

A2–DOGMAS AND CONTROVERSIES IN THE HANDLING OF NITROGENOUS WASTE

Organised by R. Wilson and T. Taylor for the Osmoregulation and Respiration Groups and co-sponsored by The Physiological Society

A2.1–From yeast ammonium transporters to human Rhesus proteins

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Ammonium transport across biological membranes is a process encountered in most if not all living cells. Ammonium is an excellent nitrogen source for many micro-organisms and plants, but it is instead a cytotoxic metabolic product in animals. Using yeast as a model, we cloned the first gene encoding a specific ammonium transporter (Mep1), allowing to define a new family of proteins (Mep/Amt) highly conserved in eubacteria, archaeobacteria, other fungi, plants and invertebrates like nematode and fruit fly. Ammonium uptake in yeast is mediated by the unglycosylated Mep1 and Mep3 proteins and by the Mep2 glycoprotein. While the three Meps are required for growth in the presence of low ammonium concentrations, Mep2 has also been assigned a particular role in ammonium sensing in the process of pseudohyphal differentiation. Although no close homologue of these ammonium transporters has yet been found in vertebrates, we have shown that Mep/Amt family members and proteins from the Rhesus (Rh) blood-group belong to the same superfamily. Despite their importance in transfusion medicine, the function of Rh proteins remained unknown. Rh type proteins are also present in primitive organisms such as green algae, sponge, nematode and fruit fly, suggesting a role widely conserved. Using heterologous expression in yeast, we have shown that human erythroid- and kidney-specific Rh proteins function as ammonium transporters. In the light of recent data, we will discuss the potential roles of proteins from the Mep/Amt/Rh superfamily.

A2.2–Not the simple way: Ammonia excretion in crabs

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It is the common belief that marine invertebrates excrete their nitrogenous waste products largely in the form of ammonia (NH_3) into the aquatic environment. The mechanism of excretion is thought to be simple diffusion along an outwardly directed NH_3 partial pressure gradient. However, our recent research on crustaceans showed that even marine *Cancer* species, bearing an extreme leaky gill epithelium, are capable of excreting

ammonia actively against a 4–8-fold inwardly directed gradient (Weihrauch et al., J. Comp. Physiol. B 169:25–37, 1999). Employing gills from the shore crab *Carcinus maenas* as a model system, we demonstrated that the branchial ammonia excretion mechanism is rather complex, involving not only Na^+/K^+ -ATPase and V-type H^+ -ATPase but also a functional microtubule network (Weihrauch et al., J. Exp. Biol. 205: 2765–2775, 2002) and is most likely mediated via exocytosis.

Recently we have discovered a Rhesus-like protein in *Carcinus maenas* gills that shows high homology to the human Rhesus associated proteins, identified as ammonium transporters (Marini et al., Nature 26: 341–344, 2000). A 1,966-nucleotide cDNA coding for this 478 amino acid protein was cloned and sequenced employing poly-A mRNA from *Carcinus* gill (GenBank Acc.: AF364404). From a hydrophobicity blot of the amino acid sequence, twelve transmembrane (TM) domains were predicted. Real-time quantitative PCR mRNA expression analysis of various tissues from *Carcinus maenas* revealed that the putative crustacean ammonium transporter (RhCM) is exclusively expressed in the ammonia excreting gills. These data were confirmed by Western blot analysis employing an RhCM-specific antibody.

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A2.3–Developmental strategies of nitrogen excretion in fish

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Nitrogen excretion during fish development is a critical aspect of early physiology because the major fuel source is obtained endogenously through absorption of the yolk amino acids. Catabolism of proteins and amino acids results in the formation of ammonia, a potentially toxic nitrogenous end product. Ammonia may be excreted to the environment, diverted away from developing tissues (i.e. stored in the yolk sac), or modified by formation of urea and/or glutamate/glutamine. Ammonia is excreted primarily as NH_3 , facilitated by an acid boundary layer next to the egg capsule or chorion. Urea excretion is dependent, in part, on a facilitated urea transporter. Early induction of urea cycle enzymes in several teleost species occurs, although interestingly, the pathway is silent in adults. In trout, the cDNAs of several urea cycle enzymes have been isolated and expression of the key enzyme, carbamoyl phosphate synthetase III is detected shortly after fertilization. Both urea and ammonia content rise prior to hatching in trout embryos, but exposure to external ammonia does not trigger

hatching. Further, peak ammonia content after hatching does not appear to be linked to exhaustive exercise and adenylate catabolism during the hatching process. Our knowledge to date suggests that trout embryos have very efficient mechanisms to cope with potential ammonia toxicity in order to prevent long-term detrimental effects on developmental maturation. There are still many gaps in our knowledge, however, particularly concerning the sites of nitrogen excretion during early life stages.

A2.4–Mechanisms of osmotic regulation in a marine elasmobranch in response to dilute seawater: a developmental perspective

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Marine elasmobranchs maintain high levels of urea and other osmolytes in their tissues in order to remain isoosmotic with their environment. Under dilute seawater stress, an increase in renal urea excretion and a decrease in tissue osmolyte concentrations have been reported in the little skate (*Raja erinacea*). Embryonic elasmobranchs undergoing rapid growth and feeding endogenously may not have the ability to synthesize urea or cope with hypoosmotic stress. We tested the hypothesis that embryonic skates (*R. erinacea*) lack the key ornithine urea cycle (OUC) enzyme carbamoyl phosphate synthetase III (CPSase III) and the capacity to osmoregulate in dilute seawater. Under control conditions, 4 month old whole embryos had a tissue ratio of urea:TMAO and other organic osmolytes of 2.3:1, while 8 month whole embryos had a ratio of 2.8:1. Both embryonic stages had significant levels of the OUC enzymes CPSase III, ornithine transcarbamoylase (OTCase), and arginase, as well as the accessory enzyme glutamine synthetase (GSase). When exposed to dilute 75% (25 ppt) vs. 100% seawater (33 ppt), skate embryos at 4 and 8 months significantly increased total urea excretion within the first 3 h of exposure but rates returned to control levels by 24 h post-exposure. TMAO concentration was significantly decreased in 4 month whole embryos after 120 h of dilute seawater exposure, whereas urea was significantly decreased in 8 month whole embryos. These findings contradict our hypothesis and suggest that embryonic skates synthesize urea and regulate tissue osmolytes in response to environmental dilution.

A2.5–Ammonia toxicity in the weather loach: a fish for all seasons

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The Chinese weather loach, *Misgurnus anguillicaudatus*, lives in rice paddies that are flooded in the spring and summer but dry up in the fall and winter. During dry periods the fish buries in the mud and breathes air, using the gut as an air-breathing organ. When buried, blood P_{CO_2} can approach 100 mmHg, but blood pH changes little. The gut and skin surfaces become alkaline and some ammonia can be lost by volatilization from these surfaces during air exposure. This fish is very tolerant of ammonia; the 96hLC₅₀ is approximately 75 mM NH_4^+Cl . Exposure to elevated levels of ammonia resulted in a switch to partial amino acid catabolism and the accumulation of alanine, with an associated decrease in ammonia production. The animal does not convert ammonia to urea or glutamine, there is no increase in glutamine synthetase activity, and ammonia simply accumulates in its tissues. Ammonia is accumulated in liver and muscle, and to a lesser extent, heart and brain. The toxic action of ammonia is probably due to membrane depolarization leading to excessive NMDA receptor activation in the brain, and convulsions and death of the animal. The NMDA receptor blocker MK-801 reduces ammonia toxicity in *Misgurnus*, as in some other vertebrates. The nature of ammonia tolerance in *Misgurnus* will be discussed.

A2.6–Exogenous Ammonia as a Growth Stimulant in Fish

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Traditionally, aquaculturists strive to keep environmental levels as low as possible because ammonia is considered a toxicant which reduces the growth of fish. However, most tests have used high levels. It is now clear that many fish species can 'detoxify' elevated internal ammonia levels by increased synthesis of amino acids, especially glutamine, and that ¹⁵N from exogenous ammonia can be incorporated into internal amino acids. This raises the possibility that under conditions where N is limiting, low levels of environmental ammonia might actually stimulate amino acid and protein synthesis, and therefore growth. Indeed about 5 years ago, we reported several studies in which prolonged exposure to very low levels (about 70 μmol/L) of elevated ammonia was accompanied by increased growth and protein accretion (1,2,3), even when ration was restricted (4).

However the focus of those studies was on other issues. We here report the results of two new 70 day growth studies, one pilot, one large scale, on juvenile rainbow trout, where ammonia levels were carefully controlled at nominal values of <10, 75 and 225 $\mu\text{mol/L}$, where feeding was carefully regulated, and where protein accretion and growth were recorded. The data suggest that moderately elevated ammonia levels may actually be beneficial to productivity in aquaculture (supported by NSERC).

1. Linton et al. (1997). *Trans. Am. Fish. Soc.* 126: 259–272
2. Linton et al. (1998) *Can. J. Fish. Aquat. Sci.* 55: 576–586
3. Reid et al. (1998) *Can. J. Fish. Aquat. Sci.* 55: 1534–1544.
4. Linton et al. (1999). *Trans. Am. Fish. Soc.* 128: 758–763.

A2.7—Ammonia makes fish slow, it's a depolarising experience

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When exposed to copper, either at low, sublethal concentrations (<0.1 $\mu\text{mol l}^{-1}$) in acid soft water or at higher concentrations (>5 $\mu\text{mol l}^{-1}$) in seawater, trout accumulate ammonia, despite being able to take up oxygen and excrete carbon dioxide at normal rates (Wilson and Taylor, 1993; Beaumont et al., 1995). On exposure to copper in acid soft water, rates of ammonia excretion are increased, in association with cortisol production, but not enough to explain the high levels of accumulation; implying that an active process eliminating NH_4^+ is being inhibited by copper (Beaumont et al., 2003). A proton pump on the apical membrane of gill epithelial cells may be inhibited by copper.

Fish swim less well in solutions of copper with their rates of sustainable swimming showing a negative correlation ($r^2=0.7$) with plasma ammonia levels (Beaumont et al., 1995). They also swim less well in solutions of ammonia (Shingles et al., 2001). High levels of tissue ammonia directly affect intermediary metabolism, favouring anaerobic energy production, but limiting aerobic metabolism. There was also some evidence for tissue hypoxia during copper exposure. However, the predominant factor affecting swimming ability was partial depolarisation of white muscle fibres, preventing their recruitment during sustained swimming (Beaumont et al., 2000).

- Beaumont et al (1995) *J. exp. Biol.* **198**, 2213–2220
 Beaumont et al (2000) *J. exp. Biol.* **203**, 2229–2236
 Beaumont et al (2003) *J. exp. Biol.* **206**, 153–162
 Shingles et al (2001) *J. exp. Biol.* **204**, 2691–2698
 Wilson and Taylor (1993) *J.Comp. Physiol. B.* **163**, 239–246.

A2.8—The effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout

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Ammonia and, to a lesser extent, urea are usually considered the primary forms of excreted nitrogen in teleost fish. Consequently, most studies examining nitrogen metabolism in fish have concentrated on these end products. However, analysis of total nitrogen excretion invariably exceeds the sum of ammonia and urea nitrogen, suggesting that nitrogen is excreted in alternative forms. The aim of the current research was to determine possible alternative nitrogen excretory products in rainbow trout (*Oncorhynchus mykiss*) that may account for this observed discrepancy, and whether they vary with ration. In fed fish, ammonia and urea together accounted for a significantly lower percentage than in fasted fish and the discrepancy increased with increasing ration. This confirmed the hypothesis that fed fish are capable of considerable excretion of nitrogen in alternate forms. Holding water was analyzed for amino acids and protein content to determine if these entities were significant sources of excreted nitrogen. Whereas amino acids accounted for a relatively small percent of the discrepancy, the contribution of protein was larger and increased with feeding. These results suggest that protein excretion, possibly in shed body mucus, may be an important component of total nitrogen balance. The biological implications of this will be discussed, as will ongoing research into the potential roles of other excretory products such as creatinine, creatine, TMA, and TMAO in teleost nitrogen balance (supported by NSERC).

A2.9—Urea transporters: What, Where and Why?

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In mammals urea is the major end-product of amino acid deamination and plays important roles in water and nitrogen balance. Central to these roles are membrane bound urea transporter proteins derived from the UT-A gene. We have determined the structure of the mouse UT-A gene and characterized five splice variants (UT-A1, UT-A2, UT-A3, UT-A4 and UT-A5). The gene consists of 24 exons spanning ~400 kb of chromosome 18. Transcription is driven by two promoters situated at the 5' end of the gene and also a promoter preceding exon 14. UT-A proteins are highly expressed in the kidney where they mediate epithelial urea flux as part of the

urinary concentrating mechanism. Expression profiling of other mouse tissues has revealed that UT-A proteins are expressed in extra renal tissues, including testis and colon. In testis, UT-A protein expression is strongly coordinated with spermatogenesis and a phloretin inhibitable urea pathway, characteristic of UT-A proteins, exists across the seminiferous tubule epithelia. We have also discovered UT-A proteins in colon and shown that a phloretin inhibitable urea pathway is present in colonic plasma membranes. We suggest that this pathway promotes diffusion of urea into the colon. Subsequent intestinal breakdown of urea provides a nitrogen source for resident commensal bacteria and contributes to maintenance of host nitrogen balance. This presentation will review recent developments in both renal and non-renal urea transport.

A2.10—Psychological Regulation of Gill Urea Transport by the Gulf Toadfish?

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An unusual pulsatile urea excretion mechanism (tUT) exists in the gills of the gulf toadfish that is believed to be involved in the behavior of this species. While studies have demonstrated the molecular and pharmacological similarity of tUT to the UT-A facilitated diffusion transport system of the mammalian kidney, the regulatory factors behind this mechanism have not yet been resolved. In mammals, AVP and glucocorticoids are two important endocrine regulators of UT-A transporters. Circulating AVT (the teleost homologue of AVP) is not involved in the control of pulsatile urea excretion in toadfish. However, sharp, periodic declines of plasma cortisol concentrations appear to play a permissive role in the activation of this mechanism. While pulsatile excretion of urea is consistently preceded by a decline in circulating cortisol, lowered cortisol concentrations do not always result in pulsatile excretion, suggesting that periodic activation of the transporter requires an additional stimulus. Recent work on the regulation of tUT has turned to the role of 5-hydroxytryptamine (5-HT; serotonin), as arterial injection of this monoamine causes physiologically sized pulses in approximately 50% of the fish injected. The injection of 5-HT also results in a significant increase in plasma cortisol concentrations, suggesting the involvement of this monoamine in the rise in cortisol preceding a pulse. Present work is investigating the pharmacology of this response through the use of 5-HT receptor agonists and antagonists.

A2.11—The influence of photoperiod on pulsatile urea excretion in toadfish (*Opsanus beta*)

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The gulf toadfish *Opsanus beta* (Batrachoididae) is one of the few teleosts to maintain a functional ornithine-urea cycle (O-UC) during adult life and possess the capability to change from ammonotelic to ureotelic within 24 hours in the laboratory. Captive *O. beta* excrete most nitrogenous waste across the gill membrane. In the lab, urea is generally excreted in daily pulses of 1.5 hrs in duration while ammonia is eliminated continually. At present, the mechanism of O-UC activation is putatively due to an elevation of plasma cortisol (stress response) which promotes the up regulation of the key OU-C enzyme glutamine synthetase. Experiments in this study examine the diel pattern of nitrogen excretion in the laboratory and in mesocosms. Under both experimental conditions toadfish were exposed to natural photoperiod, samples were collected hourly from toadfish in shelters fabricated with PVC pipe, and assayed for urea and ammonia with standard chemical techniques. In laboratory trials, conducted in 2 L containers with static seawater changed daily, urea pulses occurred at random with no correlation to light or dark cycles. In mesocosm experiments, toadfish were unrestrained in 8000 L tanks with the seagrass *Thalassia testudinum* planted on carbonate substrate effectively simulating their natural habitat. Shelters were outfitted with an underwater IR camera connected to a time-lapse video recorder to document toadfish behavior. Urea and ammonia excretion in mesocosms occurred predominately during daylight hours with peak levels near dawn or dusk. Differing results between experimental regimes are believed to reflect the degree of stress encountered by toadfish.

A2.12—Increased urea transporter expression in Dahl salt-sensitive rats—potential role in salt balance

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Dahl salt sensitive (DS) rats are a widely used genetic model of salt-sensitive hypertension, however the exact basis of this hypertension is unknown. We performed transporter profiling using semi-quantitative immunoblotting in the kidneys of Dahl salt-sensitive (DS) rats vs. Dahl salt-resistant (DR) rats using a battery of poly-

clonal antibodies targeted to all of the major renal solute transporter proteins. DS and DR rats (120–150 g) were housed in metabolic cages and received a high (2 meq/day) NaCl diet by ration feeding. Surprisingly, in the inner medulla of DS rats there were large increases in the abundances of the collecting duct urea transporters (UT) UT-A1 (212% \pm 13%, normalized to DR) and UT-A3 (223% \pm 18%) and these changes were confirmed by immunocytochemistry. In isolated perfused IMCDs, urea permeability was significantly greater in DS rats. mRNA levels (real-time RT-PCR) in DS rats (normalized to DR) were 1.80 ± 0.25 for UT-A1 ($p < 0.05$) and 1.86 ± 0.45 for UT-A3. Immunoblotting and immunocytochemistry revealed an increase in 11 β -hydroxysteroid dehydrogenase type 2 (11- β HSD2) abundance. In view of previous studies showing downregulation of collecting duct urea transporters by glucocorticoids, we hypothesized that the increased 11- β HSD2 may contribute to increased UT expression in the IMCD. Administration of carbenoxolone, a competitive inhibitor of 11- β HSD2 activity, caused significant reductions in the abundances of UT-A1 and UT-A3. We propose that the higher expression of UTs may indirectly cause a change in final sodium balance, which may contribute to the greater blood pressure observed in the DS rats.

A2.13—Facultative ammonotelism in nectar-feeding birds

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Facultative switching from excreting one form of nitrogenous waste product to another, particularly in response to a change in water availability, is widespread among animal taxa. However, this phenomenon was unknown among birds until 1994 when Preest and Beuchat found ammonotelism in Anna's hummingbird at low ambient temperatures. We tested whether facultative ammonotelism would occur in an old world nectarivore, the Palestine sunbird (*Nectarinia osea*), in response to changes in water, salt, or protein intake, or ambient temperature. In contrast to Anna's hummingbird, no ammonotelism was observed at low ambient temperatures in Palestine sunbirds. Differences between these studies may be related to differences between sunbird and hummingbird physiology or to differences in methodology. We found that ammonotelism did ostensibly occur in response to a drop in protein intake, and resulted from a reduction in uric

acid (UA) production. In addition, UA concentrations in excreted fluid were significantly less than in ureteral urine, suggesting that breakdown of UA occurred in the hindgut. Thus, this apparent ammonotelism was the combined result of a drop in UA production in response to low protein intake and of UA breakdown in the hindgut. We predict that apparent ammonotelism will occur in other birds under similar circumstances. Ammonia excretion did not respond to changes in water availability in Palestine sunbirds, despite six-fold increases in water intake rates. However, as Palestine sunbirds can modulate water uptake from the gut (McWhorter, Martinez Del Rio and Pinshow, 2003), they are unlikely to experience large changes in water turnover rates.

A2.14—The recycling (conservation) of uric acid nitrogen by the intestine of birds

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The diets of a number of avian species contain very little nitrogen, therefore the availability of this element for protein synthesis and repair may be limited. Evolution may have solved this problem with the development of uric acid as an end product of nitrogen metabolism and most birds, if not all, are uricotelic. For the purpose of excreting nitrogen, uric acid is an ideal molecule as it contains four nitrogen atoms and it has a very low aqueous solubility and therefore obligates little water for its excretion. The second of these characteristics has the potential of causing problems within the urinary system by uric acid precipitating from solution. This has been prevented by binding uric acid to a protein with this complex taking the form of small spherical structures that pass with ease along the urinary system of ducts. These small spheres are prevented from coalescing into larger spheres by the presence of a relatively large amount of protein in the urine. The coupling of the renal and gastrointestinal systems in birds channels the output of the kidneys into an area where the composition of the urine is significantly modified. In the lower gastrointestinal tract, uric acid is digested by bacteria resulting in the production of amino acids and short-chain volatile fatty acids. These compounds are absorbed into the venous blood and carried to the kidneys and liver. In the liver, the amino acids can be incorporated in proteins, enter the citric acid cycle, or be used to produce glucose through gluconeogenesis.