^{a}	0.35±0.04^{b}	0.34±0.07^{b}	0.11±0.02^{a}	0.042±0.010^{a}	0.14±0.05^{a}	0.061±0.003^{a}
Tyrosine	0.082±0.005	0.088±0.016	0.065±0.005	0.094±0.016	0.087±0.007	0.092±0.018	0.067±0.012
Valine	0.13±0.01^{a}	0.40±0.06^{b}	0.28±0.06^{ab}	0.31±0.02^{ab}	0.23±0.03^{ab}	0.25±0.04^{ab}	0.23±0.01^{ab}
TFAA	9.4±0.7^{a}	12±1^{ab}	15±2^{b}	8.0±0.7^{a}	8.9±1.0^{a}	9.4±0.3^{a}	8.0±0.2^{a}
TEFAA	0.80±0.10^{a}	2.1±0.02^{c}	1.8±0.4^{bc}	1.2±0.1^{ab}	0.85±0.09^{a}	0.98±0.08^{ab}	0.93±0.03^{a}

Values represent means±S.E.M., N=4.
Means not sharing the same letter are significant different, P<0.05.

Table 6 Contents (μmol g^{-1} muscle) of various free amino acids (FAA), total FAA (TFAA) and total essential FAA (TEFAA) in the muscle of *Pelodiscus sinensis* during the 72-h period post-feeding.

	FAA contents (μmol g^{-1} muscle)						
	Unfed	Fed					Unfed
FAA	0h	12h	24h	36h	48h	72h	72h
Alanine	0.16±0.02^{a}	0.32±0.12^{a}	0.96±0.26^{b}	0.33±0.05^{a}	0.12±0.01^{a}	0.099±0.024^{a}	0.13±0.03^{a}
β-alanine	0.028±0.002	0.033±0.007	0.028±0.005	0.026±0.001	0.028±0.004	0.036±0.006	0.032±0.003
Arginine	0.046±0.007^{a}	0.048±0.009^{a}	0.63±0.18^{b}	0.039±0.008^{a}	0.046±0.004^{a}	0.024±0.008^{a}	0.042±0.004^{a}
Aspartate	0.10±0.04^{a}	0.12±0.03^{a}	0.64±0.12^{b}	0.064±0.006^{a}	0.043±0.003^{a}	0.062±0.008^{a}	0.082±0.022^{a}
Glutamate	0.96±0.11^{a}	0.84±0.18^{a}	1.9±0.1^{b}	0.62±0.07^{a}	0.47±0.03^{a}	1.2±0.2^{a}	0.56±0.05^{a}
Glutamine	0.77±0.09^{a}	1.2±0.2^{abc}	2.0±0.3^{bc}	2.1±0.3^{c}	0.85±0.09^{a}	1.1±0.3^{ab}	0.78±0.13^{a}
Glycine	0.50±0.02^{a}	0.49±0.06^{a}	1.5±0.2^{b}	0.87±0.11^{ab}	0.53±0.16^{a}	0.64±0.08^{a}	0.64±0.15^{a}
Histidine	0.46±0.11	0.29±0.04	0.44±0.05	0.29±0.02	0.23±0.02	0.27±0.06	0.34±0.04
Isoleucine	0.15±0.02^{a}	0.45±0.03^{c}	0.13±0.03^{a}	0.39±0.06^{bc}	0.25±0.04^{ab}	0.21±0.03^{a}	0.19±0.02^{a}
Leucine	0.14±0.01^{a}	0.76±0.03^{c}	0.25±0.06^{a}	0.50±0.04^{b}	0.34±0.05^{ab}	0.22±0.07^{a}	0.35±0.03^{b}
Lysine	0.59±0.02^{ab}	1.2±0.1^{bc}	1.0±0.2^{bc}	1.6±0.2^{c}	0.57±0.10^{ab}	0.86±0.24^{ab}	0.24±0.04^{a}
Phenylalanine	0.11±0.01^{a}	0.24±0.01^{b}	0.35±0.05^{c}	0.14±0.01^{a}	0.077±0.008^{a}	0.080±0.009^{a}	0.073±0.005^{a}
Proline	0.20±0.02^{a}	0.28±0.04^{a}	0.55±0.09^{b}	0.30±0.06^{a}	0.11±0.01^{a}	0.16±0.02^{a}	0.17±0.02^{a}
Serine	0.19±0.03^{a}	0.47±0.09^{abc}	0.79±0.16^{c}	0.71±0.08^{bc}	0.19±0.07^{a}	0.20±0.04^{a}	0.31±0.03^{ab}
Taurine	6.3±0.5	5.7±0.6	6.9±1.0	5.3±0.5	6.9±1.0	6.1±0.5	5.6±0.1
Threonine	0.17±0.03^{a}	0.38±0.03^{b}	0.44±0.08^{b}	0.48±0.03^{b}	0.099±0.028^{a}	0.18±0.02^{a}	0.14±0.02^{a}
Tyrosine	0.082±0.005	0.088±0.016	0.065±0.005	0.094±0.016	0.087±0.007	0.092±0.018	0.067±0.012
Valine	0.19±0.01^{a}	0.61±0.04^{c}	0.28±0.06^{ab}	0.47±0.03^{bc}	0.34±0.03^{ab}	0.24±0.09^{ab}	0.25±0.07^{ab}
TFAA	12±1	15±1	17±2	15±1	13±1	13±1	11±1
TEFAA	1.9±0.2^{a}	4.0±0.1^{c}	3.0±0.4^{bc}	3.9±0.3^{c}	1.9±0.2^{a}	2.1±0.1^{ab}	1.6±0.1^{a}

Values represent means±S.E.M., N=4.
Means not sharing the same letter are significant different, P<0.05.

Effects of feeding on excretion of ammonia and urea

Before feeding, the ammonia and urea-N excretion rates were 0.028 and 0.068 μmol h^{-1} g^{-1} turtle. Two-way anova analysis showed that there was a significant interaction between feeding status (fed or unfed) and time interval with respect to ammonia and urea excretion rates (P interaction < 0.01). After feeding, a significant increase (with a peak of approximately 11-fold) in the rate of ammonia excretion occurred between hours 24 and 48 (Fig. 8A). In addition, there was a significant increase (with a peak of approximately 8-fold) in urea-N excretion rate between hours 0 and 36, and between hours 48 and 60 (Fig. 8B).

Calculated results for a 300 g **P. sinensis**

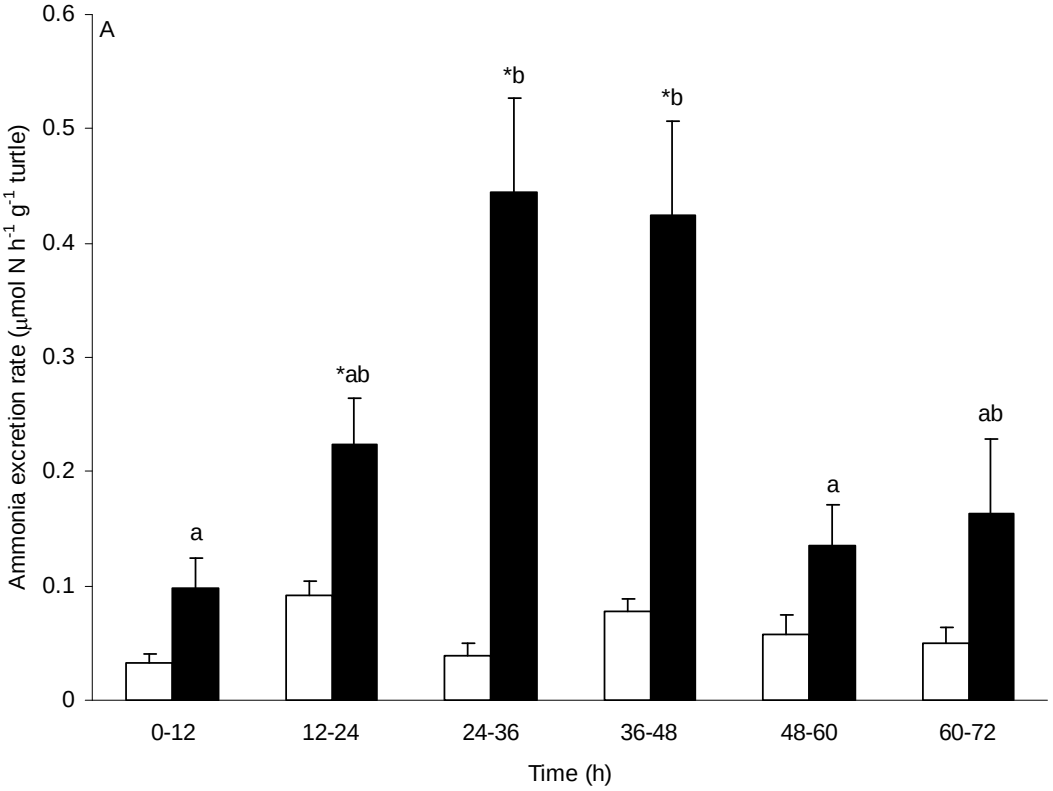
Based on results obtained from four turtles ranging from 320-505 g body mass, the mass (g) of brain, intestine, liver, muscle, and stomach per 300 g turtle were calculated as 0.34 ± 0.06 g, 3.7 ± 0.3 g, 9.4 ± 1.2 g, 74 ± 5 g, and 1.9 ± 0.3 g, respectively. The plasma volume in a 300 g turtle was estimated as 15 ± 2 ml.

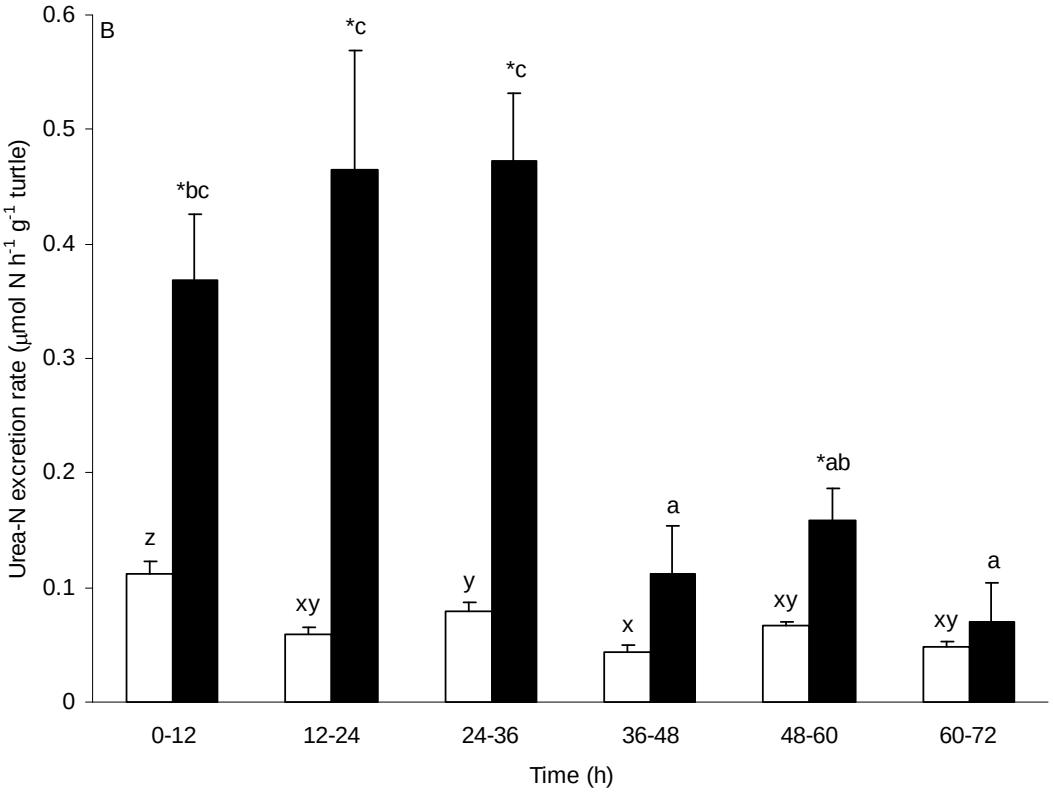
For a turtle immersed in water before feeding, the urea excretion rate was 0.034 (=0.068 μmol N/2) μmol h^{-1} g^{-1}. Because the steady state of urea content in the turtle is maintained by a balance between urea production and urea excretion, the estimated rate of urea production in a 300 g *P. sinensis* in control conditions is approximately (0.034 μmol h^{-1} g^{-1}/60 min h^{-1}) x 300 g or 0.17 μmol min^{-1}. Because there is 9.4 g of liver in a 300 g turtle, so the estimated rate of urea synthesis was 0.17/9.4 or 0.018 μmol min^{-1} g^{-1} liver, amounting to approximately 1.5% of the maximal capacity of hepatic CPS I (1.2 μmol min^{-1} g^{-1} liver).

A 300 g *P. sinensis* would have consumed 5 g of prawn meat, and in every gram of prawn meat, there was 0.031 g or 2.21 mmol N. So, in 5 g of prawn meat there was a total of 11050 μmol N. The apparent digestibility coefficient for protein

Fig. 8. Rates (μmol N h^{-1} g^{-1} turtle) of excretion of (A) ammonia and (B) urea *Pelodiscus sinensis* at 12-h intervals during the 72-h period post-feeding. White bars represent control turtles that were not fed. Black bars represent experimental turtles that were fed. Values are means + S.E.M., N=5, except for ammonia excretion rates for fed turtles from 12-24, 24-36 and 60-72 h and urea excretion rates for fed turtles from 12-24 h, N=4. * Significantly different from the corresponding unfed value, P<0.05. Means of rates not sharing the same letter are significantly different, P<0.05.

Fig. 8


A
Ammonia excretion rate (μmol N h-1 g-1 turtle)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
*ab
*b
*b
a
ab
0-12
12-24
24-36
36-48
48-60
60-72
Time (h)


B
Urea-N excretion rate (μmol N h-1 g-1 turtle)
0.6
0.5
0.4
0.3
0.2
0.1
0
z
*bc
xy
*c
y
*c
x
a
xy
*ab
xy
a
0-12
12-24
24-36
36-48
48-60
60-72
Time (h)

was taken to be 93% N (Jia et al., 2005). In this high-protein diet, it would mean that 10277 μmol N would have been assimilated. The excess ammonia excreted by a 300 g turtle between hours 12 and 48 amounted to [(0.22-0.092) + (0.45-0.039) + (0.42-0.077] μmol N h^{-1} g^{-1} x 12 h x 300 g or 3175 μmol N (from Fig. 8A). In addition, the excess urea-N excreted between hours 0 and 36 was [(0.37-0.11) + (0.46-0.058) + (0.47-0.080)] μmol N h^{-1} g^{-1} x 12 h x 300 g or 3787 μmol N (from Fig. 8B). Thus, it can be estimated that by hour 48 post-feeding, a 300 g turtle would have excreted a total of 6962 μmol excess N, which was equivalent to (6962/10277) x 100 or 68% of the N in the 5 g of prawn meat; out of this 6962 μmol N, 54% was excreted as urea-N.

The estimated rate of urea production in a 300 g *P. sinensis* in control conditions was 0.034 μmol h^{-1} g^{-1} x 24 h x 300 g or 245 μmol day^{-1}. Between hours 0 and 24, 1192 μmol excess urea would have been excreted by a 300 g turtle. At hour 24, the excess urea accumulated in various tissues and organs of a 300 g turtle can be estimated from Table 3 as [(2.3-0.8) μmol g^{-1} x 74 g muscle] + [(2.2-0.9) μmol g^{-1} x 9.4 g liver] + [(1.9-0.7) μmol g^{-1} x 1.9 g stomach] + [(2.1-0.6) μmol g^{-1} x 3.7 g intestine] + [(2.9-1.0) μmol ml^{-1} x 15 ml plasma] + [(2.5-0.7) μmol g^{-1} x 0.34 g brain] = 160 μmol. Thus, the rate of urea synthesis for a 300 g turtle during the initial 24 h after feeding was (1192 + 160 + 245) = 1597 μmol 24 h^{-1}, which represents approximately 7-fold (=1597/245) increase in the rate of urea synthesis.

DISCUSSION

P. sinensis *is ureogenic and ureotelic in water*

A full complement of OUC enzymes was detected from the liver of *P. sinensis*. Unlike *A. mutica* (Baze and Horne, 1970), the liver of *P. sinensis* possessed a high level of CPS I activity (1.2 μmol min^{-1} g^{-1} tissue), which is higher than those activities obtained by a similar assay method for the ureogenic and ureotelic marine blue spotted fan-tail ray (CPS III, 0.13-0.58 μmol min^{-1} g^{-1} tissue; Tam et al., 2003; Ip et al., 2003), the Gulf toadfish (CPS III, 0.24-0.8 μmol min^{-1} g^{-1} tissue; Anderson and Walsh, 1995), and the African lungfishes (CPS III, 0.27-0.75; Chew et al., 2003a, 2004; Loong et al., 2005), but lower than those for the crab-eating frog (CPS I, 3.8-6.6 μmol min^{-1} g^{-1} tissue; Wright et al., 2004) and the mouse (CPS I, 3.9-4.4 μmol min^{-1} g^{-1} tissue; Chew et al., 2003a; Loong et al., 2005). So, it can be concluded that *P. sinensis* is ureogenic.

The GS transferase activity present in the liver was very low, indicating that *P. sinensis* is unlikely to undergo uricotelism. This is because GS is the primary ammonia-detoxifying enzyme in the urate pathway, and is localized in mitochondria in livers of uricotelic animals. Based on the GS biosynthetic assay, no GS activity can be detected so far in liver tissues of either aquatic or semi-aquatic turtles (Campbell 1995).

During immersion, *P. sinensis* is primarily ureotelic, excreting the majority (71%) of the waste-N as urea-N. The estimated rate of urea synthesis in a 300 g *P. sinensis* in control conditions represented approximately 1.5% of the maximal capacity of CPS in the liver. Therefore, it can be inferred that there is a great potential for increased urea synthesis in this turtle at certain physiological status (e.g.

after feeding or during prolonged fasting) or when exposed to certain environmental conditions (e.g. desiccation or high environmental salinity).

Postprandial changes in tissue ammonia and urea contents

For lower vertebrates that cannot detoxify ammonia to urea, there is usually a transient surge in plasma ammonia levels after feeding (Kaushik and Teles, 1985; Wicks and Randall, 2002). For example, 8 h after feeding, the plasma ammonia concentration increases to 2.07 mmol l^{-1} in the rainbow trout, *O. mykiss* (Wicks and Randall, 2002). As for mammals, postprandial increases in plasma ammonia and amino acid levels have also been reported (Peng et al., 1972; Peters and Harper, 1987; Semon et al., 1988). For rats fed with a high protein diet, the plasma ammonia concentration increased significantly from 0.06 mmol l^{-1} to 0.13 and 0.11 mmol l^{-1} after 4 and 12 h, respectively (Semon et al., 1988). However, unlike other animals, the plasma ammonia level and tissue ammonia contents of *P. sinensis* remained unchanged throughout the 72 h post-feeding. The absence of a postprandial surge in plasma ammonia concentration could partly be a result of the high capacity for urea synthesis in its liver, which effectively detoxified ammonia to urea.

After feeding, there were significant increases in ammonia contents in the stomach (at hour 24) and the intestine (between hours 12 and 36) of *P. sinensis*. These could be due to microbial activity within the alimentary tract breaking down some of the dietary protein and amino acids. However, like the plasma, ammonia contents in the liver, muscle, and brain remained unchanged throughout the 72-h post-feeding period. In contrast, urea contents in the stomach, intestine, liver, muscle, brain and plasma increased significantly at hour 24. Thus, it can be concluded that a substantial portion of the ammonia released through the catabolism of excess amino acids was detoxified to urea in *P. sinensis* before excretion. In addition, it can be deduced that

the increase in rate of urea production was greater than the increase in rate of urea excretion; only then, would there be significant increases in tissue urea contents in the experimental animal.

Postprandial changes in contents of FAAs in the liver and the muscle

In mammals, there is net uptake of significant fractions of nearly all amino acids, except for the branched-chain amino acids, in the liver. Instead of being catabolized in the liver, the branched-chain amino acids like leucine, isoleucine, and valine are transported to the muscle, where they are used for protein synthesis or catabolized (Felig, 1975; Ishikawa, 1976). In contrast, there were significant increases in several FAAs, mainly essential ones which include some branched-chain amino acids, in the liver of *P. sinensis* between hours 12 and 36. This led to a significant increase in the TEFAA content in the liver at hours 12 and 24. However, it is uncertain at present whether these branched-chain amino acids can be metabolized in the liver of this turtle. A similar phenomenon was observed in the muscle, except that the increase in muscle TEFAA content extended from hour 12 to hour 36. Overall, these results indicated that the digestion of food and the catabolism of excess amino acids in *P. sinensis* took a longer time than similar processes in fish (Lim et al., 2004; Ip et al., 2004c).

Because the greatest changes in contents were observed in alanine and glutamate in the liver, and alanine, glycine and glutamine in the muscle, of *P. sinensis* after feeding, our results are in support of an earlier proposition that glycine and glutamine were synthesized from other amino acids consumed in excess of those required for protein synthesis (Coulson and Hernandez, 1970). Furthermore, our results indicate that alanine and glutamate might act as the vehicle of N transfer between tissues in this turtle. Taken together, it can be proposed that the defense

against postprandial ammonia toxicity in *P. sinensis* was achieved through increases in transamination and synthesis of certain non-essential amino acids in addition to an increase in urea synthesis.

Feeding resulted in increases in ammonia and urea excretion

Feeding induced an increase in the rate of nitrogenous excretion (ammonia + urea) in the subsequent 72 h in *P. sinensis*. The increase in urea excretion occurred almost immediately after feeding (between hours 0 and 36), which was apparently instrumental in preventing the build up of high levels of urea in various tissues. However, urea synthesis is an energy-intensive process; each mole of urea synthesized through the OUC with CPS I requires the hydrolysis of 4 mol of ATP. Therefore, it is logical to hypothesize that not all ammonia released from the catabolism of excess amino acids after feeding was detoxified to urea for excretion. Indeed, a significant increase in the rate of ammonia excretion occurred between hours 24 and 48. The capacity to vary the ratio of urea-N to ammonia-N for excretion is known to be present in fish (Chew et al., 2006) and in mammals (Gerardo Herrera et al., 2006) under various conditions. For the nectarivorous bat (*Glossophaga soricina*), ammonia and urea excretion increases and decreases, respectively, with decreased nitrogen intake (Gerardo Herrera et al., 2006). In the case of *P. sinensis*, the calculated results reveal that, by hour 48 post-feeding, 68% of the assimilated N from the food would have been excreted, out of which (6962 μmol N), 54% was excreted as urea-N.

Feeding induced an increase in urea synthesis

Urea can be produced through uricolysis, argininolysis, or the OUC (Campbell, 1973), but only urea production through the OUC can be regarded as a synthetic process. Because non-protein N had only a minor contribution to the total N

in prawn meat, it is unlikely that increased urea production in *P. sinensis* after feeding was a result of purine catabolism and uricolysis. For the 11050 μmol N in 5 g of prawn meat consumed by a 300 g turtle, urea, free arginine and bound arginine contributed 9.4, 360 and 1110 μmol N, respectively. Assuming that arginine was selectively and completely catabolized to urea, the sum of 1470 μmol N could account for only 62 and 39% of the excess urea-N excreted by the turtle during the initial 24 h and 36 h, respectively, post-feeding, notwithstanding urea accumulation actually occurred in the turtle.

Therefore, it can be deduced that a major portion of the urea produced by *P. sinensis* after feeding was synthesized *de novo* via the OUC, and *P. sinensis* defends against postprandial ammonia toxicity through enhanced urea synthesis, despite it being an aquatic turtle. The estimated rate of urea synthesis in a turtle during the initial 24 h after feeding was approximately 7-fold greater than that of the unfed control. The rate of 1597 μmol day^{-1} for urea synthesis in a 300 g turtle after feeding is equivalent to 1.1 μmol min^{-1}. Since there is 9.4 g of liver in a 300 g turtle, the rate of urea synthesis in the liver can be calculated as 1.1/9.4 = 0.12 μmol min^{-1} g^{-1} liver. Because the CPS I activity determined in the presence of saturating levels of substrates at optimal conditions was 1.2 μmol min^{-1} g^{-1} liver, it becomes obvious that the detoxification of ammonia released from excess protein intake through feeding demanded only 10% of the urea synthetic capacity in *P. sinensis*. Indeed, at hour 24 after feeding, there was no significant change in activities of various OUC enzymes from the liver of this turtle.

Changes in contents of certain FAAs in the brain after feeding

After feeding, the brain would be exposed to higher levels of ammonia as a result of the postprandial surge in plasma ammonia concentration, and infiltrated

ammonia would have to be detoxified to glutamine through GS (Suárez et al., 2002). Indeed, postprandial increases in brain glutamine contents have been reported in fishes (Wicks and Randall, 2002; Lim et al., 2004; Ip et al., 2004c) and mammals (Semon et al., 1988). For *P. sinensis*, a transient increase in the brain glutamine content occurred at hour 24 post-feeding, which indicates that the brain was confronted with mild postprandial ammonia toxicity despite the relatively unchanged plasma ammonia concentration. So, it can be concluded that the brain of *P. sinensis,* similar to those other vertebrates, defended against ammonia toxicity through glutamine formation.

For rats fed a high protein diet, postprandial increases in contents of many essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) in addition to glutamine and tyrosine, lead to a significant increase in the TEFAA content (Semon et al., 1988). It has been proposed that these changes in the brain lead to a decrease in intake of high protein diets in rats (Semon et al., 1988). In contrast, there was no significant change in the TEFAA content despite increases in contents of several essential FAAs in the brain of *P. sinensis* during the 72-h period post-feeding. Hence, the metabolic signals which control the intake of high protein diets in this turtle appear to be different from those in mammals.

Conclusion

Our results verify for the first time that CPS I was present in the aquatic soft-shelled turtle, *P. sinensis*. Despite aquatic turtles being considered as facultative ammonoureoteles (Campbell, 1995), a substantial portion of the ammonia released through the catabolism of excess amino acids after feeding was detoxified to urea in *P. sinensis*. The rate of urea synthesis in this soft-shelled turtle was up-regulated approximately 7-fold after feeding.

CHAPTER 2. SALINITY STRESS

MATERIALS AND METHODS

Procurement and maintenance of animals

Animals were procured and maintained as stated in Chapter 1 on page 47.

Exposure of turtles to a salinity stress

Specimens of *P. sinensis* ranging from 200 to 400 g body mass were used for this series of experiments. Food was withdrawn 5 days prior to experiments. Specimens were submerged individually in plastic aquaria tanks containing 10 volumes (w/v) of water at 25°C. Control specimens were exposed to 1‰ for 7 days. Experimental specimens were exposed to daily increases in salinity from 1‰ (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (days 4-6) to 1‰ (day 7). Gradual ascent in salinity was necessary to allow for acclimatization and survival.

Collection of water samples for analyses

Water samples (3 ml) were collected daily after stirring, acidified with 70 µl of 1 mol l^{-1} HCl and kept at 4°C until analyzed. Randomly selected water samples were collected in duplicate left at 25°C for 24 h and then acidified and stored at 4°C until analysis. These served as a control for microbial activity, and results obtained confirmed that the ammonia and urea concentration remained relatively unchanged after 24 h of incubation at 25^{o}C.

Collection of tissue samples for analyses

At days 3, 4 and 6, turtles were killed by a strong blow to the head. Blood was obtained by cardiac puncture. Blood was collected in capillary tubes for determination of haematocrit. Further blood samples were collected by cardiac puncture into heparinized syringes, and centrifuged at 5,000 *g* and 4^{o}C for 5 min to obtain the plasma. One portion of the plasma was used for measuring osmolality,

[Na^+] and [Cl^-] and the rest was deproteinized by the addition of an equal volume (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10,000 *g* and 4°C for 15 min. The resulting supernatant was kept at -25°C until analysis. The muscle, liver, stomach, intestine and brain were quickly excised. The stomach and intestine were removed, flushed well with water, and divided into 2 halves longitudinally. The excised tissues and organs (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs (Faupel et al., 1972). Frozen samples were kept at -80°C until analysis.

Determination of haematocrit

Blood collected in capillary tubes was centrifuged in a haematocrit centrifuge at 3000 rpm for 7 minutes. The haematocrit was expressed as percent packed cell volume. This was done by taking the average ratio of the length of the capillary occupied by the blood cells to that of the length occupied by the blood components and expressing it as a percentage.

Analysis of plasma osmolality and concentrations of Na^+ and Cl^-

Plasma osmolality was analyzed using a Wescor 5500 vapour pressure osmometer. Na^+ concentrations were determined by a Corning 410 flame photometer. Cl^- concentrations were determined by a Corning 925 chloride analyzer.

Determination of ammonia and urea concentrations in water samples

Ammonia and urea concentrations in water samples were determined as described in Chapter 1 on page 50.

Determination of contents of ammonia, urea and FAAs in tissue samples

Ammonia, urea and FAA contents in tissue samples were determined as described in Chapter 1 on page 47. The content of TFAA was calculated by the summation of contents of all FAAs, while the content of TEFAA was calculated as

the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Determination of activities of OUC enzymes

Activities of OUC enzymes in the livers of control- and salinity-treated turtles were determined as described in Chapter 1 on page 51.

Determination of water contents in the muscle and the liver

Liver and muscle were dissected from turtles to determine water content. The wet masses of the tissues were recorded to the nearest 0.001 g. The tissues were then dried in an oven at 95ºC until constant mass and the dry mass was recorded. The water content was estimated as the difference between wet mass and dry mass, and expressed as percent of wet mass tissue.

Determination of oxygen consumption rate

Turtles were maintained in 1‰ water or exposed to progressive increase in salinity as described above. On day 6, turtles were gently transferred to plastic respiratory chambers (18.5 cm x 11 cm x 8.5 cm) approximately 2 h prior to beginning an experiment. The chamber was filled appropriately with water (either 1‰ or 15‰ water; approximately 0.8 l), so that the total volume of the turtle plus water was 1.2 l, leaving 0.5 l of air space. The water was continuously aerated through an air stone, which was removed at the start of measurements when the chamber was sealed. Changes in aerial and aquatic P_{O_2} were monitored for approximately 2 h or until stable rates of O_2 depletion were achieved, using an Ocean Optics FOXY Fiber Optics oxygen sensing system S2000 with two FOXY-R O_2 electrodes (Ocean Optics Inc., Dunedin, FL, USA) inserted into the air and water compartments.

Statistical analyses

Results are presented as means ± standard error of the mean (S.E.M). Results in Fig. 9 were analyzed using 2-way repeated-measures ANOVA followed by least-square means (LSMEANS) to evaluate differences between means. Arcsine transformation was applied to all percentage data before statistical analysis. Results in Table 7 and those for water contents and haematocrit were analyzed using one-way analysis of variance followed by Bonferroni's multiple range test to evaluate differences between means. Results presented in the rest of tables and figures were assessed using independent t-tests. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Plasma osmolality and Na^+ and Cl^- concentrations

Plasma osmolality increased significantly by 1.3-fold in turtles exposed to 15‰ water on day 6. Simultaneously, on day 6, concentrations of Na^+ and Cl^- in the plasma increased significantly by 1.2- and 1.9-fold, respectively (Table 7).

Haematocrit and tissue water content

The haematocrit values of turtles exposed to a progressive increase in salinity on days 3, 4 and 6 salinity were 26 ± 4 (*N*=3), 29 ± 3 (*N*=4) and 29 ± 1 (*N*=5), respectively, which were not significantly different from that of the control (30 ± 1, *N*=4) in freshwater. In addition, salinity exposure had no significant effect on the liver or muscle water content (% wet mass). The percentage water content in the muscle of turtles exposed to a progressive increase in salinity on days 3, 4 and 6 salinity were 76 ± 1 (*N*=3), 74 ± 1 (*N*=4) and 75 ± 1 (*N*=5), respectively, which were comparable with the control value of 71 ± 5 (*N*=4). A similar phenomenon was observed in the liver with respective values of 57 ± 6 (*N*=3), 55 ± 4 (*N*=4) and 53 ± 2 (*N*=5) as compared with 52 ± 3 (*N*=4) of the control.

Tissue ammonia and urea contents

There were significant increases in ammonia contents in the stomach (1.6-fold) and intestine (≥1.5-fold) of turtles exposed to 10‰ water and 15‰ water on day 3 and 4, respectively (Table 8). However, there were no changes in ammonia contents in the brain, liver, muscle and plasma throughout the experimental period. In contrast, there were no changes in urea contents in turtles exposed to 10‰ water on day 3; but, the urea contents in the brain, intestine, liver, muscle, plasma and stomach increased significantly by 5.6-, 5.5-, 4.8-, 5.2-, 6.9-, and 3.8-fold, respectively, in 15‰ water on day 4. On day 6, the respective increase in 15‰ water was 7.0-, 10-,

Table 7 Osmolality (mosmol kg^{-1}) and the concentrations (mmol l^{-1}) of Na^+ and Cl^- in the plasma of *Pelodiscus sinensis* exposed to a progressive increase in ambient salinity from 1‰ to 15‰ through a 6-day period, followed with recovery in 1‰ water on day 7.

	Control	Salinity			Recovery
	Day 6 (4)	Day 3 (3)	Day 4 (4)	Day 6 (5)	Day 7 (5)
	1‰	10‰	15‰	15‰	1‰
Osmolality	285 ± 2^{a}	301 ± 5^{a}	320 ± 10^{a}	373 ± 13^{b}	291 ± 7^{a}
$[Na^+]$	129 ± 2^{a}	136 ± 6^{ab}	143 ± 5^{ab}	158 ± 7^{b}	139 ± 7^{ab}
$[Cl^-]$	77 ± 4^{a}	90 ± 5^{ab}	108 ± 8^{b}	147 ± 11^{c}	98 ± 4^{ab}

Values represent means±S.E.M., with number of determinations shown in parenthesis.

Means of contents not sharing the same letter are significantly different, $P<0.05$.

Table 8 Contents (μmol g^{-1} tissue) of ammonia in the various tissues of *Pelodiscus sinensis* exposed to progressive increase in ambient salinity from 1‰ to 15‰ through a 6-day period.

	Ammonia contents (μmol g^{-1} tissue)					
	Day 3		Day 4		Day 6	
	Control	Salinity	Control	Salinity	Control	Salinity
Tissue	1‰	10‰	1‰	15‰	1‰	15‰
Brain	1.1±0.1 (5)	1.3±0.4 (6)	1.3±0.1 (5)	1.4±0.4 (6)	1.4±0.3 (6)	1.4±0.3 (6)
Intestine	0.86±0.02 (5)	1.9±0.1* (6)	1.2±0.1 (5)	1.8±0.1* (6)	1.3±0.2 (5)	1.3±0.3 (5)
Liver	3.6±0.7 (5)	5.1±1.8 (6)	3.7±0.3 (5)	5.3±1.4 (6)	3.4±0.3 (5)	7.5±2.0 (5)
Muscle	0.36±0.05 (5)	0.91±0.25 (6)	0.69±0.11 (5)	0.92±0.26 (6)	0.71±0.21 (6)	0.89±0.33 (6)
Plasma	0.33±0.03 (5)	0.28±0.03 (6)	0.35±0.03 (5)	0.28±0.02 (5)	0.29±0.03 (5)	0.25±0.02 (6)
Stomach	0.65±0.08 (5)	1.0±0.1* (6)	0.65±0.05 (5)	1.0±0.1* (6)	0.67±0.12 (5)	0.75±0.12 (5)

Values represent means±S.E.M., with numbers of determinations represented in parenthesis.

*Significantly different from the corresponding control condition

6.7-, 9.0-, 7.0-, and 10-fold (Table 9).

Activities of OUC enzymes

Activities of CPS I, OTC, ASS+ASL and arginase from the liver of turtle exposed to 15‰ water on day 6 were not significantly different from those of the controls in freshwater (Table 10).

Ammonia and urea excretion rates

The rates of ammonia and urea-N excretion in turtles exposed to 1‰ water on day 1 were 1.5 and 3.5 μmol N day^{-1} g^{-1} turtle, respectively. There were transient decreases (by approximately 50%) in ammonia and urea excretion rates in turtles exposed to 15‰ water on day 4, but they increased back to control levels during subsequent exposure to 15‰ water on days 5 and 6 (Fig. 9). On day 7, when turtles were returned from 15‰ back to 1‰ water, there were significant increases (approximately 3-fold) in ammonia and urea excretion rates. *Pelodiscus sinensis* was ureotelic throughout the course of the experiment, with approximately 70% of the waste-N excreted as urea-N (Fig. 9).

Contents of FAAs in the muscle, liver and brain

Exposure to a progressive increase in salinity led to significant changes in levels of various FAAs, many of which were essential amino acids, in the muscle, liver and brain (Table 11, Table 12 and Table 13). There were significant increases in contents of the non-essential amino acid β-alanine and the essential amino acid histidine in the muscle. The muscle β-alanine content increased approximately 13-fold to a value of 5.2 μmol g^{-1} on days 3 and 4, whilst the muscle histidine content increased approximately 25- and 27-fold on days 3 and 4, respectively, to a value of approximately 6 μmol g^{-1} (Table 11). In the muscle, the TFAA content increased

Table 9 Contents (μmol g^{-1} tissue) of urea in the various tissues of *Pelodiscus sinensis* exposed to progressive increase in ambient salinity from 1‰ to 15‰ through a 6-day period.

	Urea contents (μmol g^{-1} tissue)					
	Day 3		Day 4		Day 6	
	Control	Salinity	Control	Salinity	Control	Salinity
Tissue	1‰	10‰	1‰	15‰	1‰	15‰
Brain	0.79±0.21 (5)	0.51±0.11 (6)	0.41±0.19 (5)	2.3±0.4* (6)	1.1±0.8 (5)	8.0±2.0* (6)
Intestine	0.64±0.14 (5)	0.47±0.13 (6)	0.42±0.18 (5)	2.3±0.4* (6)	0.97±0.51 (5)	9.9±1.7* (5)
Liver	0.60±0.18 (5)	0.49± 0.09 (6)	0.42±0.18 (5)	2.0±0.3* (6)	1.4±0.8 (5)	9.4±1.8* (5)
Muscle	0.67±0.16 (5)	0.29±0.08 (6)	0.37±0.16 (5)	1.9±0.3* (6)	1.1±0.6 (6)	9.5±1.5* (6)
Plasma	0.89±0.19 (5)	0.80±0.14 (6)	0.34±0.08 (4)	2.3±0.4* (6)	1.6±0.9 (5)	11±3* (4)
Stomach	0.86±0.20 (5)	0.37±0.08* (6)	0.46±0.20 (5)	1.7±0.2* (6)	0.93±0.56 (5)	9.4±2.0* (5)

Values represent means±S.E.M., with numbers of determinations represented in parenthesis.

*Significantly different from the corresponding control condition

Table 10 Activities (μmol min^{-1} g^{-1} liver) of carbamoyl phosphate synthetase I (CPS I), ornithine transcarbamylase (OTC), argininosuccinate synthetase + lyase (ASS+ASL) and arginase in the liver of *Pelodiscus sinensis* exposed to 15‰ water as compared with the control in 1‰ water on day 6.

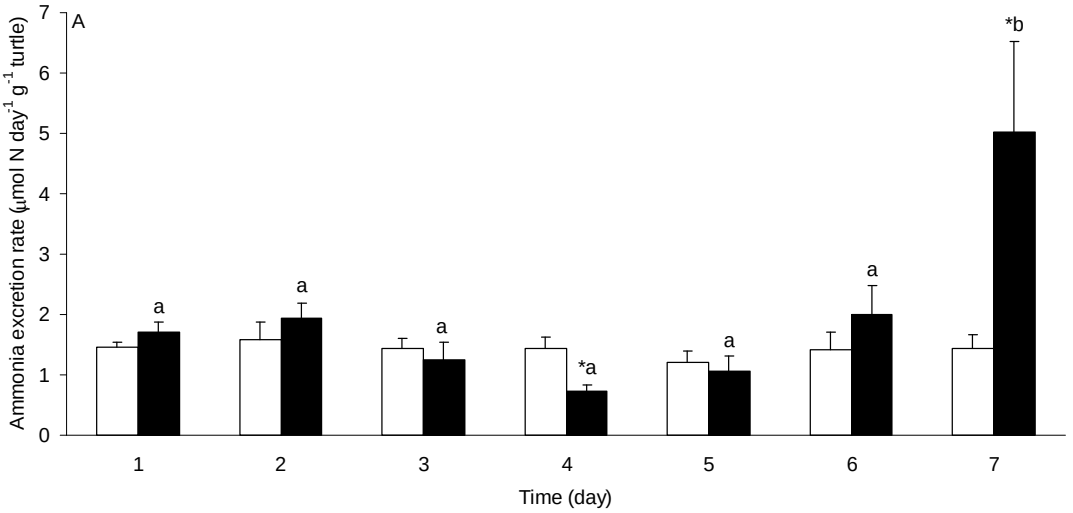
	Activities (μmol min^{-1} g^{-1} liver) Day 6	
	Control 1‰	Salinity 15‰
CPS (NH_4Cl + NAG)	0.44±0.04	0.47±0.03
OTC	61±8	66±5
ASS + ASL	0.38±0.09	0.44±0.05
Arginase	114±28	116±8

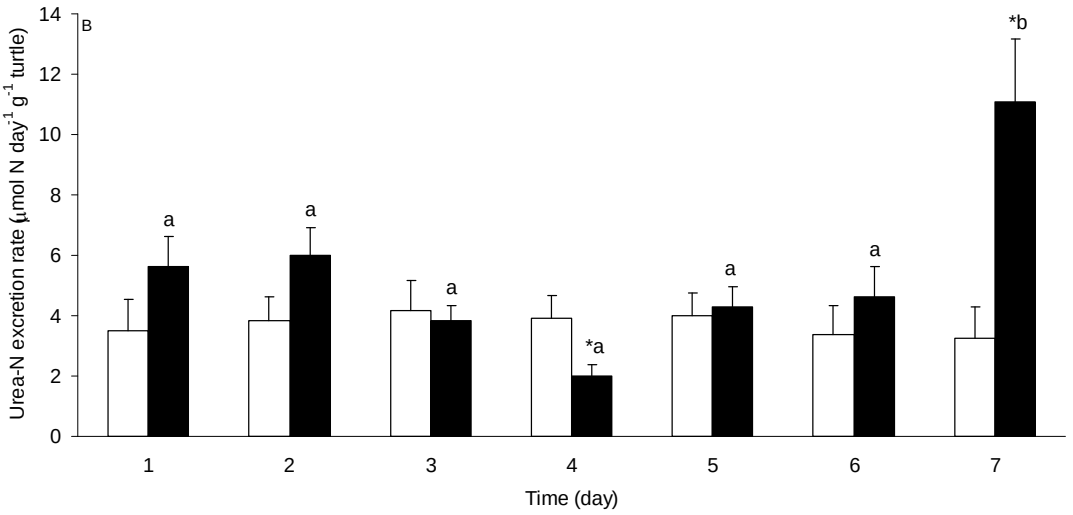
Results represent means±S.E.M.; *N*=4.

NAG, N-acetyl-L-glutamate.

Fig. 9 Rates (μmol N day^{-1} g^{-1} turtle) of excretion of (A) ammonia and (B) urea and (C) the percentage of total-N excreted as urea-N by *Pelodiscus sinensis* during exposure to a progressive increase in salinity from 1‰ to 15‰ through a 6-day period followed with a 1 day recovery in 1‰ water. White bars represent control turtles exposed to 1‰ water for 7 days. Black bars represent experimental turtles exposed to a progressive increase in salinity (1‰ on day 1 → 5‰ on day 2 → 10‰ on day 3 → 15‰ on day 4 → 15‰ on day 5 → 15‰ on day 6 → 1‰ on day 7). Values are means + S.E.M. (N=6), except for ammonia data for the experimental turtles on day 7 and urea data for the experimental turtles on days 2 and 7 (N=5). *Significantly different from the corresponding control value, $P<0.05$. Means not sharing the same letter are significantly different, $P<0.05$.

Fig. 9


A
Ammonia excretion rate (μmol N day-1 g-1 turtle)
0
1
2
3
4
5
6
7
a
a
a
*a
a
a
*b
1
2
3
4
5
6
7
Time (day)


B
Urea-N excretion rate (μmol N day-1 g-1 turtle)
0
2
4
6
8
10
12
14
a
a
a
*a
a
a
*b
1
2
3
4
5
6
7
Time (day)

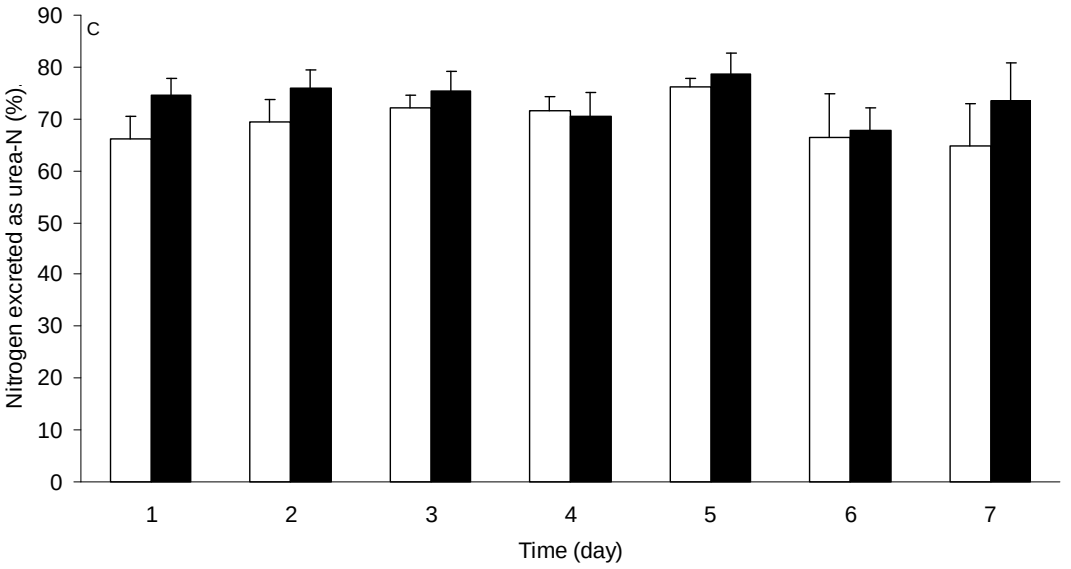

C
Nitrogen excreted as urea-N (%).
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6
7
Time (day)

Table 11 Contents (μmol g^{-1} muscle) of various free amino acids (FAAs), total FAA (TFAA) and total essential FAA (TEFAA) in the muscle of *Pelodiscus sinensis* exposed to progressive increase in salinity from 1‰ to 15‰ through a 6-day period.

	FAA contents (μmol g^{-1} muscle)					
	Control	Salinity	Control	Salinity	Control	Salinity
FAA	Day 3 (1‰)	Day 3 (10‰)	Day 4 (1‰)	Day 4 (15‰)	Day 6 (1‰)	Day 6 (15‰)
Alanine	0.15±0.01	0.38±0.03*	0.12±0.02	0.33±0.05*	0.22±0.05	0.36±0.08
β-alanine	0.41±0.03	5.2±0.5*	0.41±0.09	5.2±0.5*	0.52±0.09	3.1±1.3
Arginine	0.025±0.003	0.086±0.013*	0.032±0.008	0.089±0.019*	0.12±0.05	0.089±0.011
Aspartate	0.12±0.02	0.26±0.05*	0.17±0.02	0.23±0.02	0.16±0.05	0.22±0.06
Glutamate	1.2±0.2	1.5±0.2	1.6±0.2	1.2±0.2	1.0±0.2	0.77±0.18
Glutamine	0.83±0.16	1.1±0.3	0.64±0.14	0.81±0.21	0.77±0.15	1.0±0.1
Glycine	0.64±0.11	0.81±0.10	0.54±0.09	0.73±0.05	0.78±0.19	1.1±0.2
Histidine	0.25±0.03	6.2±0.8*	0.22±0.03	6.0±0.9*	0.47±0.08	2.1±1.1
Isoleucine	0.23±0.02	0.13±0.02*	0.21±0.03	0.17±0.04	0.19±0.03	0.11±0.02*
Leucine	0.34±0.03	0.23±0.03*	0.36±0.06	0.29±0.07	0.33±0.05	0.15±0.02*
Lysine	0.40±0.09	0.25±0.04	0.41±0.12	0.25±0.03	0.37±0.08	0.15±0.02*
Methionine	0.045±0.013	0.018±0.003	0.023±0.008	0.017±0.001	0.042±0.011	0.029±0.003
Phenylalanine	0.097±0.015	0.085±0.003	0.088±0.008	0.11±0.01	0.11±0.02	0.17±0.01
Proline	0.18±0.02	0.19±0.01	0.15±0.02	0.18±0.01	0.27±0.07	0.51±0.09
Serine	0.23±0.04	0.40±0.06	0.22±0.04	0.28±0.05	0.29±0.04	0.26±0.02
Taurine	6.5±1.1	5.4±1.1	6.1±0.9	6.2±0.5	5.5±0.4	7.1±0.5
Threonine	0.17±0.02	0.19±0.02	0.12±0.02	0.18±0.02	0.19±0.03	0.19±0.01
Tryptophan	0.073±0.007	0.027±0.008*	0.050±0.009	0.034±0.004	0.050±0.009	0.051±0.011
Tyrosine	0.067±0.014	0.11±0.02	0.060±0.008	0.23±0.07	0.31±0.02	0.43±0.02
Valine	0.34±0.05	0.20±0.02	0.29±0.04	0.25±0.06	0.28±0.05	0.20±0.03
TFAA	12±1	23±2*	12±1	23±1*	12±1	18±2*
TEFAA	1.9±0.1	7.3±0.8*	1.7±0.1	7.3±1.0*	2.0±0.1	3.1±1.1

Values represent means±S.E.M., *N*=4.
*Significantly different from corresponding control condition

Table 12 Contents (μmol g^{-1} liver) of various free amino acids (FAAs), total FAA (TFAA) and total essential FAA (TEFAA) in the liver of *Pelodiscus sinensis* exposed to progressive increase in salinity from 1‰ to 15‰ through a 6-day period.

	FAA contents (μmol g^{-1} liver)					
	Control	Salinity	Control	Salinity	Control	Salinity
FAA	Day 3 (1‰)	Day 3 (10‰)	Day 4 (1‰)	Day 4 (15‰)	Day 6 (1‰)	Day 6 (15‰)
Alanine	0.065±0.004	1.4±0.1*	0.10±0.03	1.5±0.1*	0.053±0.005	2.0±1.1
β-alanine	0.062±0.025	0.031±0.006	0.045±0.007	0.056±0.005	0.085±0.017	0.041±0.003*
Arginine	0.084±0.008	0.044±0.007*	0.089±0.016	0.063±0.022	0.054±0.022	0.11±0.01*
Aspartate	0.097±0.021	0.91±0.06*	0.12±0.02	0.96±0.09*	0.093±0.020	1.3±0.7
Glutamate	0.71±0.08	1.3±0.1*	0.62±0.06	1.7±0.3*	0.53±0.04	3.1±0.7*
Glutamine	0.46±0.37	0.36±0.02	0.14±0.06	0.31±0.05	0.064±0.014	0.54±0.18*
Glycine	0.28±0.04	1.4±0.1*	0.24±0.05	1.60±0.11*	0.21±0.03	2.5±1.1
Histidine	0.16±0.01	0.31±0.01*	0.14±0.01	0.34±0.01*	0.20±0.01	0.45±0.21
Isoleucine	0.13±0.02	0.30±0.02*	0.13±0.02	0.35±0.04*	0.13±0.02	0.36±0.14
Leucine	0.28±0.04	0.76±0.06*	0.29±0.05	0.86±0.07*	0.27±0.05	0.90±0.42
Lysine	0.16±0.02	0.70±0.09*	0.15±0.02	0.72±0.07*	0.14±0.02	1.1±0.6
Methionine	0.014±0.004	0.23±0.02*	0.012±0.007	0.22±0.02*	0.016±0.004	0.25±0.14
Phenylalanine	0.10±0.01	0.26±0.03*	0.094±0.007	0.30±0.02*	0.081±0.005	0.42±0.16
Proline	0.096±0.015	0.71±0.05*	0.093±0.012	0.65±0.03*	0.10±0.02	0.87±0.45
Serine	0.33±0.10	1.1±0.1*	0.24±0.05	1.2±0.1*	0.16±0.01	2.01±0.78
Taurine	5.5±0.6	6.3±0.7	5.5±0.7	8.4±0.5*	6.2±0.4	7.57±0.55
Threonine	0.11±0.03	0.60±0.06*	0.084±0.013	0.64±0.05*	0.078±0.013	0.85±0.41
Tyrosine	0.085±0.006	0.25±0.02*	0.080±0.008	0.25±0.02*	0.052±0.011	0.38±0.13*
Valine	0.24±0.03	0.55±0.06*	0.21±0.03	0.65±0.06*	0.20±0.03	0.82±0.30
TFAA	8.8±1.0	17±1*	8.3±0.8	20±1*	8.5±0.4	25±7
TEFAA	1.0±0.1	3.2±0.3*	0.95±0.08	3.5±0.3*	0.91±0.06	4.5±2.0

Values represent means±S.E.M., *N*=4.
*Significantly different from corresponding control condition

Table 13 Contents (μmol g^{-1} brain) of various free amino acids (FAAs), total FAA (TFAA) and total essential FAA (TEFAA) in the brain of *Pelodiscus sinensis* exposed to progressive increase in salinity from 1‰ to 15‰ through a 6-day period.

	FAA contents (μmol g^{-1} brain)					
	Control	Salinity	Control	Salinity	Control	Salinity
FAA	Day 3 (1‰)	Day 3 (10‰)	Day 4 (1‰)	Day 4 (15‰)	Day 6 (1‰)	Day 6 (15‰)
Alanine	0.29±0.03	0.93±0.08*	0.33±0.03	1.0±0.1*	0.28±0.05	0.91±0.25*
β-alanine	0.10±0.02	0.18±0.03	0.051±0.005	0.42±0.03*	0.13±0.01	0.052±0.005*
Arginine	0.29±0.02	0.24±0.01*	0.21±0.01	0.28±0.03	0.14±0.03	0.22±0.04
Aspartate	0.61±0.04	0.79±0.04*	0.60±0.02	0.94±0.12	0.58±0.05	2.32±0.09*
Glutamate	5.9±0.3	3.8±0.2*	6.9±0.1	4.0±0.4 *	6.1±0.4	8.3±1.3
Glutamine	2.4±0.2	2.0±0.3	2.6±0.1	2.7±0.7	1.9±0.2	5.4±0.9*
Glycine	0.47±0.04	0.62±0.06	0.22±0.02	0.61±0.03*	0.24±0.02	0.51±0.05*
Histidine	0.096±0.026	0.50±0.05*	0.083±0.011	1.1±0.1*	0.23±0.07	0.24±0.09
Isoleucine	0.071±0.009	0.094±0.008	0.062±0.010	0.12±0.01*	0.058±0.011	0.055±0.017
Leucine	0.12±0.01	0.23±0.02*	0.14±0.02	0.29±0.02*	0.13±0.02	0.15±0.05
Lysine	0.21±0.02	0.26±0.02	0.22±0.01	0.28±0.05	0.20±0.03	0.16±0.04
Methionine	0.071±0.004	0.036±0.002*	0.11±0.01	0.053±0.009*	0.047±0.009	0.035±0.011
Phenylalanine	0.038±0.006	0.093±0.004*	0.039±0.004	0.13±0.01*	0.034±0.003	0.17±0.03*
Proline	0.016±0.002	0.33±0.03*	0.066±0.006	0.66±0.24	0.076±0.008	0.22±0.11
Serine	0.21±0.02	0.50±0.04*	0.23±0.01	0.52±0.05*	0.20±0.04	0.48±0.09*
Taurine	2.1±0.1	1.5±0.1*	2.1±0.1	1.6±0.1*	1.8±0.1	2.2±0.1*
Threonine	0.096±0.017	0.16±0.01*	0.082±0.007	0.22±0.02*	0.065±0.011	0.17±0.02*
Tyrosine	0.029±0.001	0.069±0.008*	0.038±0.005	0.086±0.009*	0.029±0.008	0.12±0.03*
Valine	0.14±0.01	0.16±0.01	0.15±0.01	0.20±0.01*	0.11±0.02	0.10±0.03
TFAA	13±1	12±1	14±1	15±1	12±1	22±1*
TEFAA	0.77±0.04	1.5±0.1*	0.78±0.03	2.3±0.1*	0.83±0.08	1.0±0.3

Values represent means±S.E.M., *N*=4.
*Significantly different from corresponding control condition.

significantly on days 3, 4 and 6 by 1.9-, 1.9- and 1.5-fold, respectively, and the TEFAA content increased significantly by 3.8- and 4.3-fold on days 3 and 4, respectively (Table 11). As for the liver, there were also significant increases in contents of TFAA on day 3 (1.9-fold) and day 4 (2.4-fold). In addition, the respective increase in liver TEFAA contents on days 3 and 4 were 3.2- and 3.7-fold (Table 12). In the brain, there were 1.8-fold increase in contents of TFAA on day 6 and 1.9- and 2.9-fold increases in TEFAA on days 3 and 4, respectively (Table 13).

Oxygen consumption rate

The rate of O_2 uptake derived from water was not significantly different between turtles in 1‰ or 15‰ water. However, the rate of O_2 uptake derived from air increased by 2.0-fold in turtles exposed to 15‰ as compared with that of the control in 1‰ water. This resulted in a 1.9-fold increase in total rate of O_2 uptake since turtles exposed to 15‰ had a greater reliance on air-breathing (Fig. 10).

Calculated results for a 300 g **P. sinensis**

It is apparent from Table 14 that a progressive increase in salinity led to an increase in nitrogenous excretion in a 300 g *P. sinensis*. On day 3, the nitrogenous excretion increased by 1293 μmol N (Table 14). However, due to decreases in ammonia and urea excretion on the first day of exposure to 15‰ water, the cumulative increase in nitrogenous excretion reduced to 498 μmol N on day 4. Subsequently, the cumulative increase in nitrogenous excretion returned to 1084 μmol N on day 6 (Table 14). There were also increases in accumulation of ammonia and urea in various tissues of *P. sinensis*. On day 6, the excess N accumulated was 1842 μmol N (Table 14).

Fig. 10 Rates of O_2 uptake (mmol h^{-1} kg^{-1}) through air (through the lung; black bars) or through water (through the skin; white bars) in *Pelodiscus sinensis*. Control turtles were immersed in 1‰ water (*N*=6) and experimental turtles were immersed in 15‰ water on day 6 of a progressive increase in salinity (*N*=5), with S.E.M. denoted in the downward direction. The rate of total O_2 consumption (mmol h^{-1} kg^{-1}) was derived from summation of the rates of O_2 uptake through air and water, with the S.E.M. denoted in the upward direction. [a]Significantly different from the total rate of O_2 uptake in control turtles. [b]Significantly different from the rate of O_2 uptake through air in control turtles.

Fig. 10

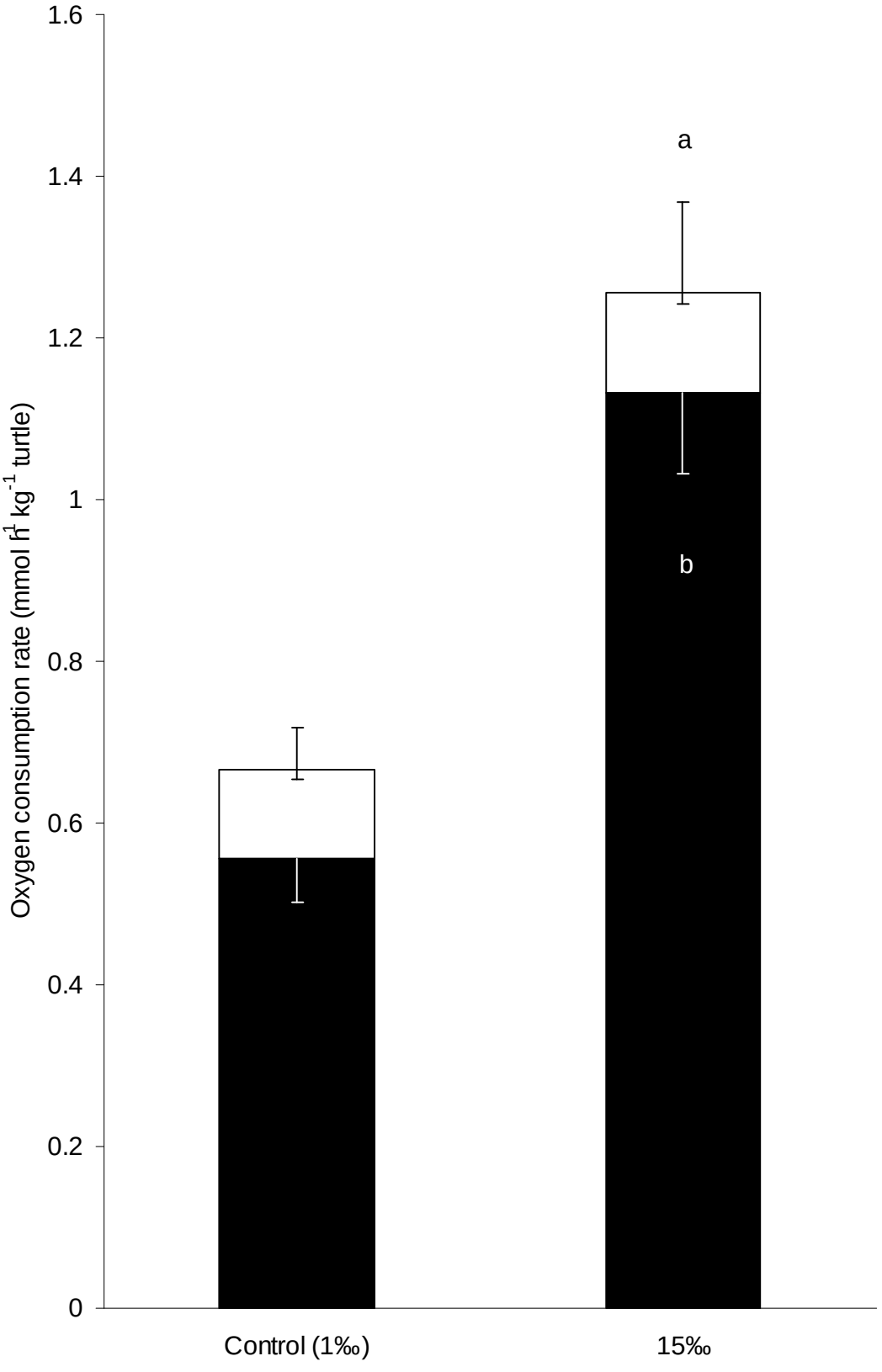

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Oxygen consumption rate (mmol h-1 kg-1 turtle)
a
b
Control (1‰)
15‰

Table 14 A nitrogen balance table (μmol N) of a 300 g *Pelodiscus sinensis*, taking into account the muscle, liver, stomach, intestine and plasma when exposed to increasing salinity from 1‰ to 15‰ over a 6-d period.

	Day 3			Day 4			Day 6		
	Control	Salinity	Difference	Control	Salinity	Difference	Control	Salinity	Difference
Excreted from *P. sinensis* (300 g)									
Ammonia-N	1343	1466	+123	1776	1687	-89	2563	2610	+47
Urea-N	3459	4629	+1170	4636	5223	+587	6852	7889	+1037
Changes in ammonia and urea excretion (A)			+1293			+498			+1084
Retained in muscle (74 g)									
Ammonia-N	27	67	+40	51	68	+17	53	65	+12
Urea-N	99	43	-56	55	284	+229	157	1409	+1252
Retained in liver (9 g)									
Ammonia-N	32	46	+14	33	48	+15	31	68	+37
Urea-N	11	9	-2	8	36	+28	25	169	+144
Retained in stomach (2 g)									
Ammonia-N	1	2	+1	1	2	+1	1	2	+1
Urea-N	3	1	-2	2	7	+5	4	37	+34
Retained in intestine (4 g)									
Ammonia-N	3	8	+5	5	7	+2	5	5	0
Urea-N	5	4	-1	3	18	+15	8	79	+71
Retained in plasma (15 ml)									
Ammonia-N	5	4	-1	5	4	-1	4	4	0
Urea-N	27	24	-3	10	70	+60	48	339	+291
Changes in ammonia and urea accumulation (B)			-5			+371			+1842
A + B			+1288			+869			+2926

DISCUSSION

Turtles of the family Trionychidae are often found in the sea or in brackish water (Minnich, 1979). *Apalone ferox* (previously as *Trionyx ferox*) and *Amyda cartilaginea* (previously as *Trionyx cartilagineus*) are sometimes found in estuaries, although the former (2-5 kg) would die within a week in 50% seawater (Dunson and Seidel, 1986). An East Indian species, *Pelochelys bibroni*, is often caught on the sea coast. *Apalone spinifera* can also be found in slightly brackish waters of New Mexico, and *T. triunguis* adults can be found along the Mediterranean coast of Israel. Larger turtles are known to be able to survive better due to the decreased surface area to volume ratio, which would limit water and electrolyte fluxes across the body surface, e.g. adult *T. triunguis* (2-5 kg) can survive in seawater for longer periods than juveniles (Dunson and Seidel, 1986). Thus, in comparison with *A. ferox* (2-5 kg; Dunson and Seidel, 1986), *P. sinensis* appears better at tolerating increased salinity because unfed *P. sinensis* (~300 g) can survive in 50% seawater for 7 days.

Increases in plasma osmolality and Na^+ and Cl^- concentrations

Turtles that reside in freshwater (10-40 mosmol kg^{-1}) maintain their plasma osmolalities hyperosmotic to the external medium (*T. scripta*, 260-320 mosmol kg^{-1}, Dantzler and Schmidt-Nielsen, 1966; *A. spinifera*, 252-282 mosmol kg^{-1}, Seidel, 1975; *Mauremys leprosa*, 362 mosmol kg^{-1}, Minnich, 1979). Similarly, *P. sinensis* has a plasma osmolality of 285 mosmol kg^{-1}, and would therefore experience hypoosmotic and hypoionic stresses in freshwater due to the continuous influx of water and loss of electrolytes. Excess water could be removed by excreting copious amounts of dilute urine, and salt loss could be minimized through salt reabsorption and/or replenished through the diet. However, *P. sinensis* would experience hyperosmotic and hyperionic stresses when exposed to 10‰ water (342 mosmol kg^{-1})

on day 3 and to 15‰ water (513 mosmol kg^{-1}) on days 4, 5 and 6. Under these conditions, the nature of the osmoregulatory problem was reversed; the turtle lost water and gained electrolytes such as Na^+ and Cl^-.

Reptilian kidneys do not function well for osmoregulatory purposes; they continue to conserve monovalent ions when the animals are confronted with hyperosmotic stress (Prange, 1985). Therefore, levels of Na^+ and Cl^- in the plasma inevitably build up to harmful levels. Salt glands allow marine turtles to secrete a hyperosmotic salt solution, so that osmotically 'free' water can be obtained in the course of drinking seawater, facilitating hypoosmotic osmoregulation. As a result, turtles with salt glands are able to maintain their plasma osmolalities at markedly lower values (*M. terrapin*, 333-459 mosmol kg^{-1}, Gilles-Baillien 1970, 1973; *C. caretta*, 316-465 mosmol kg^{-1}, Prange, 1985; Minnich, 1979 and *C. mydas*, 390 mosmol kg^{-1}, Prange and Greenwald, 1980) than that of seawater (30-34‰ water).

Pelodiscus sinensis and other members of the family Trionychidae are not known to possess salt glands (Shoemaker and Nagy, 1977; Minnich, 1979). Thus, significant increases in plasma osmolality (from 285 to 373 mosmol kg^{-1}) and concentrations of Na^+ and Cl^- (up to 158 and 147 mosmol l^{-1}, respectively) occurred in *P. sinensis* acclimated to 15‰ water. These changes could not be completely due to dehydration because the experimental animals lost only 10% of its initial weight in 15‰ water. Assuming that this loss of weight was solely due to dehydration and that a proportional amount of water was lost from the plasma, the resulting plasma osmolality, $[Na^+]$ and $[Cl^-]$ can be estimated as 316 mosmol kg^{-1}, 143 mmol l^{-1} and 86 mmol l^{-1}, respectively. Thus, it can be concluded that influxes of Na^+ and Cl^- occurred in turtles exposed to 15‰ water, but plasma $[Na^+]$ and $[Cl^-]$ were maintained at tolerable levels resulting in zero mortality. Increases in plasma $[Na^+]$ and $[Cl^-]$

contributed in part to the increased plasma osmolality, which consequently reduced the severity of osmotic water losses.

Unlike *P. sinensis*, *M. terrapin* has both a horny carapace and salt glands. After being transferred from FW to 50% seawater, the plasma osmolality of *M. terrapin* increases from 309 to 355 mosmol l^{-1}. At the same time, plasma [Na^+] increases from 129 to 156 mmol l^{-1} and plasma [Cl^-] from 88 to 113 mmol l^{-1} (Gilles-Baillien, 1970). So, judging from our results, the capacities of iono- and osmo-regulation in the *P. sinensis* and *M. terrapin* are apparently comparable. Thus, it became essential to elucidate how *P. sinensis* survived in brackish water, in spite of the lack of salt gland and the large skin area in direct contact with the external medium.

Increases in contents of FAAs in response to increased salinity

Animal cells cannot maintain an osmotic gradient across plasma membranes (Kültz, 2001). If the osmolality of the extracellular fluid (as reflected by that of the plasma) increases, water efflux from the cell would occur, leading to cell shrinkage and molecular crowding (Burg et al., 2005). So, theoretically, increases in plasma electrolyte concentrations should result in an increase in plasma volume at the expense of cell volume. However, haematocrit values (ranged from 26% to 29%) of *P. sinensis* in 1‰ and 15‰ water were comparable and exposure to 15‰ water resulted in no changes in the dry weight: wet weight ratios in the liver and the muscle. These results indicate that cell volume regulation, involving osmolytes like FAAs and/or urea, was essential to its survival in brackish water. For *M. terrapin* exposed to full strength seawater, taurine and urea are important osmolytes in the muscle, because taurine increases from 15 μmol g^{-1} to 35 μmol g^{-1} and urea from traces to 64 μmol g^{-1} (Gilles-Baillien, 1973). In comparison, the content of TFAA in the muscle

of *P. sinensis* (in 50% seawater) as a whole increased from 12 μmol g^{-1} to 23 μmol g^{-1} only on day 4, and that of urea increased to only 10 μmol g^{-1} on day 6. Thus, the osmoregulatory role of FAAs and urea in *P. sinensis* was relatively small in comparison with *M. terrapin*.

Animals usually use glycine, alanine, proline, taurine and β-alanine as osmolytes (Yancey et al., 1982). Indeed, hyperosmotic stress led to increases in contents of alanine, glycine, leucine, lysine, proline, threonine and taurine in the liver of *P. sinensis*. However, histidine and β-alanine were used by *P. sinensis* for cell volume regulation in the muscle. Together they accounted for the bulk of the increase in content of TFAA, and histidine had a major contribution to the significant increase in TEFAA content. Incidentally, histidine has also been reported as an osmolyte in the muscle of the common carp, *Cyprinus carpio* (Hegab and Hanke, 1983). Because turtles were fasted before and during the experiment, the accumulated histidine must be originated from protein degradation. The magnitude of increase in histidine content superseded those of other essential amino acids, which suggest that histidine was preferentially preserved or a unique pool of proteins enriched with histidine residues was present in *P. sinensis*. By day 6, the muscle histidine content returned to the control level even though the turtle was still under hyperosmotic stress, indicating that histidine accumulation could be a short term adaptation only.

As for the brain, FAAs which showed substantial increases in contents included alanine, glutamine, aspartate and histidine. There was also a concurrent decrease in glutamate levels, which indicate increases in transamination or glutamine synthesis. Thus, the brain of *P. sinensis* exposed to 15‰ water was confronted with ammonia toxicity, which was transiently ameliorated by a reduction in ammonia production and/or an increase in detoxification of ammonia to glutamine (Ip et al.,

2001a; Chew et al., 2005). This is in support of the proposition that increased amino acid catabolism, which resulted in an increase in ammonia production, occurred in other organs and tissues of turtles exposed to 15‰ water.

Increases in proteolysis in response to increased salinity

Increases in FAA contents can be a result of increases in their production (for non-essential amino acids) or release through proteolysis (for both essential and nonessential amino acids), or decreases in their degradation, or both. For *P. sinensis* exposed to brackish water, results obtained do not support the view that a decrease in amino acid catabolism had occurred. To the contrary, there was a significant increase in cumulative nitrogenous excretion throughout the 6-day period (Table 14), which implies that there was actually an increase in amino acid catabolism when the turtle was exposed to increased salinity.

There were significant increases in urea excretion and tissue urea contents in *P. sinensis* exposed to 15‰ water. Because *P. sinensis* is ureogenic, there should theoretically be simultaneous decreases in ammonia contents in various tissues. However, tissue ammonia contents were unaffected in most tissues studied and increased instead in the stomach and intestine. Since turtles were fasted before and during the experiment, it can be deduced that ammonia was released mainly through increased amino acid catabolism. If this indeed occurred, then contents of various FAAs and TFAA should decrease; but, the fact is TFAA and TEFAA contents in many of the tissues studied increased instead. Thus, it can be concluded indirectly that an increase in proteolysis occurred in *P. sinensis* exposed to 10 or 15‰ water. The magnitude of increase in proteolysis must be greater than that in amino acid catabolism, because only then would the contents of FAAs and TFAA increase. This

is in agreement with the proposition that cell volume regulation constitutes an important adaptation in *P. sinensis* acclimated to 15‰ water.

Increases in urea synthesis and retention in response to increased salinity

Increases in urea contents in tissues of *P. sinensis* on days 3, 4 and 6 indicate an increase in the rate of urea synthesis. In freshwater, the steady state urea contents in tissues are maintained by a balance between the rate of urea production and the rate of urea excretion. For *P. sinensis* immersed in freshwater, the urea excretion rate, and hence urea production rate, is estimated to be 3.8 μmol N day^{-1} g^{-1} (Fig. 9). The total amount of urea produced by turtles exposed to 6 days of progressive increase in salinity is equal to the summation of urea excretion throughout the 6-day period and excess urea accumulated in various tissues on day 6. Thus, the averaged daily urea production rate can be calculated as (7889 + 1252 + 144 +34 + 71 + 291) μmol N/ (6 days x 300 g), or 5.4 μmol N day^{-1} g^{-1}. That means overall the rate of urea synthesis increased (5.4/3.8) or 1.4-fold during the 6-day period. To our knowledge, this is the first report of such a phenomenon in an aquatic, freshwater turtle exposed to salinity stress. More importantly, our conclusion contradicts that of Gilles-Baillien (1970, 1973), who postulated that urea accumulation in *M. terrapin* exposed to seawater was not a result of increased urea production, but a consequence of increased urea retention in the urinary bladder.

The rate of urea synthesis in a 300 g *P. sinensis* in 1‰ water is estimated as (3.8 μmol N day^{-1} g^{-1} x 300 g)/2 or 570 μmol day^{-1} (i.e. 0.4 μmol min^{-1}). Assuming that urea production occurred mainly through de novo synthesis through the OUC in the liver, and taking that a 300 g turtle has 9.4 g of liver, the rate of urea synthesis in *P. sinensis* in freshwater can be calculated to be 0.042 μmol min^{-1} g^{-1} liver. Therefore, the rate of urea synthesis in a 300 g turtle in 15‰ water can be calculated

as 0.042 x 1.4 = 0.059 μmol min^{-1} g^{-1} liver. This is well within the hepatic OUC capacity, which had 0.44 μmol min^{-1} g^{-1} of CPS I activity. Thus, it is logical that exposure to 15‰ water did not result in significant changes in activities of OUC enzymes in *P. sinensis*.

The importance of urea as an osmolyte to *P. sinensis* in brackish water was further confirmed by the decrease in ammonia excretion on day 4 in 15‰ water, because ammonia was retained probably for urea synthesis. Simultaneously, there was a significant decrease in urea excretion in these experimental animals on day 4, which contributed to the subsequent increase in tissue urea contents. However, the capacity of *P. sinensis* to retain urea is apparently limited, and the urea excretion rate returned back to the control level as the plasma urea concentration increased. Thus, unlike marine elasmobranchs (Anderson, 2001; Yancey, 2001), *P. sinensis* cannot be regarded as ureoosmotic, and is therefore incapable of surviving in full strength seawater.

Increase in oxygen consumption rate in response to increased salinity

Protein degradation and urea synthesis are energy-dependent processes. Because increased proteolysis and urea synthesis occurred in *P. sinensis* exposed to salinity stress, there must be an increase in energy demand. Since salinity stress also led to an increase in amino acid catabolism, *P. sinensis* may have a higher metabolic rate in 15‰ water than in FW. The oxygen consumption rate in turtles immersed in 15‰ water was higher than those immersed in FW, indicating that the metabolic demands in the former were indeed greater than those in the latter.

Increases in ammonia and urea excretion during recovery in freshwater on day 7

When turtles were returned from 15‰ water to FW on day 7, there were significant increases in rates of ammonia and urea excretion. Once again, these

results confirmed that urea was retained for osmoregulatory purposes in brackish water. However, because of the lack of ammonia accumulation in the tissues and organs studied in turtles exposed to 15‰ water on day 6, it can be deduced that a major portion of the FAAs accumulated for cell volume regulation was catabolized upon return to freshwater on day 7, which resulted in increases in production and excretion of ammonia.

Conclusion

Pelodiscus sinensis is an osmoregulator that limits the entry of Na^+ and Cl^- and tolerates their increased levels. The consequential increases in plasma osmolality represent a strategy to decrease the gradient for osmotic water loss. This occurs in association with cell volume regulation through increases in accumulations of FAAs and urea, as results of increases in proteolysis and urea synthesis and retention, respectively. Exposure to salinity stress results in higher metabolic demands, which is expressed overall by a higher rate of oxygen consumption from air.

CHAPTER 3. EMERSION

MATERIALS AND METHODS

Procurement and maintenance of animals

Animals were procured and maintained as stated in Chapter 1 on page 47.

Exposure of turtles to emersion

Pelodiscus sinensis ranging from 200 to 400 g body mass were used in this series of experiments. Food was withdrawn 5 days prior to experiments. Specimens were treated individually in plastic aquaria tanks at 25°C. Average relative humidity during the experiment was 84%. Control specimens were immersed in 10 volumes (w/v) of 1‰ water for 6 days. Experimental specimens were kept in tanks with a thin film of water (50 ml of 1‰ water in a tank that was L44 cm by W29 cm by H11 cm). The film of water was in contact with the ventral surface of the turtle. However, the turtles could not drink as the water level was too shallow.

Measurement of mass changes

Masses were recorded with a Shimadzu animal balance (Shimadzu Co., Kyoto, Japan) to the nearest 0.1 g. Measurements (N=5) were taken daily for immersed and emersed turtles up to day 6.

Collection of water samples for analyses

Ammonia and urea concentrations in water samples were determined as described in Chapter 2 on page 72.

Collection of tissue samples for analyses

At days 3 and 6, turtles were killed by a strong blow to the head. Blood was obtained by cardiac puncture. Blood was collected in capillary tubes for determination of haematocrit. Further blood samples were collected into heparinized syringes, and centrifuged at 5,000 g and 4^{o}C for 5 min to obtain the plasma. A

portion of the plasma was used for analyses of osmolality and concentrations of Na^+ and Cl^-. The rest of the plasma was deproteinized by the addition of an equal volume (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10,000 *g* and 4°C for 15 min. The resulting supernatant was kept at -25°C until analysis. The muscle, liver, kidney, intestine and brain were quickly excised. The intestine was removed, flushed well with water, and divided into 2 halves longitudinally. The excised tissues and organs (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs (Faupel et al., 1972). Frozen samples were kept at -80°C until analysis.

Analyses of plasma osmolality and concentrations of Na^+ and Cl^-

Plasma osmolality and Na^+ and Cl^- concentrations were determined as described in Chapter 2 on page 73.

Determination of haematocrit

Haematocrit was determined as described in Chapter 2 on page 73.

Determination of ammonia and urea concentrations in water samples

Ammonia and urea concentrations in water samples were determined as described in Chapter 1 on page 50.

Determination of contents of ammonia, urea and FAAs in tissue samples

Contents of ammonia, urea and FAAs in tissue samples were determined as described in Chapter 1 on page 47. The content of TFAA was calculated by the summation of contents of all FAAs, while the content of TEFAA was calculated as the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Determination of activities of OUC enzymes, GS and GDH

Activities of OUC enzymes and GS in the livers of immersed and emersed turtles were determined as described in Chapter 1 on page 51.

GDH (EC 1.4.1.3) activity in the amination direction was assayed according to Ip et al. (1993). GDH activity was expressed as μmol NADH utilized min^{-1} g^{-1} tissue.

Determination of urine volume and concentrations of ammonia and urea therein

A flexible latex tubing (length 18 cm, radius 0.7 cm) was attached around the tail anterior to the cloaca of *P. sinensis* using 3M Vetbond^{TM} tissue adhesive. An opening made at the very tip of the tube was held closed by a dialysis clip (Fig. 11). Turtles were either immersed in water or exposed to emersion for 6 days as described above. Water samples were collected and acidified daily. Urine was collected daily by emptying the contents of the tubing through the opening into a 5 or 10 ml measuring cylinder. Deinoized water was introduced into the tubing through the opening to rinse the inside surface before resealing with the dialysis clip. Urine samples were acidified with 1 mol l^{-1} HCl, with the ratio of acid: urine being 7: 300, and kept at 4°C until analyzed. Ammonia and urea concentrations in the water and urine samples were determined as described on page 50.

Determination of whether ammonia or urea excretion occurred through the head or tail (urine) regions

Individual turtles were taken out of water and restrained separately on the bottom surface of a plastic box (L18.5 cm x W11.5 cm x H5.5 cm) which was turned upside down (Fig. 12).

Unlike terrestrial conditions in which turtles were deprived of drinking water, a plastic box of water (700 ml) was placed directly in front of the head in this setup. So, the experimental turtle could dip its head fully into the water at liberty for

Fig. 11 The experimental set-up for determination of urine volume and concentrations of ammonia and urea within a latex tubing

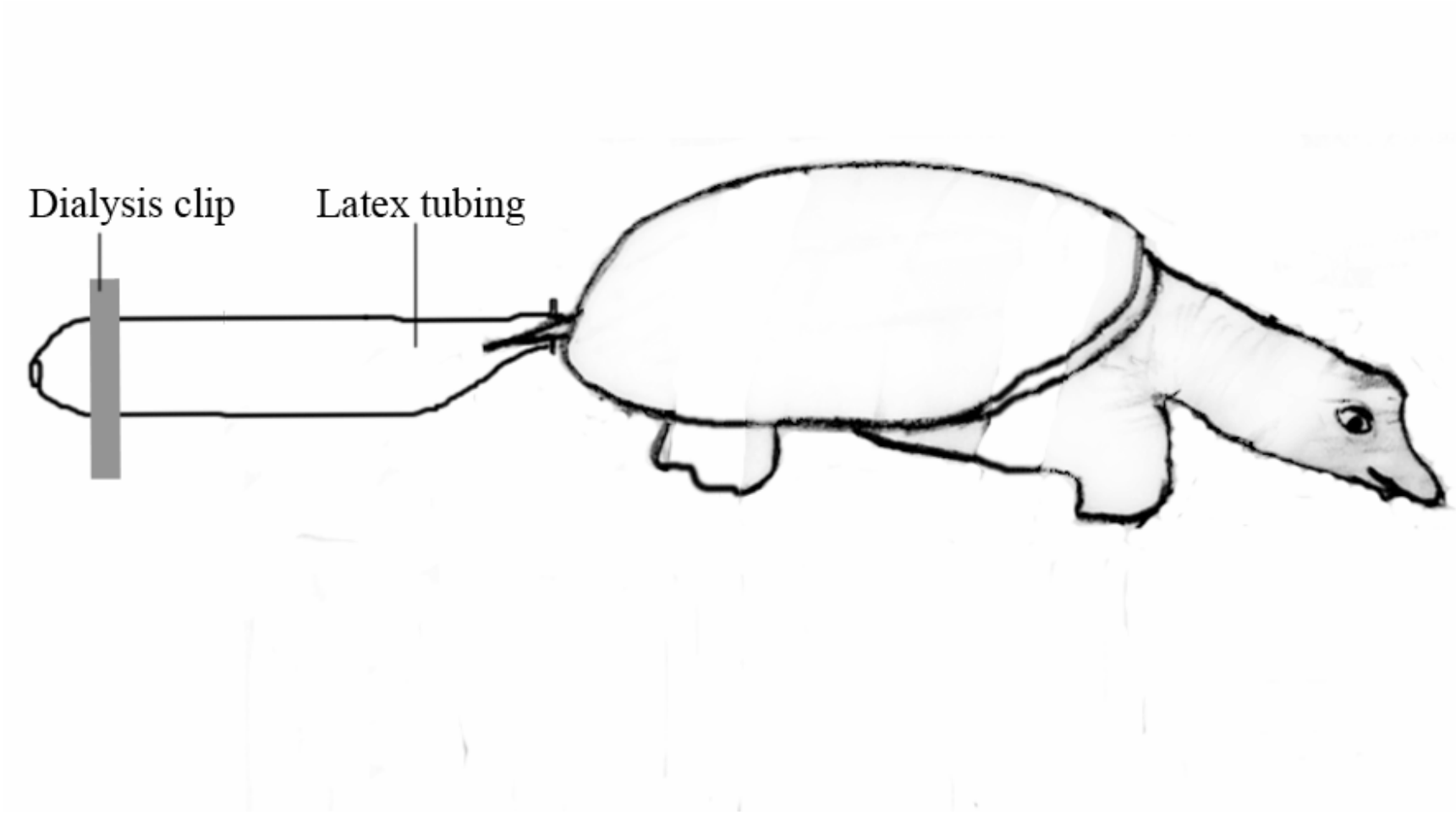
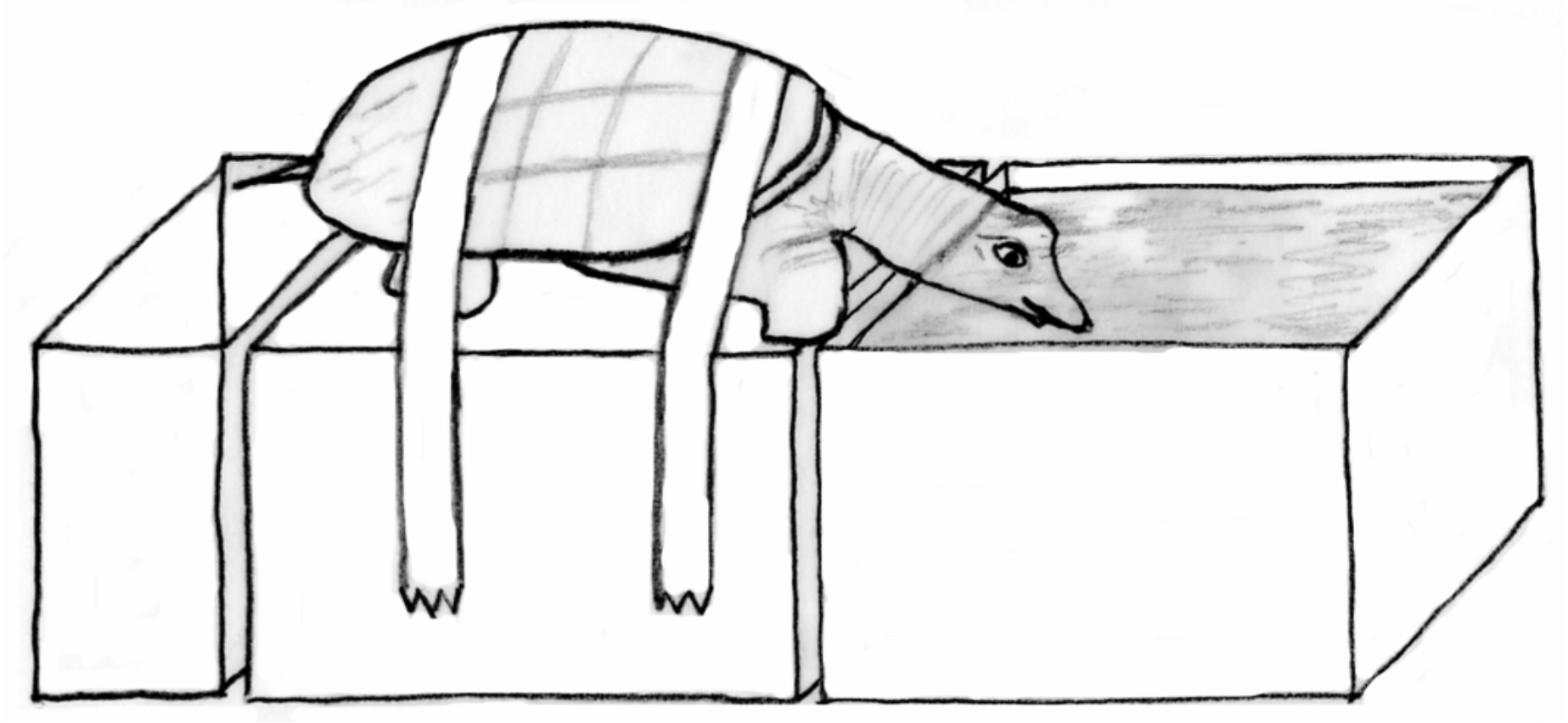


Fig. 12 The experimental set-up for the examination of whether ammonia or urea excretion occurred through the buccopharyngeal route.



drinking purpose or buccopharyngeal respiration. The water volume was determined at the end of 24 h, and water was changed daily. The change in volume less the volume due to evaporation was regarded as the volume of water consumed by the turtle. Another plastic box was placed right below the tail to collect the urine. Water and urine samples (3 ml) were collected from these two plastic boxes daily, acidified with 70 μl of 1 mol l^{-1} HCl, and kept at 4°C until analyzed. Ammonia and urea concentrations in water samples were determined on page 50.

Determination of whether urea excretion occurred through the buccopharyngeal route

Again, individual turtles (*N*=3) were restrained separately on the bottom surface of a plastic box as described above, with a plastic box of water (100 ml) placed directly in front of the head. The P_{O2} in the water was monitored continuously using an Ocean Optics FOXY Fiber Optics oxygen sensing system S2000 with a FOXY-R O_2 electrode (Ocean Optics Inc., Dunedin, FL, USA). Water samples were collected at the very beginning and right after the occurrence of a decrease in the P_{O2} in the water for the determination of urea concentration as described in Chapter 1 on page 50. The assumption was that if buccopharyngeal respiration indeed occurred, the turtle would take in water from the box and hold it in its buccopharyngeal cavity for the extraction of O_2. So, after buccopharyngeal respiration, the water regurgitated back to the box would result in a reduction in the overall P_{O2} in the water therein. Furthermore, if urea excretion indeed occurred through the buccopharyngeal route, the urea concentration in the box would increase simultaneously with the decrease in P_{O2}, right after the regurgitation of water being held in the buccopharyngeal cavity.

Determination of effects of phloretin (0.1 mmol l^{-1}) on buccopharyngeal urea excretion

The aglycon phloretin inhibits urea transport. Phloretin at a concentration of 0.1 mmol l^{-1} was incorporated into the box of water (100 ml) placed in front of the head of the turtle restrained on land (on top of a plastic box as above). After 24 h, water samples (3ml) were collected acidified with 70 μl of 1 mol l^{-1} HCl, and kept at 4°C until analyzed. Urea concentrations in water samples were determined as described in Chapter 1 on page 50.

Statistical analyses

Results are presented as means ± standard error of the mean (S.E.M). Data in Fig. 13 was analyzed using 2-way repeated-measures ANOVA followed by least-square means (LSMEANS) to evaluate differences between means. Arcsine transformation was applied to all percentage data before statistical analysis. Data in all other tables, figures and for haematocrit, masses and Na^+ and Cl^- levels were assessed using independent t-tests to evaluate differences between means. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Body mass, haematocrit, plasma osmolality and plasma [Na^+] and [Cl^-]

After 3 and 6 days of emersion, the body mass of *P. sinensis* (*N*=5) decreased by 2.0 ± 0.2% and 2.3 ± 0.5%, respectively. However, the haematocrit value for turtles exposed to terrestrial conditions for 6 days (30 ± 1, *N*=5) was not significantly different from that of the control immersed for 6 days (28 ± 3, *N*=4). Similarly, the plasma osmolality, [Na^+] and [Cl^-] for turtles exposed to terrestrial conditions for 6 days (288 ± 3 mosmol kg^{-1}, 128 ± 1 mmol l^{-1} and 84 ± 2 mmol l^{-1}, respectively, *N*=5) were not significantly different from those of the control immersed for 6 days (285 ± 2 mosmol kg^{-1}, 129 ± 2 mmol l^{-1} and 77 ± 4 mmol l^{-1} respectively, *N*=4).

Ammonia and urea excretion rates

On day 1, the rates of ammonia (Fig. 13A) and urea-N (Fig. 13B) excretion in immersed turtles (control) were 1.0 and 2.9 μmol N day^{-1} g^{-1}, respectively. So, *P. sinensis* immersed in water was primarily ureotelic, excreting approximately 74% of waste-N as urea-N. Turtles drastically reduced the rate of urea excretion during emersion, and the percentage of waste-N excreted as urea-N decreased from 74% to 20% on day 1 and increased gradually to 47% by day 6 (Fig. 13C). There was a much greater magnitude of decrease in the rate of urea excretion (decreased by 81-99%) compared to that of the rate of ammonia excretion (decreased by 34-77%) throughout the 6-day period.

Urine volume and excretion of ammonia and urea through the urine

By collecting urine into a latex tubing, it was discovered that turtles immersed in water produced 7.3-16 ml of urine daily, but those undergoing 5 days of emersion had a daily urine production of only 2.0-3.4 ml (Table 15). On day 6, anuria occurred in 5 out of 6 turtles, with one turtle producing 6.7 ml of urine (Table 15).

Fig. 13 Rates (μmol N day^{-1} g^{-1} turtle) of excretion of (A) ammonia and (B) urea and (C) the percentage of total-N excreted as urea-N by *Pelodiscus sinensis* during exposure to emersion for a 6-day period. White bars represent control turtles immersed for a 6-day period. Black bars represent experimental turtles emersed for a 6-day period. Values are means + S.E.M. (N=4). *Significantly different from the corresponding control value, P<0.05. Means not sharing the same letter are significantly different, P<0.05.

Fig. 13

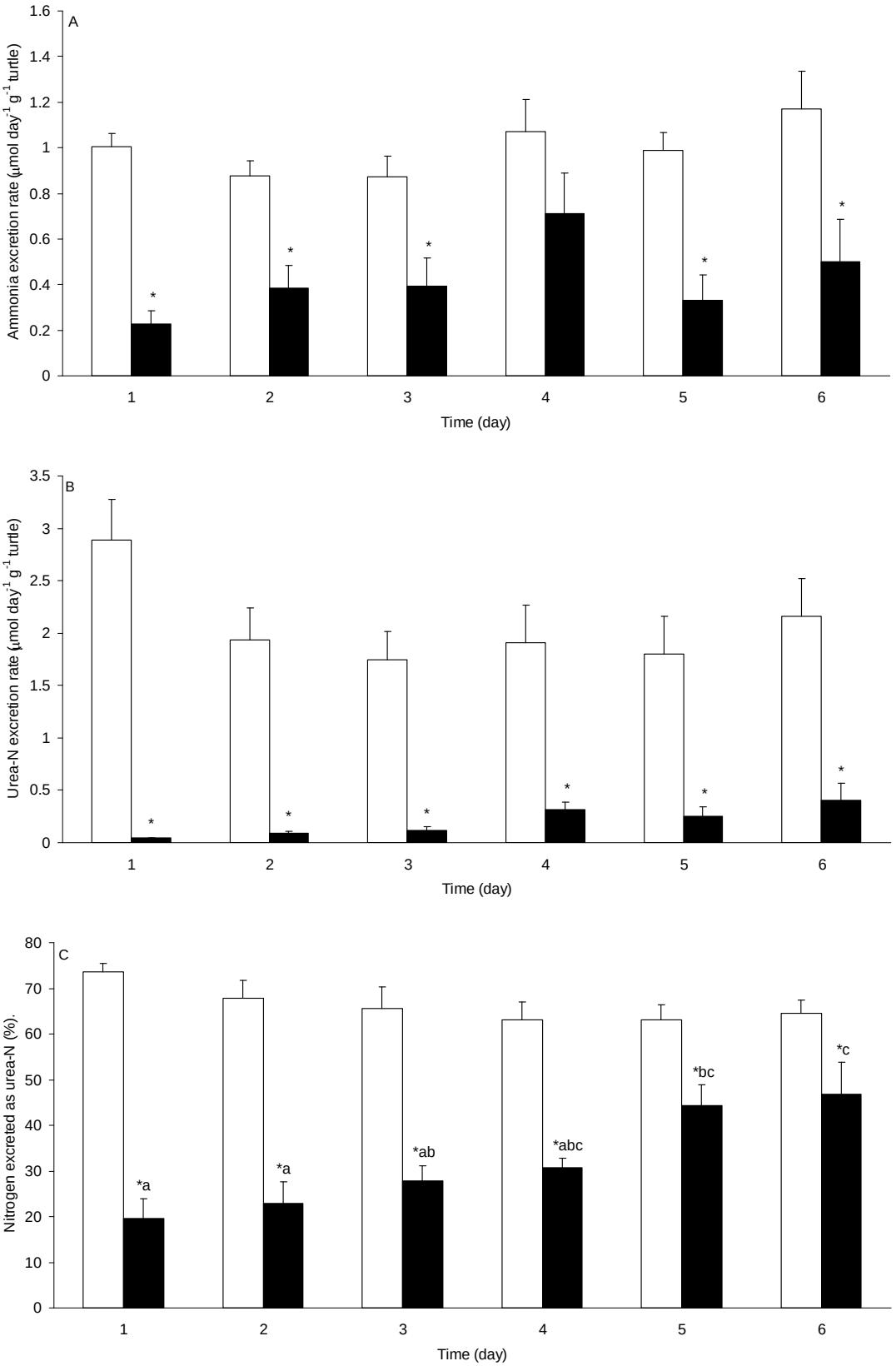

A
Ammonia excretion rate (μmol day-1 g-1 turtle)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Time (day)
B
Urea-N excretion rate (μmol day-1 g-1 turtle)
3.5
3
2.5
2
1.5
1
0.5
0
Time (day)
C
Nitrogen excreted as urea-N (%).
80
70
60
50
40
30
20
10
0
*a
*a
*ab
*abc
*bc
*c
Time (day)

Table 15 The volume (ml) of urine collected from a flexible latex tubing attached to the tail of *Pelodiscus sinensis* during 6 days of immersion (control) or emersion.

Day	Control	Emersion
1	11±4 (4)	3.4±1.6 (7)
2	16±5 (4)	2.0±0.9* (7)
3	8.1±4.5 (4)	2.9±0.5 (7)
4	15±2 (4)	2.1±0.5* (7)
5	7.3±1.9 (4)	2.2±0.4 (5)
6	15±3 (4)	6.7 (1)[#]

Values represent means±S.E.M.; with number of determinations represented in parenthesis.

*Significantly different from the corresponding control value

[#]Total *N*= 6, but no urine production in 5 out of the 6 turtles

For control turtles immersed in water, the daily rates of ammonia excretion into water (through non-urine routes) and into urine are comparable; 31-80% of the ammonia-N excreted during the 6-day period originated from the urine (Table 16). In contrast, the urea excreted through urine could account for only 0.45-15% of the total urea excreted throughout the 6 days of immersion (Table 17). Emersion resulted in significant decreases in ammonia excretion through non-urine (into water) and the urine routes on days 1, 2, 3, 4 and 6 (Table 16). It also resulted in a significant decrease in the rate of urea excretion into water on days 1, 2, 3, 4 and 6, but had no significant effects on the rate of urea excretion through the urine (Table 17).

Ammonia and urea excretion through the head and tail regions

When turtles were restrained on land with a known volume of water made available to the head, the volume of water consumed over 6 days of emersion was found to be 30-41 ml day^{-1} (Table 18). Throughout the 6-day period, the rates of ammonia excretion through the head and tail (urine route) regions were comparable, except that the rate of ammonia excretion through the tail tended to be greater than that through the head (Table 18). In contrast, the urea excretion rate through the head region was significantly greater (15- to 49-fold) than that through the urine throughout the 6 days (Table 18).

Urea excretion through the buccopharyngeal route

It was observed that turtles could hold water in the mouth and regurgitate it back to the container. So, it is probable that urea excretion through the head region could occur through the buccopharyngeal route. The definitive results which support this proposition was derived from the fact that an increase in urea concentration in the water inside the plastic container positioned in front of the turtle occurred only after a decrease in the P_{O_2} therein, which coincided with water regurgitation (Fig. 14).

Table 16 Rates (μmol N day^{-1} g^{-1} turtle) of ammonia excretion into water (non-urine route) or urine, and the percentage of ammonia-N excreted through urine, in *Pelodiscus sinensis* during 6 days of immersion (control) or emersion, with the urine being collected into a flexible latex tubing attached to the tail.

Day	Control			Emersion		
	Rate of ammonia excretion		% ammonia-N in the urine	Rate of ammonia excretion		% ammonia-N in the urine
	Into water	Into urine		Into water	Into urine	
1	0.21±0.06 (4)	0.26±0.09 (4)	52±5 (4)	0.057±0.011* (7)	0.18±0.10 (7)	46±16 (7)
2	0.45±0.25 (4)	1.0±0.3 (4)	68±17 (4)	0.070±0.028 (7)	0.12±0.05* (7)	40±15 (7)
3	0.23±0.04 (4)	0.65±0.60 (4)	31±22 (4)	0.068±0.015* (7)	0.41±0.11 (7)	81±8 (7)
4	0.17±0.03 (4)	0.75±0.25 (4)	80±3 (4)	0.19±0.08 (7)	0.19±0.08* (7)	34±12 (7)
5	0.34±0.05 (4)	0.36±0.12 (4)	48±12 (4)	0.16±0.05 (5)	0.22±0.11 (5)	43±15 (5)
6	0.36±0.11 (4)	0.78±0.32 (4)	63±12 (4)	0.024±0.011* (6)	1.3 (1)[#]	98 (1)[#]

Values represent means±S.E.M.; with number of determinations represented in parenthesis.

*Significantly different from the corresponding control value

[#]Total *N*= 6, but no urine production in 5 out of the 6 turtles

Table 17 Rates (μmol N day^{-1} g^{-1} turtle) of urea excretion into water (non-urine route) or urine, and the percentage of urea-N excreted through urine, in *Pelodiscus sinensis* during 6 days of immersion (control) or emersion, with the urine being collected into a flexible latex tubing attached to the tail.

Day	Control			Emersion		
	Rate of urea excretion		% urea-N in the urine	Rate of urea excretion		% urea-N in the urine
	Into water	Into urine		Into water	Into urine	
1	1.2±0.2 (4)	0.19±0.12 (4)	11±6 (4)	0.064±0.036* (7)	0.0030±0.0018 (7)	8.3±4.7 (7)
2	0.80±0.20 (4)	0.0043±0.0026 (4)	0.45±0.12 (4)	0.062±0.046* (7)	0.076±0.047 (7)	27±14 (7)
3	0.71±0.15 (4)	0.071±0.070 (4)	4.2±4.0 (4)	0.042±0.023* (7)	0.18±0.08 (7)	49±18 (7)
4	0.72±0.12 (4)	0.044±0.035 (4)	4.2±2.7 (4)	0.058±0.035* (7)	0.076±0.041 (7)	38±16 (7)
5	0.57±0.19 (4)	0.11±0.09 (4)	15±11 (4)	0.14±0.07 (5)	0.14±0.06 (5)	49±21 (5)
6	1.4±0.3 (4)	0.0074±0.0037 (4)	0.65±0.37 (4)	0.023±0.004* (6)	0.0030 (1)[#]	15 (1)[#]

Values represent means±S.E.M.; with number of determinations represented in parenthesis.

*Significantly different from the corresponding control value

[#]Total *N*= 6, but no urine production in 5 out of the 6 turtles

Table 18 Volumes (ml) of water consumed and rates (μmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion through the head or tail region of *Pelodiscus sinensis.*

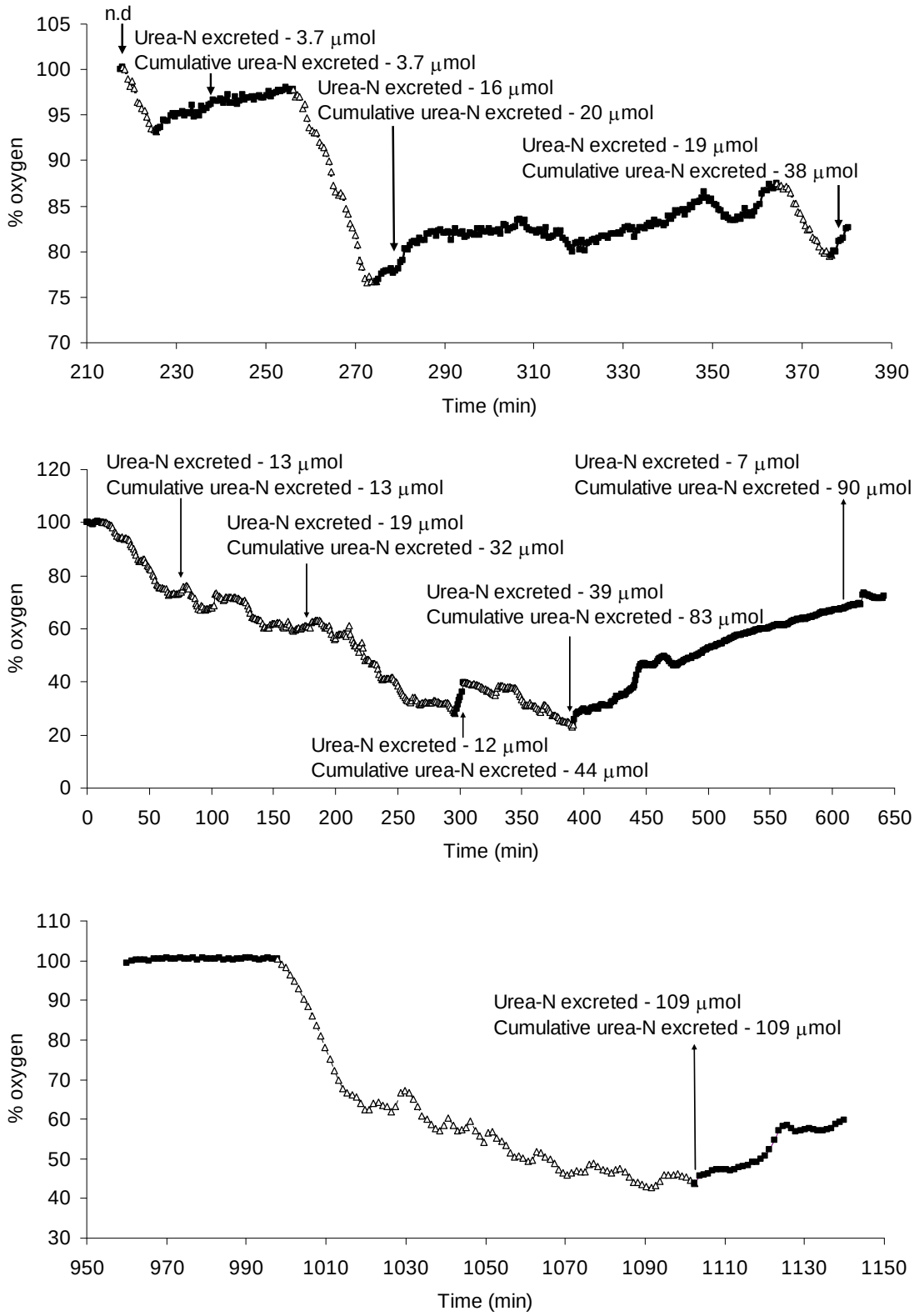
Day (*N*)	Water consumed	Ammonia			Urea		
		Rate of excretion		Total ammonia-N excretion rate	Rate of excretion		Total urea-N excretion rate
		Through the head region	Through the tail (urine)		Through the head region	Through the tail (urine)	
1 (5)	33±5	0.17±0.05	0.37±0.06*	0.54±0.10	0.95±0.07	0.038±0.011*	0.99±0.08
2 (8)	35±12	0.32±0.10	0.35±0.08	0.68±0.06	2.0±0.5	0.11±0.05*	2.1±0.5
3 (7)	41±15	0.24±0.06	0.42±0.16	0.66±0.13	1.7±0.4	0.11±0.08*	1.8±0.5
4 (7)	34±9	0.34±0.17	0.87±0.43	1.2±0.5	1.8±0.2	0.037±0.025*	1.9±0.2
5 (6)	32±6	0.37±0.14	0.65±0.29	1.0±0.3	1.6±0.3	0.11±0.07*	1.8±0.3
6 (7)	30±7	0.33±0.11	0.61±0.31	0.94±0.37	1.3±0.3	0.036±0.015*	1.4±0.3

Values represent means±S.E.M; with number of determinations represented in parenthesis.

*Significantly different from the value of the corresponding head region

Fig. 14 The quantity of urea excreted (μmol N) immediately after a decrease in oxygen level (%) in 100 ml of freshwater (1‰) made available to three different *Pelodiscus sinensis*. Dashed lines with open triangle markers represent periods when the head was immersed in the water. Solid lines with square markers represent periods when the head was not in the water. Each graph shows the profile for an individual turtle. n.d., not detectable.

Fig. 14


n.d
Urea-N excreted - 3.7 μmol
Cumulative urea-N excreted - 3.7 μmol
Urea-N excreted - 16 μmol
Cumulative urea-N excreted - 20 μmol
Urea-N excreted - 19 μmol
Cumulative urea-N excreted - 38 μmol
% oxygen
Time (min)
Urea-N excreted - 13 μmol
Cumulative urea-N excreted - 13 μmol
Urea-N excreted - 19 μmol
Cumulative urea-N excreted - 32 μmol
Urea-N excreted - 12 μmol
Cumulative urea-N excreted - 44 μmol
Urea-N excreted - 39 μmol
Cumulative urea-N excreted - 83 μmol
Urea-N excreted - 7 μmol
Cumulative urea-N excreted - 90 μmol
% oxygen
Time (min)
Urea-N excreted - 109 μmol
Cumulative urea-N excreted - 109 μmol
% oxygen
Time (min)

Buccopharyngeal urea excretion in *P. sinensis* was inhibited by 0.1 mmol l^{-1} phloretin. Urea excretion rate decreased significantly from 0.95±0.02 μmol urea-N day^{-1} g^{-1} mass turtle observed in controls (*N*=5) to 0.040±0.02 μmol urea-N day^{-1} g^{-1} mass turtle in turtles treated with phloretin (*N*=3).

OUC enzyme activities

There were no significant changes in activities of OUC enzymes from the liver of turtles after 3 days of emersion. However, 6 days of emersion led to significantly decreases in activities of ASS + ASL and arginase by 36% and 28%, respectively (Table 19).

Tissue ammonia and urea contents

There were no significant changes in the ammonia contents in all the tissues and organs studied throughout the 6 days of emersion, except for those in the plasma and the brain which increased significantly by 1.4- and 1.6-fold, respectively, on day 6 only (Table 20). In contrast, there were significant increases in urea contents in all tissues and organs examined. On day 3, the urea contents in the brain, intestine, kidney, liver, muscle and plasma increased by 2.5-, 3.1-, 2.3-, 2.3-, 2.7-, 2.9-fold and level off thereafter (Table 21).

Tissue FAAs contents

In general, 6 days of emersion had no significant effects on the contents of various FAAs, TFAA and TEFAA in the liver, muscle and plasma of *P. sinensis* (Table 22, Table 23 and Table 24). However, there was a significant increase in the glutamine content in the brain on days 3 (1.6-fold) and 6 (2.1-fold). On day 6, there was also a significant decrease in the content of TEFAA in the brain (Table 25).

Table 19 Activities (μmol min^{-1} g^{-1} liver) of carbamoyl phosphate synthetase (CPS I), ornithine transcarbamylase (OTC), argininosuccinate synthetase + lyase (ASS+ASL), arginase, glutamine synthetase (GS) and glutamate dehydrogenase (GDH) in the direction of reductive amination in the liver of *Pelodiscus sinensis* exposed to emersion.

	Activities (μmol min^{-1} g^{-1} liver)			
	Day 3		Day 6	
	Control	Emersion	Control	Emersion
CPS				
NH_4Cl	n.d.	n.d.	n.d.	n.d.
NH_4Cl + NAG	0.45±0.05	0.56±0.04	0.26±0.04	0.25±0.04
NH_4Cl + NAG + UTP	0.38±0.04	0.48±0.04	0.21±0.04	0.22±0.04
OTC	98±9	100±9	97±15	81±9
ASS+ASL	0.41±0.07	0.43±0.05	0.36±0.04	0.23±0.02*
Arginase	171±12	205±14	208±5	149±12*
GS	1.7±0.7	1.2±0.2	1.0±0.2	0.96±0.16
GDH	90±7	87±11	85±34	77±15

Values represent means±S.E.M., *N*=4.
*Significantly different from the corresponding control value
NAG, N-acetyl-L-glutamate; UTP, uridine triphosphate; n.d., not detectable (detection limit = 0.001 μmol min^{-1} g^{-1} liver).

Table 20 Ammonia content (μmol g^{-1} tissue) in various tissues of *Pelodiscus sinensis* exposed to a 6-day period of emersion. Controls were immersed in 1‰ water for the period of the experiment.

	Ammonia content (μmol g^{-1} tissue)			
	Day 3		Day 6	
	Control	Emersion	Control	Emersion
Brain	0.80± 0.13	0.69±0.10	0.41±0.07	0.66±0.06*
Intestine	1.2± 0.2	1.3±0.1	0.81±0.09	1.1±0.2
Kidney	1.8± 0.4	2.2±0.2	1.1±0.1	1.4±0.1
Liver	3.0± 0.6	1.6±0.2	1.3±0.2	1.6±0.2
Muscle	0.48± 0.11	0.66±0.08	0.37±0.05	0.43±0.06
Plasma	0.37± 0.05	0.46±0.01	0.32±0.05	0.44±0.03*

Values represent means±S.E.M., *N*=6.

*Significantly different from the corresponding control value.

Table 21 Urea content (μmol g^{-1} tissue) in various tissues of *Pelodiscus sinensis* exposed to a 6-day period of emersion. Controls were immersed in 1‰ water for the period of the experiment.

	Urea content (μmol g^{-1} tissue)			
	Day 3		Day 6	
	Control	Emersion	Control	Emersion
Brain	1.2±0.5	3.0±0.4*	1.1±0.2	3.0±0.3*
Intestine	1.2±0.4	3.7±0.5*	1.3±0.3	3.5±0.3*
Kidney	1.5±0.5	3.5±0.5*	1.1±0.3	3.5±0.3*
Liver	1.3±0.4	3.0±0.4*	1.2±0.3	2.8±0.3*
Muscle	1.2±0.4	3.2±0.4*	1.4±0.3	3.4±0.2*
Plasma	1.2±0.4	3.5±0.4*	1.4±0.3	4.3±0.5*

Values represent means±S.E.M., *N*=6.

*Significantly different from the corresponding control value.

Table 22 Contents (μmol g^{-1} liver) of various free amino acids (FAA), total faa (TFAA) and total essential faa (TEFAA) in the liver of *Pelodiscus sinensis* exposed to emersion for a 6-day period.

	FAA content (μmol g^{-1} liver)			
	Day 3		Day 6	
FAA	Control	Emersion	Control	Emersion
Alanine	0.14±0.03	0.090±0.036	0.084±0.017	0.055±0.008
β-alanine	0.032±0.007	0.047±0.021	0.053±0.008	0.035±0.008
Arginine	0.036±0.003	0.031±0.006	0.028±0.006	0.027±0.004
Aspartate	0.18±0.03	0.079±0.037	0.092±0.025	0.070±0.014
Glutamate	0.90±0.07	1.0±0.3	0.71±0.11	0.76±0.14
Glutamine	0.25±0.13	1.0±0.7	0.096±0.052	0.070±0.013
Glycine	0.26±0.01	0.23±0.03	0.30±0.05	0.23±0.03
Histidine	0.21±0.03	0.17±0.01	0.22±0.02	0.17±0.02
Isoleucine	0.10±0.03	0.077±0.017	0.11±0.03	0.084±0.032
Leucine	0.20±0.05	0.14±0.04	0.19±0.05	0.15±0.05
Lysine	0.30±0.09	0.15±0.05	0.18±0.02	0.058±0.023*
Methionine	0.012±0.003	0.011±0.002	0.014±0.002	0.011±0.001
Phenylalanine	0.10±0.01	0.076±0.010*	0.081±0.010	0.074±0.012
Proline	0.23±0.12	0.090±0.010	0.13±0.02	0.081±0.013
Serine	0.30±0.03	0.26±0.06	0.21±0.06	0.25±0.07
Taurine	7.0±1.0	5.7±0.7	5.6±0.9	5.4±1.3
Threonine	0.13±0.01	0.090±0.017	0.12±0.02	0.074±0.020
Tyrosine	0.073±0.005	0.059±0.016	0.062±0.010	0.052±0.008
Valine	0.18±0.04	0.11±0.03	0.16±0.04	0.12±0.04
TFAA	10±1	9.2±1.6	8.3±1.1	7.6±1.6
TEFAA	1.0±0.2	0.64±0.09	0.85±0.13	0.56±0.14

Values represent means±S.E.M., *N*=4.

*Significantly different from the corresponding control value.

Table 23 Contents (μmol g^{-1} muscle) of various free amino acids (FAA), total faa (TFAA) and total essential faa (TEFAA) in the muscle of *Pelodiscus sinensis* exposed to emersion for a 6-day period.

	FAA content (μmol g^{-1} muscle)			
	Day 3		Day 6	
FAA	Control	Emersion	Control	Emersion
Alanine	0.24±0.04	0.27±0.02	0.23±0.04	0.22±0.02
β-alanine	0.40±0.03	0.44±0.09	0.60±0.06	0.37±0.09
Arginine	0.16±0.03	0.14±0.02	0.15±0.03	0.11±0.02
Aspartate	0.22±0.10	0.15±0.05	0.22±0.02	0.12±0.02*
Glutamate	0.79±0.37	0.54±0.16	1.0±0.3	0.59±0.1
Glutamine	0.66±0.09	0.94±0.14	0.50±0.1	0.81±0.16
Glycine	0.70±0.04	0.94±0.07*	0.81±0.13	0.93±0.09
Histidine	0.40±0.03	0.47±0.07	0.54±0.04	0.31±0.06*
Isoleucine	0.13±0.03	0.097±0.025	0.19±0.05	0.13±0.04
Leucine	0.22±0.06	0.16±0.05	0.30±0.08	0.21±0.07
Lysine	0.47±0.08	0.39±0.07	0.39±0.07	0.25±0.04
Methionine	0.032±0.005	0.030±0.001	0.038±0.007	0.025±0.004
Phenylalanine	0.12±0.01	0.11±0.01	0.12±0.02	0.10±0.02
Proline	0.28±0.03	0.33±0.02	0.35±0.03	0.27±0.03
Serine	0.21±0.04	0.21±0.04	0.18±0.04	0.20±0.01
Taurine	6.0±1.2	4.7±0.8	4.7±0.1	5.2±1.0
Threonine	0.17±0.02	0.17±0.02	0.18±0.03	0.14±0.02
Tryptophan	0.040±0.006	0.044±0.009	0.056±0.014	0.043±0.009
Tyrosine	0.21±0.07	0.34±0.07	0.39±0.16	0.30±0.09
Valine	0.20±0.04	0.14±0.04	0.25±0.06	0.18±0.06
TFAA	12±2	11±1	11±1	10±1
TEFAA	1.8±0.2	1.6±0.2	2.0±0.2	1.4±0.3

Values represent means±S.E.M., *N*=4.

*Significantly different from the corresponding control value.

Table 24 Contents (μmol ml^{-1} plasma) of various free amino acids (FAA), total faa (TFAA) and total essential faa (TEFAA) in the plasma of *Pelodiscus sinensis* exposed to emersion for a 6-day period.

	FAA content (μmol ml^{-1} plasma)			
	Day 3		Day 6	
FAA	Control	Emersion	Control	Emersion
Alanine	0.083±0.031	0.087±0.013	0.095±0.020	0.068±0.008
β-alanine	0.059±0.032	0.0096±0.0030	0.025±0.012	0.021±0.007
Arginine	0.055±0.016	0.058±0.012	0.074±0.015	0.062±0.011
Aspartate	0.012±0.004	0.0062±0.0009	0.0068±0.0019	0.0068±0.0017
Glutamate	0.092±0.029	0.058±0.004	0.060±0.021	0.067±0.017
Glutamine	0.13±0.04	0.15±0.03	0.11±0.02	0.13 ±0.03
Glycine	0.22±0.09	0.19±0.06	0.28±0.05	0.21±0.05
Histidine	0.14±0.05	0.084±0.014	0.11±0.03	0.094±0.017
Isoleucine	0.12±0.06	0.086±0.031	0.16±0.05	0.12±0.04
Leucine	0.26±0.15	0.14±0.06	0.25±0.08	0.19±0.07
Lysine	0.15±0.05	0.15±0.03	0.19±0.02	0.11±0.01*
Methionine	0.013±0.007	0.014±0.005	0.018±0.003	0.012±0.003
Phenylalanine	0.074±0.021	0.089±0.013	0.096±0.010	0.086±0.017
Proline	0.041±0.015	0.048±0.015	0.082±0.024	0.045±0.011
Serine	0.096±0.044	0.11±0.04	0.097±0.021	0.081±0.011
Taurine	0.17±0.10	0.027±0.006	0.099±0.027	0.056±0.022
Threonine	0.071±0.024	0.070±0.018	0.099±0.011	0.059±0.013
Tryptophan	0.025±0.007	0.035±0.004	0.051±0.003	0.038±0.007
Tyrosine	0.039±0.014	0.051±0.005	0.061±0.009	0.053±0.008
Valine	0.21±0.11	0.13±0.05	0.23±0.07	0.17±0.06
TFAA	2.0±0.7	1.6±0.3	2.2±0.4	1.7±0.3
TEFAA	1.0±0.4	0.79±0.21	1.2±0.3	0.86±0.23

Values represent means±S.E.M., *N*=4.

*Significantly different from the corresponding control value.

Table 25 Contents (μmol g^{-1} brain) of various free amino acids (FAA), total faa (TFAA) and total essential faa (TEFAA) in the brain of *Pelodiscus sinensis* exposed to emersion for a 6-day period.

	FAA content (μmol g^{-1} brain)			
	Day 3		Day 6	
FAA	Control	Emersion	Control	Emersion
Alanine	0.28±0.03	0.31±0.09	0.19±0.04	0.22±0.02
β-alanine	0.16±0.04	0.055±0.011	0.054±0.005	0.039±0.004
Arginine	0.10±0.02	0.11±0.01	0.12±0.02	0.12±0.01
Aspartate	0.66±0.12	0.63±0.05	0.55±0.02	0.65±0.05
Glutamate	4.8±0.9	5.7±0.4	4.8±0.5	5.8±0.3
Glutamine	1.9±0.2	3.0±0.3*	1.5±0.2	3.1±0.6*
Glycine	0.26±0.04	0.17±0.01	0.28±0.05	0.24±0.04
Histidine	0.30±0.06	0.14±0.03*	0.11±0.01	0.089±0.012
Isoleucine	0.039±0.015	0.025±0.004	0.048±0.015	0.032±0.007
Leucine	0.090±0.033	0.064±0.008	0.098±0.026	0.074±0.015
Lysine	0.27±0.04	0.24±0.01	0.25±0.02	0.17±0.01*
Methionine	0.063±0.015	0.048±0.010	0.056±0.010	0.081±0.010
Phenylalanine	0.071±0.012	0.052±0.012	0.042±0.004	0.049±0.008
Proline	0.11±0.02	0.087±0.007	0.067±0.013	0.081±0.008
Serine	0.20±0.04	0.20±0.05	0.12±0.02	0.17±0.01
Taurine	2.1±0.2	1.6±0.1	1.6±0.1	1.5±0.1
Threonine	0.080±0.011	0.057±0.005	0.064±0.011	0.050±0.002
Tyrosine	0.052±0.007	0.041±0.012	0.032±0.009	0.046±0.015
Valine	0.063±0.023	0.041±0.006	0.062±0.017	0.045±0.011
TFAA	12±1	12±1	10±1	13±1
TEFAA	0.91±0.13	0.62±0.04	0.68±0.03	0.51±0.02*

Values represent means±S.E.M., *N*=4.

*Significantly different from the corresponding control value.

Calculated results for a 300 g P. sinensis

The construction of a balance sheet (Table 26) for the excretion and retention of N during emersion for a 300 g turtle reveals that emersion indeed resulted in a decrease in nitrogenous excretion. The reduction in N-waste amounted to 2002 and 3950 μmol-N on day 3 and day 6 respectively, but the respective excess N accumulated were 415 and 445 μmol-N only (Table 26).

Table 26 A nitrogen balance table (μmol N) of a 300 g *Pelodiscus sinensis*, taking into account the muscle, liver, intestine and plasma when exposed to emersion for a 6-day period.

	Day 3			Day 6		
	Control	Emersion	Difference	Control	Emersion	Difference
Excreted from *P. sinensis*						
Ammonia-N	825	302	-523	1795	764	-1031
Urea-N	1968	74	-1894	3730	366	-3364
Reduction in nitrogenous excretion (A)			-2417			-4395
Retained in muscle (74 g)						
Ammonia-N	36	49	+13	27	32	+5
Urea-N	179	474	+295	208	506	+298
Retained in liver (9 g)						
Ammonia-N	27	14	-13	11	15	+4
Urea-N	24	54	+30	21	50	+29
Retained in intestine (4 g)						
Ammonia-N	5	5	0	3	5	+2
Urea-N	9	29	+20	10	28	+18
Retained in plasma (15 ml)						
Ammonia-N	6	7	+1	5	7	+2
Urea-N	37	106	+69	43	130	+87
Increase in nitrogenous accumulation (B)			+415			+445
(A) + (B)			-2002			-3950

DISCUSSION

The osmotic stress of emersion

In the past, the rates of evaporative water loss in turtles were determined in the laboratory under non-physiological conditions; the external surfaces of the animals were completely dried and dry air was flushed over animals during measurements (e.g. Bentley and Schmidt-Nielsen, 1970). In a more recent study, Peterson and Greenshields (2001) exposed *T. scripta* to 10 days of dehydration in a relative humidity of 40% at 25ºC. At the end of the 10-day period, the reduction in body mass of *T. scripta* amounted to 19-32%, and the plasma osmolality increased from 275 to 402 mosmol l^{-1} (Peterson and Greenshields, 2001). The hatchlings of the leatherback sea turtle, *Dermochelys coriacea*, also dehydrated rapidly when denied access to seawater; the haematocrit increased significantly from 30 ± 1% to 39 ± 1% and plasma [Na^+] increased significantly from 138 ± 3 to 166 ± 11 mmol l^{-1} within a 12-h period (Reina et al., 2002). In contrast, *P. sinensis* exhibited only a slight change (2-2.3%) in the body mass after 3 or 6 days of emersion. Thus, it was not surprising that 6 days of emersion had no significant effects on its haematocrit and plasma osmolality, [Na^+] and [Cl^-]. Taken together, these results indicate that this soft-shelled turtle is well-adapted to emersion in its natural habitat, in spite of having an integument with relatively high water permeability.

Decreased production of urine, and the apparent change from ureotely to ammonotely during emersion

Because *P. sinensis* lost so little mass, and hence water, during emersion, it must have special adaptations which limited the loss of water. Firstly, it might be able to decrease pulmonary water loss by increasing oxygen extraction from air. This strategy has been observed in the chuckwalla, *S. obesus*, the royal snake, *S. cliffordii*

and the asp *C. cerastes* (Bentley, 1976). Secondly, and more importantly, it must be able to decrease the loss of water through urination, as observed in other testudines exposed to dehydrating conditions. Dehydration decreases glomerular filtration rate and increases tubular reabsorption of water in *T. scripta*, causing anuria in more severe cases (Dantzler and Schmidt-Nielsen, 1966). Anuria also occurred in *C. longicollis* after 20 days of dehydration. During dehydration, the volume of urine in the bladder of *C. longicollis* decreased from a mean of 32 to 2.0 ml, showing that water was reabsorbed from the bladder (Rogers, 1965).

Indeed the urine volume of *P. sinensis* decreased from 7-15 ml (control) to 2-3 ml during 5 days of emersion. By day 6, many (5 out of 6) of the experimental objects had no urine output. During the 6 days of emersion, there was a significant decrease in daily excretion of nitrogenous waste (ammonia-N + urea-N). The magnitude of decrease in the rate of urea excretion (by 82-99%) was much greater than that of ammonia excretion (by 29-76%) throughout the 6-day period. This could be due to two factors acting separately or in combination. Firstly, urea synthesis through the hepatic OUC might be suppressed during emersion. Results obtained subsequently were in support of this proposition. Secondly, different routes might be involved in ammonia and urea excretion, and emersion somehow hindered urea excretion more than ammonia excretion. For example, it is possible that ammonia was excreted continuously through the ventral skin in contact with a film of water, while urea was excreted mainly through the urine, the production of which was suppressed leading to a reduction in the excretion of urea, during emersion. However, subsequent results proved it otherwise.

Routes of ammonia and urea excretion in turtles immersed in freshwater

By collecting the urine into a latex tubing, it was discovered that approximately 31-80% of the ammonia excreted by *P. sinensis* immersed in freshwater was found in the urine, but surprisingly, the percentage of urea excreted through the urine was relatively small (0.45-15%). Our results indicate for the first time that the majority of urea excreted by *P. sinensis* immersed in freshwater took a non-urine route.

Because soft-shelled turtles are known to be capable of buccopharyngeal respiration under water, experiments were designed to examine the possibility that urea excretion occurred through the buccopharyngeal route in *P. sinensis*. By restraining the turtle on land for 6 days, with a known volume of water positioned in front of the extensible head and a container at the posterior end to receive urine, we demonstrated that a major portion of the urea was indeed excreted through the head region. Although the body of turtle was completely exposed to air, the conditions differed from those of emersion because the turtle could drink at liberty. Indeed, experimental turtles consumed 30-41 ml of water daily during the 6-day period. Taken together, the sum of nitrogenous (ammonia-N + urea-N) excretion through the head and the urine under such an experimental condition (from Table 18) could account for >90% (Fig. 14) of the nitrogenous excretion in turtles immersed in freshwater. So, it can be concluded that the experimental turtle did not only drink water but also held water in, and subsequently "regurgitated" water from, the buccopharyngeal cavity for purposes of O_2 extraction and urea excretion.

To confirm that urea was excreted through the buccopharyngeal lining of *P. sinensis*, we made an attempt to determine the oxygen level in the water placed within a plastic container adjacent to the head of the turtle. It was observed that turtles could

indeed hold water in the mouth and regurgitate it back to the container. When regurgitation of water occurred, there was a decrease in the oxygen level in the water, and simultaneously, there was an increase in the urea concentration therein. Thus, it can be concluded that *P. sinensis* was able to excrete urea through the buccopharyngeal lining, which has never been reported previously for other turtles and reptiles. Under such experimental conditions, pulmonary respiration should theoretically be sufficient to cater for gaseous exchange needed for survival in air; therefore, holding water in and regurgitating water from the buccopharyngeal cavity appears to be an activity dedicated for the excretion of urea.

Buccopharyngeal urea excretion in *P. sinensis* apparently involved urea transporter(s) because it can be inhibited by the aglycon phloretin. The first urea transporter cDNA was isolated from rabbit kidney inner medulla using an expression cloning approach that utilized heterologous expression of mRNA in *Xenopus* oocytes (You et al., 1993). This cDNA coded a 397-amino-acid glycoprotein termed UT-A2. Expression in *Xenopus* oocytes confirmed that the protein translocated urea in a facilitative manner and was inhibited by phloretin. Soon after this, a homologous cDNA was isolated from bone marrow that encoded a urea transporter that shared characteristics of UT-A2, but with only 60% amino acid identity (Olivès et al, 1996). It has now transpired that these two proteins are the products of two genes, known as UT-A and UT-B. In mouse and human, these genes are arranged in tandem on chromosome 18 (Olivès et al, 1995, 1996; Fenton et al., 1999). Both UT-A and UT-B family members are expressed in the kidney. UT-A is differentially expressed along the nephron (Shayakul et al., 1997), whereas UT-B is expressed in blood vessel of the descending vasa recta (Xu et al., 1997). Products of both genes have been detected in several other tissues including liver, brain, and colon (You et al., 1993; Olivès et al,

1996. At present, no information is available concerning UT-A and UT-B genes in *P. sinensis*; but, taken together, our results suggest that UT-A was present and expressed in the buccopharyngeal epithelium of this turtle and the resulting urea transporter was involved in facilitated urea excretion. It would imply that the kidney of this turtle is not the major organ involved in the excretion of urea.

An explanation for the apparent change from ureotely to ammonotely during emersion

During emersion, there was no water to flush the buccopharyngeal epithelium. Consequently, nitrogen excretion did not occur as evidenced by the drastically reduced urea excretion rates (1.5-18.2% of the control value). During emersion, there was a reduction in urine production but the ammonia concentration in the urine remained relatively unchanged. As a result, emersion reduced ammonia excretion rates by 34-77% over 6 days. Because of this, the experimental turtle apparently changed from ureotely to ammonotely during emersion. From these results, it can be deduced that the reduction in ammonia excretion through the urine and buccopharyngeal route was partially compensated for by an increase in ammonia excretion through the skin of the ventral carapace in constant contact with water. The compensation was gradual and took 2-3 days, indicating that the transition between the renal route and the cutaneous route could be time-dependent. The integument of soft-shelled turtles is known to be permeable to respiratory gases (Girgis, 1961; Wang et al., 1989) and therefore it is logical that it also constitutes an effective route for ammonia (NH_3) excretion.

The decrease in urea excretion was the greatest on day 1 (1.5% of the control value), but the urea excretion rate recovered to 19% of the control value on day 6. Thus, it is probable that some urea excretion occurred through the skin of the ventral

carapace during an extended period of emersion, and it would be important to investigate the expression of urea transporters in the skin of these experimental turtle in the future.

Decrease in urea synthesis during emersion

Indeed 6 days of emersion led to significant increases in urea contents in all tissues and organs studied. However, the excess urea-N accumulated was only 414 μmol N on day 3 and 432 μmol N on day 6, while the respective decreases in urea-N excretion amounted to 1894 and 3364 μmol N (Table 26). So, the excess urea accumulated could only account for 22% and 13% of the deficit in urea excretion on day 3 and day 6, respectively, and it is therefore logical to deduce that there was a decrease in the rate of urea synthesis in *P. sinensis* during 6 days of emersion. If urea had been produced at a constant rate, a proportional amount of urea should theoretically be accumulated in the turtle.

The urea excretion rate, and hence urea production rate, of *P. sinensis* in freshwater can be estimated to be 2.1 μmol N day^{-1} g^{-1}. This is because tissue urea contents are maintained at steady states by a balance between urea production and urea excretion during immersion. On day 6 of emersion, the averaged daily urea production rate can be calculated from the daily urea excretion and excess urea accumulated in various tissues on day 6, i.e., (121 + 506 + 50 +28 + 130) μmole N/ (6 days x 300 g), or 0.46 μmol N day^{-1} g^{-1}. That means the averaged daily urea excretion rate decreased by [(2.1-0.46)/2.1] x 100 = 78% during 6 days of emersion. Judging by enzyme activities, both CPS I and ASS + ASL could be rate-limiting in the hepatic OUC in *P. sinensis*. The decreases in ASS + ASL (by 40%) and arginase (by 30%) activities supports the proposition that the rate of urea synthesis was decreased during emersion. So, it can be concluded that urea did not act as an important osmolyte to

reduce evaporative water loss in *P. sinensis* during emersion, as has been suggested for certain animals, many of which aestivate under arid conditions (Shoemaker et al., 1969; Horne, 1971). Similarly, the role of urea in reducing evaporative water loss in the aestivating giant African snail, *Achatina fulica*, has been questioned recently (Hiong et al., 2005).

Possible decrease in ammonia production during emersion

CPS I utilizes ammonia as one of the substrates to produce urea. Hence, there should be increases in ammonia contents in various tissues and organs, if decreased urea synthesis occurred concurrently with an unchanged or decreased rate of ammonia excretion during emersion. However, ammonia contents increased significantly in the plasma and brain on day 6 only, and the accumulated ammonia was inadequate to account for the decreased urea accumulation. Therefore, it can be deduced indirectly that ammonia production, which occurred mainly through amino acid catabolism under fasting conditions, had been suppressed. Because there were no significant increases in contents of TFAA and TEFAA in various tissues and organs, it can also be deduced that the release of amino acid through proteolysis was also proportionally reduced in *P. sinensis* during emersion. However, results indicate that, partial catabolism of certain amino acids to alanine (Ip et al., 2001b; Chew et al., 2001, 2003b) was not involved in reducing ammonia production in *P. sinensis*, because there was no accumulation of alanine during emersion. While 6 days of emersion did not result in extraordinary increases in glutamine synthesis in extra-cranial tissues in *P. sinensis* as in some tropical air-breathing fishes exposed to air (Jow et al., 1999; Ip et al., 2001a; Chew et al., 2001; Tay et al., 2003), its brain was capable of detoxifying ammonia to glutamine as in many other vertebrates (Cooper and Plum 1987; Peng et al., 1998; Ip et al., 2005).

Conclusion

Pelodiscus sinensis is ureotelic in water; urea excretion occurred mainly through the buccopharyngeal epithelium, while ammonia excretion could occur through the buccopharyngeal route, the kidney and the skin. During 6 days of emersion, it reduced water loss through a reduction in urine production, which in part resulted in no change in haematocrit and plasma osmolality, $[Na^+]$ and $[Cl^-]$. Despite a decrease in urine production, *P. sinensis* was able to maintain ammonia excretion rates at 23-66% of immersed controls, possibly through the skin of the ventral carapace. However, urea excretion was drastically reduced. Although tissue urea contents increased significantly, the rate of urea synthesis decreased and there were decreases in activities of certain OUC enzymes. A decrease in urea synthesis occurred without an accumulation of ammonia, TFAA and TEFAA, indicating that there was a suppression in ammonia production through amino acid catabolism and a reduction in proteolysis.

CHAPTER 4. ACUTE AMMONIA TOXICITY

MATERIALS AND METHODS

Procurement and maintenance of animals

Animals were procured and maintained as stated in Chapter 1 on page 47.

Intraperitoneal injection with a lethal dose of NH_4Cl and the protective effects of MK801 or MSO

Intraperitoneal injection was performed through an area of the skin between the hind leg and the soft carapace without using anesthesia, and the whole process took less than 1 min. Preliminary experiments indicated that 100% mortality would occur within a 24-h period after *P. sinensis* was injected intraperitoneally with 12.5 μmol NH_4Cl g^{-1} turtle. In contrast, no mortality was observed in turtles injected with 0.9% NaCl. Upon respiratory failure and the loss of reflex, which occurred usually within 1-3 h after the injection with this dose of NH_4Cl, the brain was excised and freeze-clamped in liquid nitrogen. Brain samples were stored at -80°C until the analyses of ammonia, glutamate and glutamine contents.

To evaluate whether MK801 (Sigma-Aldrich Chemical Co., St Louis, MI) had a protective effect against acute ammonia toxicity in *P. sinensis*, turtles (*N*=10) were injected intraperitoneally with 1.6 μg MK801 g^{-1} turtle (pH 7.0) 15 min prior to the injection with 12.5 μmol NH_4Cl g^{-1} turtle. The protective dose of MK801 for mammal is 2 μg g^{-1} animal, and the equivalent dose for *P. sinensis* was estimated as 1.6 μg g^{-1} turtle taking into consideration the extra mass of the carapace. Preliminary results obtained indicated that injection of 1.6 μg MK801 g^{-1} turtle alone had no observable effects. Controls (*N*=10 each) were injected with 0.9% NaCl solution in place of MK801 before the injection with NH_4Cl. The mortality rate and time of death for succumbed turtles were recorded during the subsequent 24 h.

Similarly, the possible protective effect of MSO (Sigma-Aldrich Chemical Co., St Louis, MI) was evaluated by the intraperitoneal injection with 82 μg MSO g^{-1} turtle (pH 7.0) 15 min prior to the injection with 12.5 μmol NH_4Cl g^{-1} turtle. The dose (82 μg g^{-1} turtle) of MSO used in this study was equivalent to the protective dose of 100 μg MSO g^{-1} for mammals. Preliminary results obtained indicate that injection of 82 μg MSO g^{-1} turtle alone had no observable effect. Controls (*N*=10 each) were injected with 0.9% NaCl solution in place of MSO before the injection with NH_4Cl. The mortality rate and time of death for succumbed turtles were recorded during the subsequent 24 h. A separate batch of turtle was injected with 82 μg MSO g^{-1} followed with 12.5 μmol g^{-1} NH4Cl, and euthanized for the collection of the brains exactly 1 h after the injection with MSO before they succumbed to ammonia toxicity. Brain samples were freeze-clamped in liquid nitrogen and stored at -80^{o}C until the analyses of ammonia, glutamate and glutamine contents.

Determination of water contents in the brain

The water content in the brain of turtles succumbed to a lethal dose (12.5 μmol g^{-1} turtle) of ammonium chloride and turtles injected with 0.9% NaCl (*N*=3 each) were determined as described in Chapter 2 on 74.

Determination of contents of ammonia, glutamine and glutamate

The brains collected above were used for the determination of contents of ammonia, glutamine and glutamate. Ammonia was determined as described in Chapter 1 on page 47. An aliquot of the supernatant as obtained for ammonia assay was also used to determine contents of glutamine and glutamate. Glutamine was determined by the method of Mecke (1985). Glutamate was assayed as modified from the protocol for GDH in the deaminating direction in Ip et al. (1993). Results were expressed as μmol g^{-1} brain.

Intraperitoneal injection with a sub-lethal dose of NH_4Cl and the collection of water and tissues samples

Experimental specimens were injected intraperitoneally with a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle). Control specimens were injected with an equivalent volume of 0.9% saline. After injection, specimens were submerged individually in plastic aquaria tanks containing 10 volumes (w/v) of water at 25°C.

Collection of water samples for analyses

Water samples (3 ml) were collected every 6 h, acidified with 70 μl of 1 mol l^{-1} HCl and kept at 4°C until analyzed. Randomly selected water samples were collected in duplicate left at 25°C for 24 h and then acidified and stored at 4°C until analysis. These served as a control for microbial activity, and results obtained confirmed that the ammonia and urea concentration remained relatively unchanged after 24 h of incubation at 25°C.

Collection of tissue samples for analyses

At hours 0.5, 1, 3, 6, 12 and 24, turtles were killed by a strong blow to the head. Blood samples were collected by cardiac puncture into heparinized syringes, and centrifuged at 5,000 *g* and 4°C for 5 min to obtain the plasma. The plasma was deproteinized by the addition of an equal volume (v/v) of ice-cold 6% trichloroacetic acid and centrifuged at 10,000 *g* and 4°C for 15 min. The resulting supernatant was kept at -25°C until analysis. The muscle, liver, stomach, intestine and brain were quickly excised. The stomach and intestine were removed, flushed well with water, and divided into 2 halves longitudinally. The excised tissues and organs (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs (Faupel et al., 1972). Frozen samples were kept at -80°C until analysis.

Determination of ammonia and urea concentrations in water samples

Ammonia and urea concentrations in water samples were determined as described in Chapter 1 on page 50.

Determination of contents of ammonia, urea and FAAs in tissues samples

Contents of ammonia, urea and FAAs in tissue samples were determined as described in Chapter 1 on page 47. The content of TFAA was calculated by the summation of contents of all FAAs, while the content of TEFAA was calculated as the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Determination of enzymes activities

Activities of GS in the brain, liver and muscle of saline- and NH_4Cl-injected *P. sinensis* were determined as described in Chapter 1 on page 51.

Glutamate dehydrogenase (EC 1.4.1.3) activity was assayed in the aminating and deaminating directions according to Ip et al. (1993). For the amination reaction, GDH activity was expressed as μmol NADH utilized min^{-1} g^{-1} tissue. For the deamination reaction, GDH activity was expressed as μmol formazan formed min^{-1} g^{-1} tissue.

Determination of whether increased ammonia excretion occurred through the urine or other parts of the body

A flexible latex tubing (length 18 cm, radius 0.7 cm) was attached around the tail anterior to the cloaca of *P. sinensis* using 3M Vetbond™ tissue adhesive. An opening made at the very tip of the tube was held closed by a dialysis clip (Fig. 11). Turtles were either injected with 0.9% NaCl (control) or injected intraperitoneally with 7.5 μmol NH_4Cl g^{-1} turtle followed by immersion in 10 volumes (w/v) of 1‰

water. Water samples were collected and acidified after 24 h. At the same time, urine was collected by emptying the contents of the tubing through the opening into a 5 or 10 ml measuring cylinder. Urine samples were acidified with 1 mol l^{-1} HCl, with the ratio of acid: urine being 7: 300, and kept at 4°C until analyzed. Ammonia and urea concentrations in the water and urine samples were determined as described in Chapter 1 on page 50.

In a separate experiment, individual turtles were taken out of water, injected intraperitoneally with 7.5 μmol NH_4Cl g^{-1} turtle, and restrained separately on the bottom surface of a plastic box (L18.5 cm x W11.5 cm x H5.5 cm) which was turned upside down. A plastic box of water (700 ml) was placed directly in front of the head in this setup. So, the experimental turtle could dip its head fully into the water at liberty for drinking purpose or buccopharyngeal respiration. Water samples (3 ml) were collected from the plastic box after 24 h, acidified with 70 μl of 1 mol l^{-1} HCl, and kept at 4°C until analyzed. Ammonia and urea concentrations in water samples were determined as described in Chapter 1 on page 50.

Statistical analyses

Results are presented as means ± standard error of the mean (S.E.M). Data in Fig. 15 was analyzed using 2-way repeated-measures ANOVA followed by least-square means (LSMEANS) to evaluate differences between means. Arcsine transformation was applied to percentage data before statistical analysis. Data in tables were assessed using independent t-tests to evaluate differences between means. Differences with $P<0.05$ were regarded as statistically significant.

RESULTS

Contents of ammonia, glutamate and glutamine in the brains of turtles succumbed to a lethal dose of NH_4Cl

After turtles were injected intraperitoneally with a lethal dose of NH_4Cl (12.5 μmol g^{-1} turtle), they died between 1 and 3 h (Table 27). The brain ammonia content of turtles that succumbed to acute ammonia toxicity increased 14-fold to 21 μmol g^{-1} (Table 28). In addition, the brain glutamine content increased 2-fold to 4.4 μmol g^{-1}, while the brain glutamate content decreased by 48% (Table 28).

Effects of acute ammonia toxicity on brain water content

The water contents (N =3) in brains of turtles succumbed to 12.5 μmol g^{-1} of NH_4Cl (84 ± 1%) and those injected with 0.9% NaCl (85 ± 1%) were comparable.

Effects of MK801 and MSO in prevention of mortality

MK801 (1.6 μg g^{-1} turtle) reduced the mortality (by 50%) of *P. sinensis* injected with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl over a 24-h period, and extended the time to death for those which succumbed to ammonia toxicity (Table 27).

MSO (82 μg g^{-1} turtle) did not reduce the mortality of turtles injected with 12.5 μmol g^{-1} turtle of NH_4Cl, but significantly extended the time to death by 4.6-fold (Table 27). For turtles which finally succumbed to acute ammonia toxicity after the injection with 82 μg MSO g^{-1} followed with 12.5 μmol NH_4Cl g^{-1}, the brain ammonia, glutamate and glutamine contents were comparable to those which succumb to acute ammonia toxicity without MSO intervention (Table 28). In order to examine if the protective effects of MSO were derived from a suppression of glutamine synthesis and hence glutamine accumulation, the brains of turtles injected with 82 μg MSO g^{-1} followed with 12.5 μmol NH_4Cl g^{-1} were obtained 1 h after the injection with NH_4Cl.

Table 27 Mortality within 24 h and time to death in *Pelodiscus sinensis* injected with NaCl (0.9%), MK801 (1.6 μg g^{-1} turtle) or MSO (82 μg g^{-1} turtle) followed with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl 15 min afterwards.

	Mortality within 24 h	Time to death (h)	
		Range	Average
Saline + NH_4Cl (10)	10	1.1 – 2.7	2.1±0.1[a]
MK801 + NH_4Cl (10)	5	2.3 – 11	4.2±1.7[a]
MSO + NH_4Cl (10)	10	2.7 – 14	7.9±1.7[b]

Average time to death within 24 h are represented as means±S.E.M.

Number of turtles injected at the start of the experiment are represented in parenthesis

Averages not sharing the same letter are significantly different, $P<0.05$.

Table 28 Contents (μmol g^{-1} brain) of ammonia, glutamate and glutamine in *Pelodiscus sinensis* 1 h after the injection with 0.9% NaCl (control), at the time of death after the injection with NaCl (0.9%) or MSO (82 μg g^{-1} turtle) followed with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl 15 min afterwards, or 1 h after the injection with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl subsequent to a dose of MSO injected 15 min earlier.

	Contents (μmol g^{-1} brain)		
	Ammonia	Glutamate	Glutamine
Saline, at 1 h post-saline injection (4)	1.5±0.1[a]	6.5±0.2[d]	2.2±0.1[e]
Saline + NH_4Cl, at time of death between 1.1 – 2.0 h (5)	21±1[b]	3.4±0.1[c]	4.4±0.2[f]
MSO + NH_4Cl, at time of death between 2.7 – 3.3 h (5)	22±1[b]	3.5±0.2[c]	4.7±0.2[f]
MSO + NH_4Cl, at 1 h post-NH_4Cl injection before death (4)	17±2[b]	6.2±0.2[d]	7.4±0.4[g]

Values represent means±S.E.M., with the number of determinations represented in parenthesis

Means of contents not sharing the same letter are significantly different, $P<0.05$.

Surprisingly, the brain glutamine and glutamate contents of these turtles were significantly higher than those of turtles that had succumbed to 12.5 μmol NH_4Cl g^{-1} with or without MSO intervention (Table 28). MSO apparently reduced the rate of ammonia buildup in the brain of the experimental turtle (Table 28).

Effects of injection with a sub-lethal dose of NH_4Cl on tissue ammonia and urea contents

No mortality was recorded among the 24 turtles injected intraperitoneally with a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle). Intraperitoneal injection with this sub-lethal dose of NH_4Cl led to significant increases in ammonia contents, which peaked within the first hour but returned to control levels at hour 24, in all the tissues studied (Table 29). At 1 h post-injection with NH_4Cl, the ammonia contents of the brain increased to 16 μmol g^{-1} brain, before recovering subsequently back to the control level (Table 29). The greatest ammonia content (35 μmol g^{-1} tissue) was recorded for the liver at hour 1. At their peaks, ammonia contents in the brain, intestine, liver, muscle, plasma and stomach increased 11-, 9.4-, 11-, 14-, 20- and 15-fold, respectively.

In contrast, significant increases (1.2- to 2.5-fold) in urea contents in the brain, intestine, liver, muscle, plasma and stomach were relatively small during the first hour post-injection with NH_4Cl (Table 30). Between hour 6 and hour 24, urea contents of all the tissues studied increased to 1-2 μmol g^{-1} tissue (Table 30), while the ammonia contents were in the process of returning back to normal (Table 29).

Effects of injection with a sub-lethal dose of NH_4Cl on tissue FAA contents

Intraperitoneal injection with 7.5 μmol NH_4Cl g^{-1} turtle resulted in significant changes in contents of many amino acids, including essential ones, in the brain (Table 31), liver (Table 32) and muscle (Table 33). In the brain, glutamine content increased

Table 29 Contents (μmol g^{-1} tissue) of ammonia in various tissues of *Pelodiscus sinensis* during the 24-h period after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	Ammonia contents (μmol g^{-1} tissue)											
	0.5h		1h		3h		6h		12h		24h	
Tissue	Saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl
Brain	1.4±0.1	15±1*	1.5±0.1	16±1*	1.4±0.4	8.4±1*	1.4±0.1	5.3±1.4*	0.55±0.05	2.8±0.4*	1.5±0.2	2.1±0.3
Intestine	1.7±0.1	16±2*	1.7±0.1	13±1*	1.2±0.1	10±1*	1.6±0.1	7.2±1.3*	1.1±0.1	3.8±0.7*	1.7±0.2	1.7±0.2
Liver	3.9±0.8	22±4*	3.1±0.5	35±4*	3.5±0.2	13±1*	3.9±0.5	15±3*	3.2±0.5	7.0±1.7	4.1±0.4	4.8±0.6
Muscle	0.71±0.15	10±2*	0.71±0.07	10±1*	0.69±0.05	6.2±1.2*	0.53±0.01	7.0±2.2*	0.47±0.03	2.9±0.7*	0.76±0.21	1.3±0.3
Plasma	0.55±0.04	11±1*	0.46±0.02	7.3±0.4*	0.26±0.04	3.3±0.4*	0.22±0.04	2.9±0.5*	0.18±0.03	0.51±0.03*	0.50±0.03	0.41±0.02
Stomach	0.94±0.09	12±2*	0.82±0.02	12±1*	0.80±0.04	6.6±0.7*	0.72±0.04	7.6±1.8*	0.75±0.09	3.4±0.9*	0.97±0.22	1.1±0.1

Values are means ± S.E.M., N=4.

*Significantly different from the corresponding saline-injected value (P<0.05).

Table 30 Contents (μmol g^{-1} tissue) of urea in various tissues of *Pelodiscus sinensis* during the 24-h period after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	Urea contents (μmol g^{-1} tissue)											
	0.5h		1h		3h		6h		12h		24h	
Tissue	Saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl	Saline	NH_4Cl	saline	NH_4Cl	saline	NH_4Cl
Brain	0.29±0.10	0.37±0.04	0.30±0.06	0.76±0.16*	0.24±0.03	0.62±0.36	0.30±0.09	0.96±0.30	0.13±0.02	1.2±0.3*	0.53±0.12	1.5±0.1*
Intestine	0.20±0.07	0.46±0.06*	0.43±0.07	0.73±0.11	0.23±0.03	0.66±0.32	0.39±0.09	1.1±0.2*	0.37±0.08	1.2±0.3*	0.54±0.03	1.3±0.1*
Liver	0.57±0.18	0.69±0.13	0.58±0.03	1.1±0.1*	0.16±0.05	0.75±0.23*	0.17±0.06	1.2±0.4*	0.29±0.08	1.0±0.2*	0.58±0.18	1.0±0.1
Muscle	0.33±0.11	0.54±0.10	0.60±0.08	0.92±0.21	0.35±0.07	0.99±0.28	0.48±0.12	1.4±0.3*	0.45±0.10	1.8±0.3*	0.50±0.15	1.9±0.1*
Plasma	0.39±0.13	0.46±0.05	0.68±0.08	0.90±0.23	0.27±0.05	0.70±0.23	0.28±0.08	1.4±0.3*	0.61±0.10	0.89±0.14	0.62±0.15	1.4±0.2*
Stomach	0.26±0.07	0.53±0.11	0.38±0.07	0.73±0.13*	0.32±0.09	0.87±0.48	0.34±0.11	1.4±0.3*	0.46±0.09	1.5±0.3*	0.55±0.16	1.4±0.1*

Values are means ± S.E.M., N=4.

*Significantly different from the corresponding saline-injected value (P<0.05).

Table 31 Contents (μmol g^{-1} brain) of various free amino acids (FAA), total faa (TFAA) and total essential FAA (TEFAA) in the brain of *Pelodiscus sinensis* during the 24-h period after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	FAA contents (μmol g^{-1} brain)											
	0.5h		1h		3h		6h		12h		24h	
FAA	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl
Ala	0.30±0.02	0.55±0.03*	0.40±0.08	0.58±0.03	0.35±0.03	1.3±0.1*	0.41±0.07	1.3±0.1*	0.49±0.08	1.1±0.1*	0.29±0.03	1.1±0.1*
β-ala	0.053±0.001	0.042±0.003*	0.052±0.003	0.050±0.007	0.058±0.006	0.13±0.03	0.062±0.013	0.12±0.02*	0.12±0.01	0.14±0.04	0.091±0.021	0.21±0.05
Arg	0.17±0.01	0.16±0.01	0.17±0.01	0.16±0.01	0.16±0.01	0.19±0.01	0.23±0.04	0.19±0.01	0.31±0.01	0.25±0.03	0.27±0.02	0.26±0.03
Asp	0.76±0.03	0.70±0.01	0.81±0.05	0.66±0.04*	0.72±0.06	0.57±0.09	0.76±0.06	0.64±0.05	0.92±0.07	0.90±0.10	0.57±0.02	1.0±0.1*
Glu	6.1±0.3	6.1±0.1	6.5±0.2	5.8±0.3	6.7±0.4	2.8±0.1*	6.6±0.3	3.7±0.3*	6.9±0.4	2.8±0.2*	6.2±0.4	4.7±0.2*
Gln	2.3±0.2	3.5±0.1*	2.2±0.1	4.4±0.2*	2.4±0.2	5.1±0.2*	2.2±0.1	8.0±1.2*	1.9±0.1	6.5±0.2*	2.5±0.2	5.4±0.7*
Gly	0.23±0.02	0.21±0.01	0.27±0.05	0.27±0.03	0.24±0.02	0.61±0.06*	0.29±0.05	0.52±0.03*	0.46±0.01	0.65±0.08	0.32±0.08	0.59±0.06*
His	0.069±0.006	0.060±0.003	0.070±0.008	0.060±0.012	0.070±0.008	0.41±0.07*	0.053±0.010	0.40±0.06*	0.087±0.012	0.55±0.07*	0.11±0.02	0.85±0.25*
Ile	0.030±0.002	0.040±0.002*	0.030±0.001	0.060±0.021	0.035±0.002	0.10±0.01*	0.040±0.007	0.080±0.007*	0.052±0.003	0.098±0.011*	0.057±0.007	0.12±0.01*
Leu	0.055±0.005	0.075±0.005*	0.064±0.004	0.079±0.010	0.076±0.005	0.24±0.03*	0.064±0.005	0.20±0.01*	0.077±0.010	0.25±0.03*	0.11±0.02	0.30±0.01*
Lys	0.81±0.02	4.3±0.4*	0.96±0.03	4.7±0.2*	0.79±0.06	0.48±0.05*	0.48±0.19	0.36±0.04	0.17±0.01	0.40±0.05*	0.21±0.02	0.45±0.02*
Phe	0.075±0.015	0.065±0.008	0.071±0.003	0.067±0.007	0.059±0.003	0.14±0.02*	0.059±0.005	0.15±0.01*	0.066±0.010	0.19±0.02*	0.040±0.003	0.17±0.01*
Pro	0.060±0.004	0.077±0.007	0.066±0.005	0.074±0.002	0.060±0.004	0.76±0.05*	0.035±0.012	0.55±0.06*	0.017±0.002	0.77±0.14*	0.036±0.013	0.81±0.04*
Ser	0.20±0.02	0.20±0.02	0.23±0.04	0.24±0.03	0.22±0.02	0.52±0.05*	0.21±0.01	0.42±0.01*	0.27±0.03	0.56±0.04*	0.21±0.02	0.56±0.05*
Tau	2.2±0.1	2.1±0.1	2.1±0.1	2.1±0.1	2.1±0.2	2.1±0.2	2.0±0.1	2.0±0.1	2.1±0.1	1.9±0.2	2.0±0.1	2.0±0.2
Thr	0.087±0.018	0.062±0.006	0.087±0.007	0.075±0.014	0.081±0.009	0.24±0.02*	0.079±0.014	0.21±0.02*	0.12±0.02	0.24±0.02*	0.076±0.010	0.28±0.03*
Tyr	0.073±0.019	0.072±0.013	0.058±0.007	0.038±0.002*	0.045±0.005	0.094±0.009*	0.054±0.005	0.11±0.01*	0.056±0.011	0.14±0.02*	0.036±0.005	0.14±0.01*
Val	0.080±0.012	0.095±0.007	0.077±0.014	0.092±0.002	0.091±0.006	0.17±0.02*	0.094±0.005	0.14±0.01*	0.11±0.01	0.17±0.02*	0.045±0.004	0.21±0.01*
TFAA	11±1	15±1*	12±1	15±1*	12±1	11±1	12±1	11±1	12±1	11±1	13±1	14±1
TEFAA	1.2±0.1	4.7±0.4*	1.4±0.1	5.1±0.2*	1.2±0.1	1.8±0.1*	0.87±0.16	1.5±0.1*	0.67±0.04	1.9±0.2*	0.73±0.01	2.4±0.3*

Values are means ± S.E.M., *N*=4.

*Significantly different from the corresponding saline-injected value.

Table 32 Contents (μmol g^{-1} liver) of various free amino acids (FAA), total faa (TFAA) and total essential FAA (TEFAA) in the liver of *Pelodiscus sinensis* during the 24-h period after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	FAA contents (μmol g^{-1} liver)							
	3h		6h		12h		24h	
FAAs	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl
Ala	0.095±0.022	1.5±0.2*	0.083±0.011	1.3±0.2*	0.071±0.012	1.2±0.2*	0.091±0.035	0.91±0.07*
β-ala	0.057±0.004	0.035±0.002*	0.060±0.005	0.043±0.007	0.056±0.011	0.024±0.003*	0.047±0.007	0.031±0.004
Asp	0.065±0.004	0.68±0.21*	0.075±0.021	0.68±0.12*	0.059±0.010	0.70±0.15*	0.11±0.02	0.53±0.04*
Glu	0.71±0.13	1.5±0.3*	1.0±0.2	1.7±0.4	1.1±0.2	1.2±0.2	0.62±0.06	1.2±0.1*
Gln	0.051±0.007	0.79±0.37	0.32±0.17	0.84±0.44	0.067±0.016	0.24±0.03*	0.089±0.027	0.18±0.02*
Gly	0.21±0.01	1.3±0.2*	0.33±0.08	1.4±0.2*	0.39±0.08	1.5±0.1*	0.29±0.05	1.2±0.1*
His	0.12±0.01	0.32±0.04*	0.12±0.01	0.38±0.04*	0.11±0.01	0.34±0.02*	0.15±0.01	0.24±0.02*
Ile	0.097±0.008	0.12±0.02	0.11±0.02	0.10±0.02	0.10±0.02	0.19±0.04	0.12±0.01	0.18±0.01*
Leu	0.14±0.01	0.34±0.04*	0.15±0.03	0.31±0.05*	0.14±0.04	0.52±0.07*	0.27±0.01	0.42±0.02*
Lys	0.20±0.01	0.55±0.07*	0.16±0.03	0.55±0.09*	0.19±0.02	0.63±0.04*	0.14±0.02	0.38±0.03*
Phe	0.11±0.01	0.13±0.02	0.11±0.02	0.13±0.01	0.10±0.01	0.22±0.03*	0.098±0.008	0.15±0.01*
Pro	0.017±0.002	0.42±0.04*	0.018±0.002	0.39±0.04*	0.019±0.002	0.72±0.13*	0.087±0.013	0.63±0.04*
Ser	0.22±0.02	0.85±0.13*	0.24±0.03	0.80±0.09*	0.30±0.06	0.97±0.07*	1.3±0.1	0.63±0.06
Tau	6.4±0.2	6.5±0.6	5.8±0.3	5.2±1.2	5.6±0.6	6.9±0.7	5.9±0.6	6.7±0.7
Thr	0.13±0.01	0.31±0.04*	0.12±0.02	0.29±0.03*	0.20±0.06	0.48±0.08*	0.078±0.017	0.40±0.03*
Tyr	0.066±0.005	0.15±0.02*	0.087±0.008	0.13±0.02*	0.075±0.004	0.22±0.03*	0.083±0.006	0.19±0.02*
Val	0.14±0.02	0.23±0.03*	0.16±0.02	0.20±0.03	0.14±0.03	0.40±0.08*	0.21±0.02	0.33±0.02*
TFAA	8.7±0.4	15±1*	8.9±0.6	14±1*	8.6±1.1	16±1*	8.8±0.8	14±1*
TEFAA	0.81±0.05	1.7±0.2*	0.81±0.13	1.6±0.2*	0.88±0.15	2.4±0.3*	0.92±0.07	1.8±0.1*

Values are means ± S.E.M. (*N*=4).
*Significantly different from the corresponding saline-injected value.

Table 33 Contents (μmol g^{-1} muscle) of various free amino acids (FAA), total faa (TFAA) and total essential FAA (TEFAA) in the muscle of *Pelodiscus sinensis* during the 24-h period after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	FAA contents (μmol g^{-1} muscle)							
	3h		6h		12h		24h	
FAAs	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl	Saline	NH_4Cl
Ala	0.24±0.02	0.38±0.01*	0.28±0.04	0.53±0.14	0.22±0.03	0.46±0.12	0.14±0.02	0.36±0.06*
β-ala	0.66±0.14	4.7±1.3*	0.37±0.07	4.0±0.3*	0.46±0.12	3.4±0.7*	0.43±0.08	4.3±0.3*
Arg	0.080±0.016	0.15±0.02*	0.074±0.018	0.13±0.01*	0.065±0.008	0.11±0.02	0.023±0.003	0.089±0.005*
Asp	0.20±0.04	0.17±0.03	0.19±0.04	0.26±0.08	0.27±0.03	0.20±0.03	0.12±0.02	0.16±0.05
Glu	0.90±0.15	0.52±0.09	1.1±0.2	0.56±0.13*	0.95±0.16	0.58±0.06	1.5±0.2	0.64±0.11*
Gln	0.61±0.05	1.3±0.2*	0.87±0.14	1.4±0.2	0.53±0.04	1.1±0.1*	0.76±0.13	1.0±0.2
Gly	0.84±0.10	0.90±0.10	0.98±0.03	1.0±0.1	1.0±0.1	1.1±0.4	0.58±0.09	0.87±0.15
His	0.40±0.08	6.7±2.0*	0.23±0.03	5.6±0.5*	0.24±0.04	4.9±1.0*	0.20±0.01	7.7±0.5*
Ile	0.25±0.03	0.14±0.03	0.19±0.01	0.11±0.02*	0.26±0.05	0.081±0.013*	0.20±0.04	0.089±0.013*
Leu	0.16±0.01	0.22±0.05	0.15±0.02	0.18±0.03	0.17±0.05	0.13±0.03	0.35±0.07	0.16±0.02*
Lys	0.080±0.007	0.62±0.09*	0.083±0.012	0.50±0.08*	0.055±0.004	0.39±0.06*	0.43±0.12	0.29±0.04
Phe	0.11±0.01	0.16±0.01*	0.10±0.01	0.16±0.01*	0.10±0.01	0.13±0.02	0.089±0.013	0.12±0.01
Pro	0.048±0.002	0.50±0.07*	0.043±0.001	0.45±0.03*	0.055±0.007	0.33±0.05*	0.15±0.01	0.25±0.01*
Ser	0.22±0.04	0.26±0.04	0.27±0.05	0.27±0.06	0.20±0.03	0.21±0.02	0.25±0.05	0.25±0.03
Tau	5.2±0.2	6.3±0.5	6.7±0.2	5.5±1.0	6.7±0.7	6.4±0.9	5.5±0.5	5.4±1.4
Thr	0.21±0.02	0.21±0.01	0.20±0.01	0.22±0.03	0.23±0.03	0.18±0.02	0.17±0.03	0.16±0.01
Try	0.020±0.004	0.081±0.008*	0.022±0.002	0.075±0.017	0.028±0.002	0.060±0.020	0.064±0.007	0.043±0.004*
Tyr	0.056±0.012	0.078±0.008	0.077±0.008	0.089±0.009	0.074±0.010	0.072±0.009	0.054±0.008	0.074±0.010
Val	0.17±0.01	0.24±0.05	0.17±0.02	0.19±0.03	0.18±0.04	0.13±0.02	0.31±0.05	0.13±0.02*
TFAA	10±1	24±3*	12±1	21±1*	12±1	20±1*	11±1	22±1*
TEFAA	1.4±0.1	8.4±1.9*	1.1±0.1	7.0±0.5*	1.3±0.2	6.0±1.0*	1.8±0.1	8.7±0.5*

Values are means ± S.E.M. (*N*=4).
*Significantly different from the corresponding saline-injected value.

significantly 1.5- to 3.6-fold during the 24 h post-injection, with a significant decrease in glutamate content between hours 3 and 24 (Table 31). The brain TFAA content increased significantly at hours 0.5 and 1 (by 1.4- and 1.3-fold, respectively) while the brain TEFAA content increased significantly throughout the 24-h period post-injection with NH_4Cl (Table 31). In the liver (Table 32) and the muscle (Table 33), TFAA and TEFAA contents increased significantly throughout the 24 h post-injection with NH_4Cl. There was also a significant increase (~2-fold) in glutamine content in the muscle at hours 3 and 12 (Table 33).

Effects of injection with a sub-lethal dose of NH_4Cl on enzyme activities

The GS activity in the liver of *P. sinensis* increased significantly by 2.2-fold in the liver 12 h post-injection with NH_4Cl, but those in the brain and muscle were unaffected (Table 34). There were significant decreases in the GDH deaminating activity in the brain and the liver, and a significant increase in the GDH aminating activity in the brain at hour 12 (Table 34).

Effects of injection with a sub-lethal dose of NH_4Cl on ammonia and urea excretion rates

Turtles injected with saline intraperitoneally excreted approximately 60% of waste-N as urea-N, and were therefore primarily ureotelic (Fig. 15C). However, turtles injected with 7.5 μmol NH_4Cl g^{-1} turtle switched from ureotely to ammonotely during the subsequent 24-h period, with the percentage of waste-N excreted as urea-N decreased to a minimum of 19% between hours 18 and 24 (Fig. 15C). This is despite a significant increase in the rate of urea excretion between hours 0-6 and 6-12 by 1.4- and 3.0-fold respectively (Fig. 15B), as the NH_4Cl injection caused a much larger significant increase (3.4 to 8.0-fold) in the rate of ammonia excretion, especially between hours 12 and 18 (Fig. 15A).

Table 34 Activities of glutamine synthetase (GS; μmol γ-glutamylhydroxamate min^{-1} g^{-1} tissue) and glutamate dehydrogenase in the aminating (GDHa; μmol NADH utilised min^{-1} g^{-1} tissue) and deaminating (GDHd; μmol formazan formed min^{-1} g^{-1} tissue) directions from the brain, liver and muscle of *Pelodiscus sinensis* at hours 12 and 24 after being injected intraperitoneally with NaCl (0.9%, control) or a sub-lethal dose of NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

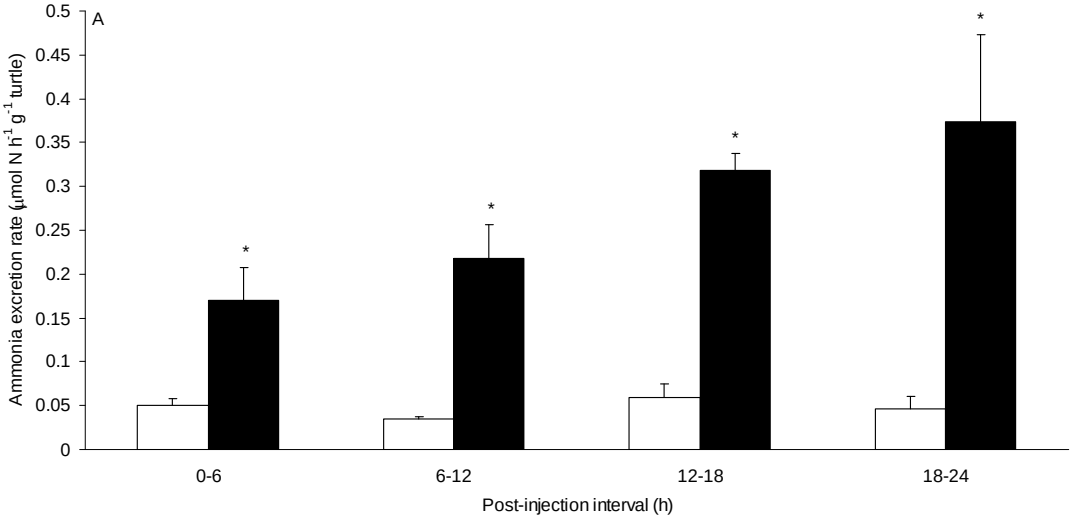
		Activities (μmol min^{-1} g^{-1} tissue)			
		12h		24h	
Enzyme	Tissue	Saline	NH_4Cl	saline	NH_4Cl
GS	Brain	83±3	88±5	107±9	96±7
	Liver	1.1±0.2	2.4±0.4*	0.75±0.19	0.80±0.23
	Muscle	0.36±0.05	0.39±0.15	0.33±0.06	0.30±0.04
GDHa	Brain	76±2	87±2*	47±3	49±9
	Liver	51±4	44±3	23±2	29±6
	Muscle	0.12±0.02	0.14±0.02	0.17±0.02	0.16±0.02
GDHd	Brain	0.36±0.01	0.27±0.01*	0.35±0.01	0.31±0.04
	Liver	4.1±0.2	2.8±0.1*	3.5±0.2	3.5±0.2
	Muscle	0.0066±0.0038	0.0055±0.0014	0.051±0.010	0.051±0.007

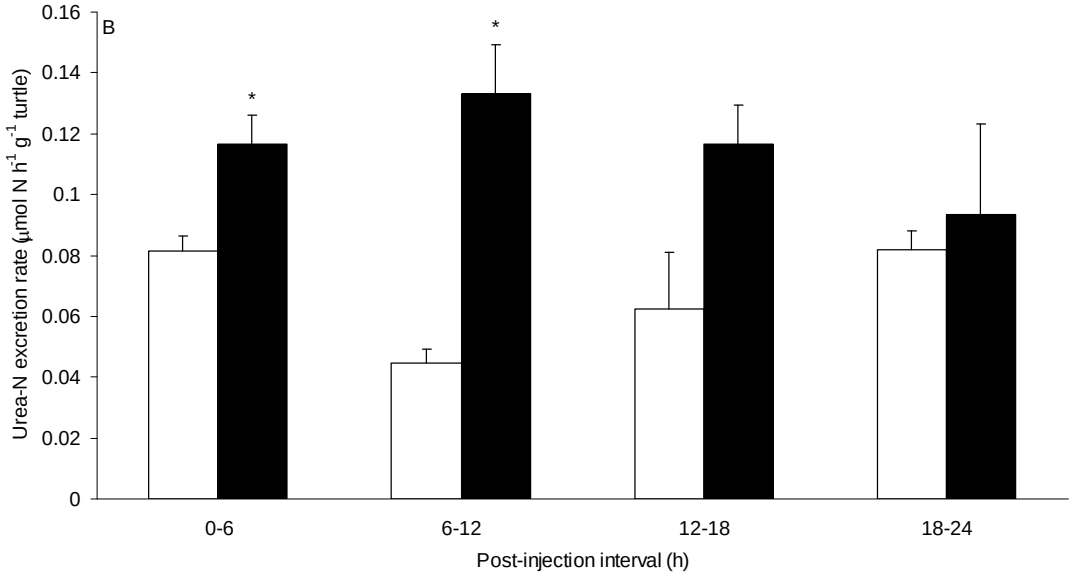
Values are means ± S.E.M. (*N*=4); except 24h NH_4Cl-injected (*N*=3).

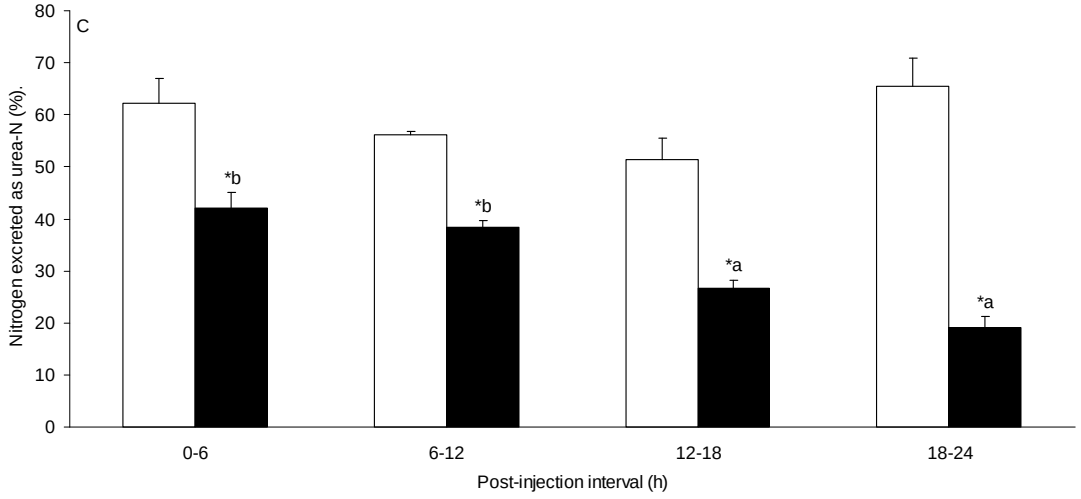
*Significantly different from the corresponding saline-injected value, $P<0.05$.

Fig. 15. Rates (μmol N h^{-1} g^{-1} turtle) of excretion of (A) ammonia and (B) urea and (C) the percentage of total-N excreted as urea-N by *Pelodiscus sinensis* in the 24-h period post-injection. White bars represent saline-injected turtles (control). Black bars represent ammonium chloride-injected turtles. Values are means + S.E.M. (N=4). *Significantly different from the corresponding control value, P<0.05. Means of values not sharing the same letter are significantly different, P<0.05.

Fig. 15


A
Ammonia excretion rate (μmol N h-1 g-1 turtle)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
0-6
6-12
12-18
18-24
Post-injection interval (h)


B
Urea-N excretion rate (μmol N h-1 g-1 turtle)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
0-6
6-12
12-18
18-24
Post-injection interval (h)


C
Nitrogen excreted as urea-N (%).
80
70
60
50
40
30
20
10
0
*b
*a
0-6
6-12
12-18
18-24
Post-injection interval (h)

Routes of increased ammonia excretion

For turtles injected intraperitoneally with 7.5 μmol NH_4Cl g^{-1} turtle, there appears to be increased ammonia excretion into the water (through non-urine routes) and urine (Table 35). In contrast, the rate of urea excretion through the urine route appears to remain the same as that observed in control turtles (Table 35). To differentiate whether increased ammonia and urea excretion occurred through the buccopharyngeal epithelium or the skin, turtles were restrained on land but with water made available to the head region. It was discovered that increased ammonia excretion occurred through the buccopharyngeal route (Table 36). However, the increased ammonia excretion was not able to account for the amount of ammonium-N injected into the turtle (Table 36). Therefore, these results indirectly confirmed that increased ammonia excretion also took place through the skin covering the whole body surface including the carapace.

Calculated results for a 300 g **P. sinensis**

Since 7.5 μmol NH_4Cl was injected per gram turtle, a 300 g turtle would have been injected with 2250 μmol NH_4Cl. By hour 24, 86% (1943 μmol N) of the injected ammonia had been excreted, out of which 1601 μmol N or 82% was in the form of ammonia (Table 37). Taking into consideration the nitrogen accumulated in various tissues, a total of 2235 μmol N or 99% of the injected ammonia had been accounted for (Table 37).

Table 35 Rates (μmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion into water (non-urine route) or urine in *Pelodiscus sinensis* during the subsequent 24 h after being injected intraperitoneally with a sub-lethal dose (7.5 μmol g^{-1} turtle) of NH_4Cl, with the urine being collected into a flexible latex tubing attached to the tail.

	Rate of excretion (μmol N day^{-1} g^{-1} turtle)			
	Control (4)		NH_4Cl injection (5)	
	Into water	Into tubing	Into water	Into tubing
Ammonia	0.21±0.06	0.26±0.09	2.7±0.6*	0.87±0.12*
Urea	1.2±0.2	0.19±0.12	4.0±0.2*	0.31±0.18

Values represent means±S.E.M., with the number of determinations stated in parenthesis.

*Significantly different from the corresponding control value.

Table 36 Rates (μmol N day^{-1} g^{-1} turtle) of ammonia and urea excretion through the head region of *Pelodiscus sinensis* during the subsequent 24 h after being injected intraperitoneally with a sub-lethal dose (7.5 μmol g^{-1} turtle) of NH_4Cl.

	Rate of excretion (μmol N day^{-1} g^{-1} turtle)	
	Control (5)	NH_4Cl injection (3)
Ammonia	0.17±0.05	1.6±0.5*
Urea	0.95±0.07	4.5±1.8*

Values represent means±S.E.M.; with the number of determinations stated in parenthesis.

*Significantly different from the corresponding control value.

Table 37 A nitrogen balance table (μmol N) for a 300 g *Pelodiscus sinensis* at hour 24 after the intraperitoneal injection with 0.9% NaCl (control) or 2250 μmol NH_4Cl (7.5 μmol NH_4Cl g^{-1} turtle).

	24 h		
	Saline	NH_4Cl	Difference
Excreted from *P. sinensis* (300 g)			
Ammonia-N	342	1943	+1601
Urea-N	486	828	+342
Changes in ammonia and urea excretion (A)			+1943
Retained in muscle (74 g)			
Ammonia-N	56	96	+40
Urea-N	74	281	+207
Retained in liver (9 g)			
Ammonia-N	37	43	+6
Urea-N	10	18	+8
Retained in stomach (2 g)			
Ammonia-N	2	2	+0
Urea-N	2	6	+4
Retained in intestine (4 g)			
Ammonia-N	7	7	+0
Urea-N	4	10	+6
Retained in plasma (15 ml)			
Ammonia-N	8	6	-2
Urea-N	19	42	+23
Changes in ammonia and urea accumulation (B)			+292
A + B			+2235

DISCUSSION

Extremely high levels of ammonia in the brain of turtles succumbed to a lethal dose of NH_4Cl

After the injection of a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl into the peritoneal cavity, all the turtles succumbed to ammonia toxicity within 2.7 h. The ammonia content in the brain of the succumbed turtle increased 14-fold and reached 21 μmol g^{-1} brain, which is 1.5-fold greater than that (14 μmol g^{-1} brain) reported for the giant mudskipper known to have very high environmental ammonia tolerance. It can therefore be concluded that the brain of *P. sinensis* can tolerate high levels of ammonia. The glutamine content in the brains of the succumbed turtles also increased significantly, but surprisingly the magnitude of increase (from 2.2 to 4.4 μmol g^{-1} brain) was much lower than that of ammonia. The brain of *P. sinensis* had high GS activity, but the rate of increased glutamine synthesis apparently could not cope with the rate of ammonia infiltration, resulting in the drastic increase in the ammonia content in the brain. Because the brain glutamine content increased to 8 μmol g^{-1} brain at hour 6 in turtles injected with a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle) and recovered slowly thereafter, it can be concluded that ammonia per se, and not glutamine synthesis and its accumulation, was the major cause of death in *P. sinensis* confronted with acute ammonia toxicity. The extraordinarily high levels of ammonia in the brain would lead to membrane depolarization (Sugden and Newsholme, 1975), which could be the main cause of ammonia toxicity in this turtle. Since membrane depolarization can lead to the activation of NMDA receptors (Fan and Szerb, 1993; Hermenegildo et al., 2000), it became imperative to determine whether MK801 had a protective effect on *P. sinensis* against acute ammonia toxicity (see below).

MK801 and MSO had protective effects on P. sinensis *injected with a lethal dose of* NH_4Cl

In rats, the mortality due to acute ammonia toxicity *in vivo* is prevented by the administration of a wide range of NMDA receptor antagonists (Marcaida et al., 1992; Hermenegildo et al., 1996). Studies using *in vivo* cerebral microdialysis show that acute ammonia exposure results in the activation of NMDA receptor-coupled nitric oxide-cyclic GMP signal transduction pathway in the rat brain (Hermenegildo et al., 2000). Administration of MK801, a NMDA receptor antagonist, at a dosage of 2 μg g^{-1} leads to significant improvement in clinical grading and slowing of electroencephalogram activity and provide significant protection in mouse given a lethal dose of ammonia (Marcaida et al., 1992). However, Ip et al. (2005) reported recently that MK801 (2 μg g^{-1} fish) had no protective effect on mudskippers, *P. schlosseri* and *B. boddarti* injected with lethal doses of ammonium acetate. Rather, MK801 exacerbated ammonia toxicity in *B. boddarti* because the mortality increased from 70% to 100%.

MK801 (1.6 μg g^{-1} turtle) clearly had a protective effect on *P. sinensis* injected with 12.5 μmol g^{-1} turtle of NH_4Cl because it reduced the mortality drastically to 50% within a 24-h period. Thus, our results reveal for the first time that activation of NMDA receptors could be the explanation for acute ammonia toxicity in the brains of *P. sinensis*.

MSO is an irreversible inhibitor (Folbergrova, 1964) of GS in the mammalian system. In patients suffering from hyperammonemic encephalopathy, MSO ameliorates ammonia toxicity by decreasing the levels of glutamine synthesized, and therefore prevents cell swelling due to glutamine accumulation. Hence, death is prevented via the prevention of brain edema (Brusilow, 2002; Butterworth, 2002).

However, it is unlikely that brain edema was the cause of death in *P. sinensis*. This is supported by the fact that glutamine levels were found at moderate levels of 4.4 μmol g^{-1} brain and that there was no change in water contents in the brain, which indicates a lack of swelling. In the case of rats injected intraperitoneally with CH_3COONH_4, the protective effects of MSO can also be a result of the prevention of glutamate release and the avoidance of activation of NMDA receptors (Kosenko et al., 1994).

MSO (at a dosage of 100 μg g^{-1} animal) that has protective effects on mammals, exerted a slight protective effect on *P. sinensis* (at a dosage of 82 μg g^{-1} turtle) against acute ammonia toxicity. It did not reduce the mortality of the experimental turtles but extended the time to death to 7.9 h. However, a close examination of the brain ammonia and glutamine contents reveals that the protective effects of MSO might not be related to the suppression of glutamine synthesis and accumulation. Instead, our results suggest that MSO, via an unknown mechanism, could have activated the aminating GDH activity or inhibited the deaminating GDH activities in the brain of *P. sinensis*. Comparing the brains of turtles injected with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl at the time of death (1.1–2 h) and those of turtles injected with 82 μmol MSO g^{-1} turtle followed with 12.5 μmol NH_4Cl g^{-1} turtle and euthanized at 1 h post-injection, MSO apparently reduced the rate of ammonia buildup through increased conversion of ammonia to glutamate. The increased rate of glutamate formation was apparently adequate to sustain a higher rate of glutamine synthesis without reducing the glutamate content in the presence of MSO.

A sub-lethal dose of NH_4Cl led to extremely high levels of ammonia transiently in the brain

The ammonia content in the brain of *P. sinensis* increased to an extremely high level (16 μmol g^{-1} brain) 1 h after the injection with 7.5 μmol NH_4Cl g^{-1} turtle.

This could be the highest level reported for brains of vertebrates confronted with a sub-lethal dose of ammonia through injection. Such a level of ammonia is intolerable in mammals; 1 to 3 μmol ammonia g^{-1} brain results in encephalopathy (Cooper and Plum, 1987; Felipo and Butterworth, 2002). These results confirm that the brain of *P. sinensis* had high tolerance to ammonia at cellular and sub-cellular levels because no mortality was recorded among turtles injected with 7.5 μmol NH_4Cl g^{-1} turtle. The transient nature of the ammonia accumulation indicates that *P. sinensis* must have adaptations to ameliorate acute ammonia toxicity and/or to remove ammonia.

At the cellular level, an increase in intracellular NH_4^+ would lead to changes in membrane potential (Sugden and Newsholme, 1975) which would result in the reversal of glutamate transport and hence an increase in the extracellular glutamate concentration (Szatkowski et al., 1990). In addition, membrane depolarization can lead to the removal of the Mg^{2+} block on NMDA receptors and result in their activation (Fan and Szerb, 1993). Therefore, our results suggest that *P. sinensis* could have special abilities to control the intracellular ammonia level in its brain despite drastic increases in brain ammonia contents (intracellular + extracellular). NH_4^+ can replace K^+ in the facilitated diffusion of K^+ through K^+ channels and/or active transport of K^+ through Na^+, K^+-ATPase; both these processes have direct or indirect deleterious effects on the membrane potential of a cell. In view of the high levels of ammonia in the brains of *P. sinensis* injected intraperitoneally with 7.5 μmol NH_4Cl, g^{-1} turtle, it can be deduced that membrane potentials were resilient to NH_4^+ interference so that activation of NMDA receptors occurred only at very high extracellular ammonia concentrations, and hence very high brain ammonia content.

Increased synthesis of glutamine, accumulations of essential amino acids and changes in activities of some enzymes in turtles injected with a sub-lethal dose of NH_4Cl

The GS transferase activity in the brain is the highest among the three tissues studied. Indeed increased glutamine synthesis was adopted as a mechanism to ameliorate ammonia toxicity in *P. sinensis*, although the brain GS activity remained unchanged at hours 12 and 24 post-injection with 7.5 μmol NH_4Cl g^{-1} turtle, as in the case of other vertebrates (Cooper et al., 1985; Lavoie et al., 1987). The brain glutamine increased to 8 μmol g^{-1} brain in turtles injected with 7.5 μmol NH_4Cl g^{-1} turtle. Since there was no mortality among the experimental turtles, it can be concluded that the magnitude of the resultant astrocyte swelling did not result in a deleterious cranial pressure. This could be due to the presence of more cranial space in reptilian skulls (Bellairs and Attridge, 1975) as compared with mammalian ones.

Synthesis of glutamine requires glutamate as a precursor, and indeed there was a simultaneous decrease in the brain glutamate content in turtles injected with 7.5 μmol NH_4Cl g^{-1} turtle. There was an increase and a decrease in the aminating and deaminating GDH activities, respectively, in the brain of turtles 12 h after the injection with 7.5 μmol g^{-1} NH_4Cl, indicating a possible increase in the rate of glutamate production. However, the rate of glutamine synthesis must be greater than the rate of glutamate formation; only then would there be a decrease in brain glutamate content. These results suggest that more ammonia was detoxified to the amide-N than the amino-N of glutamine. Since there was an increase in the brain TEFAA content, and since essential FAAs could not be synthesized by the experimental turtles during fasting, it is logical to conclude that a suppression of

amino acid catabolism occurred therein, reducing the production of endogenous ammonia and hence alleviating the possibility of ammonia intoxication.

By the same analysis, reduction of ammonia production also occurred in the liver and muscle of turtles injected intraperitoneally with 7.5 μmol g^{-1} turtle of NH_4Cl. This is supported by a significant decrease in GDH activity in the deamination direction in the liver at hour 12, which could have reduced the rate of ammonia production.

The significant increases in enzymes activity at hour 12 returned to levels comparable to controls at hour 24. This suggested that these enzymes may have been modulated by covalent modification or changes in the levels of allosteric modulators as the time frame is too short for it to be due to increased production of enzymes. A future avenue to examine would be to determine the mechanisms involved in the regulation of enzyme activities.

Only a small portion of the ammonia injected into the turtle was detoxified to urea

Pelodiscus sinensis is ureogenic and ureotelic in freshwater. It is known to have a high capacity for urea synthesis through the hepatic OUC, and can increase the rate of urea synthesis by 7-fold after feeding as mentioned in Chapter 1. However, intraperitoneal injection with 7.5 μmol NH_4Cl g^{-1} turtle resulted in only slight increases in urea contents in various tissues. The excess amount of urea synthesized during the 24-h period was (342 + 207 + 8 + 4 + 6 + 23) = 590 μmol urea-N = 295 μmol urea. Thus, only 590/2250 x 100% or 26% of the excess ammonia injected into the turtle was detoxified to urea-N. In control turtles, urea contents were maintained at steady states and therefore urea excretion rate would be equal to urea production rate (= 486 μmol urea-N day^{-1}/2 or 243 μmol urea day^{-1}). Thus, the increase in urea synthesis was a mere (295 + 243)/ 243 or 2.2-fold.

One hour post-injection with a sub-lethal dose of NH_4Cl, ammonia accumulated to an extraordinarily high level (35 μmol g^{-1} liver) in the liver and recovered toward the control level thereafter. In comparison, significant increases in urea contents in various tissues occurred only from 6 h onward, reaching a peak at hour 24, but by then, 71% of the ammonia injected into the turtle had been excreted. Thus, the accumulated urea probably had an endogenous origin. The exit of endogenous ammonia from liver cells could have been impeded by the high concentration of extracellular ammonia, leading to an increase in urea synthesis therein. Our results therefore support the proposition that increased urea synthesis might not be an effective mechanism to detoxify exogenous ammonia, because it has to penetrate through the plasma and mitochondrial membranes before being detoxified through CPS I and the OUC (Ip et al., 2004b).

Excess ammonia was excreted mainly as ammonia per se within the 24-h period

A dosage of 7.5 μmol NH_4Cl g^{-1} turtle implies that a total of 2250 μmol ammonia would have been injected into a 300 g turtle. During the 24 h post-injection with NH_4Cl, the rate of ammonia excretion increased 3.4-8.0-fold. By hour 24, 1943 μmol N or 86% would have been excreted, out of which only 342 μmol N or 15% was excreted as urea-N. It is probable that the distribution of exogenous ammonia in the extracellular spaces predisposed and facilitated its being excreted as ammonia. Consequently, *P. sinensis* became ammonotelic during the 24 h post-injection with NH_4Cl. These results indicate that detoxification of ammonia to urea was not essential to the survival of *P. sinensis* confronted with acute ammonia toxicity in spite of its being ureogenic.

Increased ammonia excretion occurred mainly through the skin

Increased urea excretion in turtles injected with 7.5 μmol g^{-1} turtle of NH_4Cl occurred through the buccopharyngeal route only. However, increased ammonia excretion in these experimental turtles involved both the urine and non-urine routes. Our results confirm directly that increased ammonia excretion through non-urine routes, like increased urea excretion, could also occur through the buccopharyngeal epithelium. Because there was a greater percentage of the exogenous ammonia being detoxified to and excreted as urea in the experimental turtles restrained on land, it can be concluded indirectly that increased ammonia excretion actually also occurred through the skin when turtles were immersed in water. When water was lacking, ammonia excretion through the skin was impeded, and therefore a greater portion of the ammonia injected into the turtle had to be detoxified to urea.

Conclusion

Pelodiscus sinensis is unique in having extremely high tolerance to ammonia at cellular and subcellular levels in the brain. Acute ammonia toxicity was apparently a result of activation of NMDA receptors and not astrocyte swelling as a consequence of increased glutamine synthesis and accumulation. The main strategy that *P. sinensis* adopts to deal with the exogenous ammonia insult is to excrete the bulk of the injected ammonium chloride-N as ammonia. In addition, turtles decreased endogenous ammonia production by decreasing amino acid catabolism. Some ammonia was also detoxified to glutamine and glutamine formation in the brain and liver was increased through modulation of enzymes activity.

INTEGRATION, SYNTHESIS AND CONCLUSIONS

Advantages and disadvantages of having a soft-shell

Unlike other testudines, *P. sinensis* has a carapace covered with soft, leathery skin. The carapace of *P. sinensis*, like those of other soft-shelled turtles, is much flatter than hard-shelled testudines. This enables *P. sinensis* to seek refuge and hide under rocks or in crevices, and also facilitate burying itself into sand. During captivity, it was observed that *P. sinensis* constantly squeezed itself below various types of objects at the bottom of the tank. *Pelodiscus sinensis* is also relatively more agile, in part because of its lighter carapace. It can swim with reasonably high speed, and has an extremely fast snapping action when disturbed.

In shallow waters, the long snorkel-like snout of *P. sinensis* allows it to breathe air while keeping its eyes underwater. However, when the water level is deep, pulmonary respiration requires swimming to the water surface, which means greater exposure to predators. Thus, its ability to endure prolonged submersion is an advantage and this ability is due in part to its soft, leathery skin. *Pelodiscus sinensis* can perform cutaneous respiration in water or on land as its skin is permeable to respiratory gases. *Pelodiscus sinensis* can also hold water in its mouth to perform buccopharyngeal respiration, especially during forced submergence. This implies that the buccopharyngeal cavity must be better vascularized and more effective in gaseous exchange than the external skin. However, why the turtle holds water in its mouth instead of air, especially when air has 30-fold more oxygen, remains an enigma.

If the general skin surface of the carapace is permeable to respiratory gases, it is probable that it is also permeable to ammonia gas (NH_3). That would mean a certain portion of the nitrogenous waste could be excreted directly through the skin as ammonia without being detoxified to urea. *Pelodiscus sinensis* has a full complement

of OUC enzymes with high CPS I activity (1.2 μmol min^{-1} g^{-1} liver) in its liver, and is therefore ureogenic. However, urea synthesis is energy-intensive; 4 mol of ATP are required for the synthesis of 1 mol of urea. So, under certain conditions, the capacity to excrete ammonia through the skin could be an advantage to *P. sinensis* because it would save energy by doing so. Although *P. sinensis* is primarily ureotelic during immersion, it excreted 29% of the waste-N as ammonia.

Being a respiratory organ, the skin of soft-shelled turtles cannot be absolutely impermeable to water. Because *P. sinensis* can also be found in swamps and marshes where waters are brackish, its soft shell would impose a greater problem of osmotic water loss than the hard-shell of other testudines. This is compounded with the fact that *P. sinensis* does not possess salt glands. To resolve this, urea and FAAs could be utilized as osmolytes, and this could be one of the reasons why soft-shelled turtles adopt a transition between ureotely and ammonotely, instead of transitions between ureotely and uricotely or ammonotely and uricotely. In fact, being an aquatic turtle, there is no physiological need for *P. sinensis* to adopt uricotelism as a means to defend against ammonia toxicity.

Permeation of water through the skin of the soft-shell also means that *P. sinensis* would be exposed to greater dehydration stress during emersion. Emersion can occur when the ponds or creeks dry up during hot spells or when the turtle emerges from the waters to bask. A reduction in urine production is an important adaptation to reduce water loss, but this would impede nitrogenous waste excretion. Therefore, *P. sinensis* must resort to physiological and biochemical adaptations to ameliorate ammonia toxicity during emersion.

Increased ammonia excretion could indeed occur through the skin under certain conditions

During emersion, there was a reduction in urine production in *P. sinensis* but the ammonia concentration in the urine remained relatively unchanged. So, the reduction in ammonia excretion through the urine was partially compensated for by an increase in ammonia excretion through the skin of the ventral carapace in constant contact with water. Further evidence that increased ammonia excretion could occur through the skin was obtained from turtles injected intraperitoneally with a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle) and restrained on land with water made available to the head only. Under such conditions, there was an increase in the amount of ammonia being detoxified to urea, which indicates indirectly that the skin played an important role in ammonia excretion. In this situation, urea formation becomes a necessity to prevent ammonia from building up to deleterious levels within the body. In conclusion, the skin of *P. sinensis*, and possibly other soft-shelled turtles, is apparently permeable not only to the normal respiratory gases but also gaseous ammonia (NH_3).

Buccopharyngeal nitrogenous excretion: A novel discovery

The majority of urea excreted by *P. sinensis* immersed in freshwater took a non-urine route, because the percentage of urea excreted through the urine was relatively small (0.45-15%). A major portion of the urea was excreted through the head region, where buccopharyngeal respiration took place. During buccopharyngeal respiration, the turtle held water in its mouth and regurgitated it subsequently. A major portion of urea excretion apparently took place in the mouth because urea must have been excreted into the water before regurgitation. Since such a phenomenon has

never been reported previously for other turtles and reptiles, it is a novel and important discovery.

Two important conclusions can be drawn from these results. First, although "urine" usually refers to liquid produced by the kidney of animals, it implicitly implies that the excreted liquid contains "urea". Therefore, strictly speaking, in addition to the water released through the cloaca, the water being regurgitated through the mouth should also be regarded as urine because it contains high concentrations of urea. Second, holding water in and regurgitating water from the buccopharyngeal cavity appears to be an activity dedicated primarily to the excretion of urea.

What may be the adaptive significance of buccopharyngeal nitrogenous excretion? At present, there is no definitive answer to this, but the author surmises that *P. sinensis* could have developed this capacity in order to invade the brackish water environment. While conquering the brackish water environment provided more resources for growth and reproduction, exposure to water of high salinity can be stressful and deleterious. If urea, as a major component of the nitrogenous wastes, was to be excreted only through the urine, *P. sinensis* would need to continuously take in water even in waters of high salinity. As a result, there would be an accelerated osmotic loss of water and gain in ions as the brackish water goes through the intestine. This situation can be detrimental since reptilian kidneys, including those of *P. sinensis*, are incapable of excreting monovalent ions or producing hyperosmotic urine. So, by excreting urea through the buccopharyngeal route, which involves the regurgitation and not the drinking of the ambient water, the problem related to intake of brackish water can be resolved.

While the salt glands of marine turtles are specially designed for the excretion of ions, the buccopharyngeal lining of *P. sinensis* represents a unique organ for the

excretion of urea. It is therefore important to establish in the future whether other turtles or reptiles have acquired a similar ability to excrete urea through the buccopharyngeal route. Future experiments can be performed by isolating the buccopharyngeal epithelium to examine its properties of urea transport in an Ussing-type apparatus. Because five types of urea transporters are known to date, future efforts can be made to elucidate if there is a concentration of certain types of urea transporter in the buccopharyngeal epithelium. Urea transporters (SHUT) are present in the kidney of marine elasmobranchs, but they function to retain urea. In addition, phloretin-sensitive, Na^+-urea counter-transporter, which transports urea toward the serosal side, is present in their gills. As for teleosts, UT-A2-like protein is known to be expressed in the gills of the Gulf toadfish, *Opsanus beta*, which facilitates the pulsatile release of urea across the gills. Urea transporter could also be present in the belly skin of the crab-eating frog, *Rana cancrivora*. Thus, it is not completely unexpected that urea transporter is expressed in the buccopharyngeal epithelium of *P. sinensis*.

A lack of buccopharyngeal nitrogenous excretion during emersion resulted in apparent ammonotely

Emersion for 5 days resulted in a decrease in the urine volume of *P. sinensis* and by day 6, many (5 out of 6) of the experimental turtles entered a state of anuria. There was an apparent shift from ureotely to ammonotely in the experimental turtles during emersion due to a drastic decrease in urea excretion. This decrease was due to a lack of water to flush the buccopharyngeal epithelium, which is the route of urea excretion. Thus, 6 days of emersion led to significant increases in urea contents in all tissues and organs studied, but accumulation of urea was apparently unrelated to the reduction of evaporative water loss (see below).

Multiple physiological roles of urea and increased urea synthesis

The normal dietary intake of protein by animals provides amino acids in excess of the amounts required for the synthesis of new protein to sustain protein turnover. These excess amino acids are catabolized, releasing ammonia and resulting in a momentary increase in ammonia level in the animal. Indeed, feeding induced an increase in urea synthesis in *P. sinensis* to defend against postprandial ammonia toxicity. The estimated rate of urea synthesis in a turtle during the initial 24-h period post-feeding was approximately 7-fold greater than that of the unfed control. However, there were no significant changes in activities of various OUC enzymes from the liver of this turtle at hour 24 post-feeding, because the detoxification of ammonia released from the catabolism of excess amino acids demanded only 10% of the urea synthetic capacity in *P. sinensis*. Consequently, the plasma ammonia level and tissue ammonia contents of *P. sinensis* remained unchanged throughout the 72-h period post-feeding. In contrast, urea contents in all the tissues studied increased significantly at hour 24, indicating that a substantial portion of the ammonia released through catabolism of excess amino acids was detoxified to urea. Feeding induced an almost instantaneous increase in urea excretion (between hours 0 and 36), which was instrumental in preventing the build-up of high levels of urea in various tissues. By hour 48 post-feeding, 68% of the assimilated N from the food was excreted, out of which (6962 μmol N), 54% was excreted as urea-N.

Exposure to brackish water for 6 days induced increases in urea contents in tissues of *P. sinensis*, indicating the occurrence of an increase in urea synthesis. For turtles immersed in freshwater, the urea production rate is estimated to be 3.8 μmol N day^{-1} g^{-1}. But for turtles exposed to brackish water, the averaged daily urea production rate was 5.4 μmol N day^{-1} g^{-1}. Overall, this means that the rate of urea

synthesis increased 1.4-fold during the 6-day period, which was well within the hepatic OUC capacity, which had 0.44 μmol min^{-1} g^{-1} of CPS I activity. Urea could be an important osmolyte for *P. sinensis* in brackish water in spite of its limited capacity to effectively retain urea.

In contrast, emersion for 6 days resulted in a suppression of hepatic urea synthesis in *P. sinensis*. Although 6 days of emersion led to significant increases in urea contents in all tissues and organs studied, the excess urea accumulated could only account for only 13-22% of the deficit in urea excretion. Indeed, calculated results reveal that the averaged daily urea excretion rate decreased by 82-99% during 6 days of emersion. The decreases in ASS + ASL (by 40%) and arginase (by 30%) activities further confirm this proposition. Thus, urea does not act as an important osmolyte to reduce evaporative water loss in this turtle during emersion, although it might have a role to play in osmoregulation during exposure to brackish water.

Intraperitoneal injection with 7.5 μmol NH_4Cl g^{-1} turtle resulted in only slight increases in urea contents in various tissues. In total, only 21% of the ammonia injected into the turtle was detoxified to urea-N and the increase in urea synthesis was a mere 1.8-fold. The accumulated urea might have an endogenous origin because exogenous ammonia has to penetrate through the plasma and mitochondrial membranes before being detoxified by CPS I. A major portion of the ammonia injected into the turtle was excreted as ammonia per se, and the rate of ammonia excretion increased 4-7-fold. By hour 24, 81% of the ammonia injected into the turtle was excreted, out of which only 12% was excreted as urea-N. The distribution of exogenous ammonia in the extracellular spaces could have predisposed and facilitated its being excreted as ammonia. Consequently, *P. sinensis* became ammonotelic during the 24-h period post-injection with NH_4Cl, and it can be concluded that

detoxification of ammonia to urea was not essential to the survival of *P. sinensis* confronted with acute ammonia toxicity despite it being ureogenic.

In conclusion, the urea-synthesizing capacity in the liver of *P. sinensis* is high, and urea synthesis is involved in detoxifying endogenous, and not exogenous, ammonia in *P sinensis*. The rate of urea synthesis can be regulated, and it becomes higher and lower during exposure to brackish water and terrestrial conditions, respectively.

The role of FAAs in defense against ammonia toxicity in extra-cranial tissues

After feeding, the greatest changes in contents were observed for alanine and glutamate in the liver, and alanine, glycine and glutamine in the muscle, of *P. sinensis*. Glycine and glutamine could be synthesized from other amino acids consumed in excess of those required for protein synthesis, and alanine and glutamate might act as the vehicle of N transfer between tissues. Taken together, it can be proposed that the defense against postprandial ammonia toxicity in *P. sinensis* was achieved through increases in transamination and synthesis of certain non-essential amino acids in addition to an increase in urea synthesis.

Six days of emersion did not result in an increase in glutamine synthesis, or synthesis of other non-essential amino acids (e.g. alanine) in extra-cranial tissues in *P. sinensis* as in some tropical air-breathing fishes exposed to air. This could be a result of the drastic reduction in ammonia production in turtles exposed to terrestrial conditions.

The injection of a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle) into the peritoneal cavity of *P. sinensis* resulted in accumulations of glutamine, and some other non-essential amino acids in the muscle and liver. Thus, the defense against ammonia toxicity under such conditions could involve increased transamination.

FAAs can act as osmolytes for cell volume regulation in brackish water

Exposure of *P. sinensis* to brackish water resulted in increases in plasma osmolality and electrolyte concentrations, but there were no changes in haematocrit values in the plasma and dry weight: wet weight ratios in the liver and the muscle. Hence, cell volume regulation, involving osmolytes like FAAs and/or urea, was essential to its survival in brackish water. However, the content of TFAA in the muscle of *P. sinensis* (in 50% seawater) as a whole increased from 12 μmol g^{-1} muscle to 23 μmol g^{-1} muscle only on day 4, and that of urea increased to only 10 μmol g^{-1} muscle on day 6. Thus, the osmoregulatory roles of FAAs and urea in *P. sinensis* were relatively small as compared with other euryhaline and/or ureogenic animals. The FAAs involved in cell volume regulation were alanine, glycine, leucine, lysine, proline, threonine and taurine in the liver, and histidine and β-alanine in the muscle. The novel finding here is that the magnitude of increase in histidine content superseded those of other essential amino acids, which suggest that histidine was preferentially preserved or a unique pool of proteins enriched with histidine residues was present in *P. sinensis*.

Increases in FAA contents can be a result of increases in their production (for non-essential amino acids) or release through proteolysis (for both essential and non-essential amino acids), or decreases in their degradation, or both. For *P. sinensis* exposed to brackish water, results obtained do not support the view that a decrease in amino acid catabolism had occurred. To the contrary, there was a significant increase in cumulative nitrogenous excretion throughout the 6-day period, which implies that there was actually an increase in amino acid catabolism when the turtle was exposed to increased salinity. Since turtles were fasted before and during the experiment, it can be deduced that ammonia was released mainly through increased amino acid

catabolism. If this indeed occurred, then contents of various FAAs and TFAA should decrease; but, the fact is TFAA and TEFAA contents in many of the tissues studied increased instead. Thus, it can be concluded that an increase in proteolysis occurred in *P. sinensis* exposed to brackish water (10 or 15‰ water). The magnitude of increase in proteolysis must be greater than that in amino acid catabolism, because only then would the contents of FAAs and TFAA increase. This is in support of the conclusion that cell volume regulation constitutes an important adaptation in *P. sinensis* acclimated to brackish water.

Amelioration of ammonia toxicity through reduction in ammonia production

CPS I utilizes ammonia as one of the substrates to produce urea. Hence, during emersion, there should be an increase in ammonia contents in various tissues and organs, when a decrease in urea synthesis occurred with the rates of ammonia excretion decreased or remaining unchanged. However, ammonia contents increased significantly in the plasma and brain on day 6 only, and the accumulated ammonia was inadequate to account for the decreased urea accumulation. Therefore, ammonia production, which occurred mainly through amino acid catabolism under fasting conditions, could have been suppressed. Because there were no significant increases in contents of TFAA and TEFAA in various tissues and organs, it can also be concluded that the release of amino acid through proteolysis was also proportionally reduced in *P. sinensis* during emersion.

The TEFAA contents in the brain, liver and muscle of *P. sinensis* injected with a sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle) increased significantly. Since essential FAAs could not be synthesized by the experimental turtles, it is logical to conclude that a suppression of amino acid catabolism occurred in these tissue and organs, reducing the production of endogenous ammonia and hence alleviating the

possibility of ammonia intoxication. This is supported by a significant decrease in GDH activity in the deamination direction in the liver at hour 12, which could have reduced the rate of ammonia production.

Detoxification of ammonia to glutamine in the brain

The brain of *P. sinensis,* similar to those of other vertebrates, ameliorated ammonia toxicity through glutamine formation. For *P. sinensis*, a transient increase in the brain glutamine content occurred at hour 24 post-feeding, indicating that the brain was confronted with mild postprandial ammonia toxicity in spite of the absence of a significant increase in plasma ammonia concentration after feeding. However, there was no significant change in the TEFAA content despite increases in contents of several essential FAAs in the brain of *P. sinensis* during the 72-h post-feeding period. Hence, the metabolic signals which control the intake of high protein diets in this turtle are different from those in mammals.

For turtles exposed to brackish water, in addition to alanine, aspartate and histidine, there was a significant increase and a significant decrease in contents of glutamine and glutamate, respectively. Thus, it can be concluded that an increase in transamination or glutamine synthesis had occurred, and that the brain of *P. sinensis* exposed to 15‰ water was confronted with ammonia toxicity, which was transiently ameliorated by a reduction in ammonia production and/or an increase in detoxification of ammonia to glutamine. These results prelude to the proposition that an increase in amino acid catabolism, which resulted in an increase in ammonia production and urea synthesis, occurred in *P. sinensis* exposed to 15‰ water.

Six days of emersion did not result in an increase in glutamine synthesis in extra-cranial tissues in *P. sinensis* as in some tropical air-breathing fishes exposed to

air. This could be a result of the drastic reduction in ammonia production in turtles exposed to terrestrial conditions.

Through the intraperitoneal injection with a lethal (12.5 μmol g^{-1} turtle) or sub-lethal dose of NH_4Cl (7.5 μmol g^{-1} turtle) into turtles, it was confirmed that ammonia was detoxified to glutamine in the brain. However, increased synthesis and accumulation of glutamine was not the cause of death in turtles confronted with acute ammonia toxicity (see below). Ammonia toxicity can also be ameliorated by an increase in transamination or decreased amino acid catabolism in the brain of *P. sinensis*.

Extreme ammonia tolerance in the brain

MK801 (1.6 μg g^{-1} turtle), a NMDA receptor antagonist, reduced the mortality in *P. sinensis* injected with a lethal dose (12.5 μmol g^{-1} turtle) of NH_4Cl. Thus, our results reveal for the first time that activation of NMDA receptors could be the cause of death during acute ammonia toxicity in *P. sinensis*. Although MSO (82 μg g^{-1} turtle), an inhibitor of GS, extended the time to death in turtles confronted with acute ammonia toxicity, it has no effect on the mortality rate within a 24-h period. The limited protective effect of MSO was apparently unrelated to its capacity in inhibiting GS activity, and acute ammonia toxicity in *P. sinensis* was not a result of increased glutamine synthesis and accumulation.

Turtles succumbed to acute ammonia toxicity (12.5 μmol NH_4Cl g^{-1} turtle) had a brain ammonia content of 21 μmol g^{-1} brain. For those injected intraperitoneally with a sub-lethal dose (7.5 μmol g^{-1} turtle) of NH_4Cl, the brain ammonia content increased to 16 μmol g^{-1} brain at hour 1 post-injection. This could be the highest level reported for brains of vertebrates confronted with a sub-lethal dose of ammonia through injection. *P. sinensis* could probably regulate the

intracellular ammonia level in its brain despite drastic increases in ammonia content therein and thus avoid the deleterious effects of ammonia. It is also probable that membrane potentials of cells in its brain were resilient to NH_4^+ interference so that activation of NMDA receptors occurred only at very high extracellular ammonia concentrations. In conclusion, the brain of *P. sinensis* had extraordinary high tolerance of ammonia at the cellular and subcellular levels.

Evolution of mechanisms of ammonia toxicity from fish to mammals

The giant mudskipper, *P. schlosseri*, has very high environmental ammonia tolerance. The brain ammonia content reaches 14 μmol g^{-1} in fish succumbed to a lethal dose of NH_4Cl injected into its peritoneal cavity. MK801 and MSO had no protective effects in this mudskipper injected with a lethal dose of NH_4Cl. Thus, NMDA receptor activation and increased glutamine synthesis and accumulation are not the major cause of death in this fish intoxicated with ammonia. The brain of *P. sinensis* has even higher ammonia tolerance than that of *P. schlosseri*; death occurs at extremely high ammonia contents of 21 μmol g^{-1} brain. However, unlike *P. schlosseri*, activation of NMDA receptors is involved in ammonia toxicity, albeit the involvement of astrocyte swelling and brain edema as a result of glutamine accumulation can be ruled out. One of the common factors here is the presence of extra space in the cranial cavity, which presumably prevents brain herniation when swelling occurs therein, but this is not the case for mammalian brains. Another common factor is that the brains of *P. sinensis* and *P. schlosseri* have high ammonia tolerance; because of this, the rates of increase in glutamine synthesis and accumulation, and consequently the magnitude of astrocyte swelling, are moderate.

At present, it would appear that brain cells of lower vertebrate have membrane potentials resilient to NH_4^+ interference; only then can the brain ammonia content

builds up to 16 μmol g^{-1} as in the case of *P. sinensis*. In this specific case, NMDA receptors are activated as a downstream event of membrane depolarization only at very high extracellular ammonia concentrations. In contrast, mammals generally are unable to tolerate high levels of ammonia; levels of brain ammonia at 1-3 mmol l^{-1} would lead to high mortality. Because of this, ammonia infiltrated into or produced by the brain must be detoxified immediately to glutamine in order to maintain the brain ammonia at a tolerable level. This naturally results in glutamine accumulation, edema and brain stem herniation, contributing to the mean cause of death in patients suffers from HE. In addition, activation of NMDA receptor-coupled nitric oxide-cyclic GMP signal transduction pathway in the brain also contributes to ammonia toxicity in mammals, resulting in death.

Increased NMDA receptor activation by extracellular glutamate can result from not only an increased in extracellular glutamate concentration, but also a depolarization of membrane potential. Membrane depolarization occurs when inward movement of NH_4^+ substitutes the outward movement of K^+ through background K^+ -channels. Thus, it is important to evaluate the K^+ specificity of background K^+-channels in ammonia tolerant vertebrates like *P. sinensis* and *P. schlosseri* as compared with those in mammals. Because NH_4^+ can also substitute K^+ during the functioning of Na^+, K^+-ATPase, it is also essential to determine the substrate specificity for K^+ for these ATPases in these animals. Research in these directions will provide definitive explanations for ammonia toxicity and extreme ammonia tolerance, and results obtained may have important bio-medical applications.

Future implications

The discovery of buccopharyngeal nitrogenous excretion has implications for the treatment of renal failure. It is important to characterize the urea transporter gene

and understand why it is expressed in the buccopharyngeal region. This understanding can then be applied to humans with renal failure. The ability to switch on urea transporters in the gut will remove urea through an extra-renal route and negate the need for dialysis.

The other novel discovery was that *P. sinensis* could tolerate an extremely high level of ammonia in its brain. Understanding the cellular and sub-cellular mechanisms of tolerance would have implications for the treatment of hepatic encephalopathy. The ability to tolerate high levels of ammonia in human brain would prevent death arising as a consequence of hyperammonemia during acute or chronic liver failure.

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