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AMMONIA, UREA AND H^+ DISTRIBUTION AND THE EVOLUTION OF UREOTELISM IN AMPHIBIANS

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Summary

In theory, the distribution of ammonia across cell membranes ($Tamm_i/Tamm_e$) between intracellular and extracellular fluids (ICF and ECF) may be determined by the transmembrane pH gradient (as in mammals), the transmembrane potential (as in teleost fish), or both, depending on the relative permeability of the membranes to NH_3 and NH_4^+ (pNH_3/pNH_4^+). The resting distributions of H^+ (*via* [^{14}C]DMO), ammonia and urea between plasma and skeletal muscle, and the relative excretion rates of ammonia-N and urea-N, were measured in five amphibian species (*Bufo marinus*, *Ambystoma tigrinum*, *Rana catesbeiana*, *Necturus maculosus* and *Xenopus laevis*). Although $urea_i/urea_e$ ratios were uniformly close to 1·0, $Tamm_i/Tamm_e$ ratios correlated directly with the degree of ammoniotelism in each species, ranging from 9·1 (*Bufo*, 10% ammoniotelic) to 16·7 (*Xenopus*, 79% ammoniotelic). These values are intermediate between ratios of about 30 (low pNH_3/pNH_4^+) in ammoniotelic teleost fish and about 3 (high pNH_3/pNH_4^+) in ureotelic mammals. The results indicate that amphibians represent a transitional stage in which ammonia distribution is influenced by both the pHi–pHe gradient and the membrane potential, and that a reduction in cell membrane permeability to NH_4^+ (i.e. increased pNH_3/pNH_4^+) was associated with the evolution of ureotelism. Hyperosmotic saline exposure increased urea excretion 10-fold in *Xenopus*, while ammonia excretion remained unchanged. $Tamm_i/Tamm_e$ fell, but this response was attributable to an abolition of the pHi–pHe gradient, rather than a physiological change in the cell membrane pNH_3/pNH_4^+.

Introduction

Ammonia is both a respiratory gas and a weak base. With a pK of approximately 9·5, it exists mainly as the ammonium ion (NH_4^+) in biological fluids at normal pH

Key words: ammonia, urea, intracellular pH, amphibian, *Bufo, Rana, Ambystoma, Necturus, Xenopus.*

values *in vivo* (6·5–8·0). Only a small fraction exists as the dissolved non-ionic gas (NH_3). The distribution of a weak base, such as ammonia, across biological membranes may be described by the theory of non-ionic diffusion (Jacobs & Stewart, 1936; Milne *et al.* 1958). In brief, the theory states that cell membranes are highly permeable to NH_3 but virtually impermeable to the charged form NH_4^+. NH_3 can diffuse freely between intra- and extracellular fluids and, under steady-state conditions, plasma NH_3 (P_{NH_3}) levels will come into equilibrium between the two compartments. The NH_4^+ concentration of each compartment is thereby dictated by its pH. Thus, the distribution of ammonia (Tamm) is a function of the intracellular (pHi) to extracellular pH (pHe) gradient:

$$\frac{\text{Tamm}_i}{\text{Tamm}_e} = \frac{1 + 10^{(pK - pHi)}}{1 + 10^{(pK - pHe)}}. \quad (1)$$

Mammals are ureotelic and have very low levels of ammonia in their body fluids (Campbell, 1973). A large body of evidence confirms that the non-ionic diffusion theory adequately describes the distribution of ammonia between extracellular and intracellular fluids of muscle and many other mammalian tissues *in vivo* (Stabenau *et al.* 1959; Visek, 1968; Meyer *et al.* 1980; Mutch & Banister, 1983). Thus, with a pHi–pHe gradient of 0·4–0·6 units, $\text{Tamm}_i/\text{Tamm}_e$ is about 2·5–4·0 in mammals.

Teleost fish are ammoniotelic and have somewhat higher levels of ammonia in their body fluids. Until very recently, the same principles were thought to govern the Tamm distribution in teleost fish tissues (Randall & Wright, 1987; Dobson & Hochachka, 1987). However, Wright *et al.* (1988) and Wright & Wood (1988) have now shown that $\text{Tamm}_i/\text{Tamm}_e$ is about 30·0 in teleost muscle *in vivo* at approximately the same pHi–pHe gradient (0·4–0·6 units). This distribution cannot be explained by the principles of non-ionic diffusion. However, assuming that teleost cell membranes have significant permeability to NH_4^+, then it is well described by the Nernst equation:

$$E_M = -\frac{RT}{zF}\ln\frac{[NH_4^+]_i}{[NH_4^+]_e} = \frac{-RT}{zF}\ln\frac{[\text{Tamm}]_i - [NH_3]_i}{[\text{Tamm}]_e - [NH_3]_e}, \quad (2)$$

where R, T, z and F have their usual meaning, and E_M, the membrane potential, has a typical teleost white muscle value of −83 mV. Wright *et al.* (1988) and Wright & Wood (1988) presented a model whereby NH_4^+ was in electrochemical equilibrium across the cell membrane, resulting in large standing P_{NH_3} gradients from ICF to ECF in the tissues of teleost fish. Ammonia would thereby cross the cell membrane in both ionized (NH_4^+) and non-ionized (NH_3) forms, perhaps a correlate of the need to move large quantities of this end-product across cell membranes in these ammoniotelic animals.

Distribution according to the pH gradient and distribution according to the membrane potential represent the two extreme ends of a spectrum of possible equilibrium states (see Fig. 3). Boron & Roos (1976) and Roos & Boron (1981) derived a general equation describing the equilibrium distribution of a weak base

subject to both transmembrane electrical and transmembrane pH gradients. For ammonia, this equation may be written:

$$\frac{\text{Tamm}_i}{\text{Tamm}_e} = \frac{[H^+]_i + K}{[H^+]_e + K} \times \frac{(pNH_3/pNH_4^+) - [F \times E_M/\boldsymbol{R} \times T(1-\gamma)] \times ([H^+]_e/K)}{(pNH_3/pNH_4^+) - [F \times E_M \times \gamma/\boldsymbol{R} \times T(1-\gamma)] \times ([H^+]_i/K)}, \quad (3)$$

where K is the NH_3/NH_4^+ dissociation constant, pNH_3 is the permeability to NH_3, pNH_4^+ is the permeability to NH_4^+, and:

$$\gamma = \exp(E_M F/\boldsymbol{R}T). \quad (4)$$

Thus, $\text{Tamm}_i/\text{Tamm}_e$ is a function of the permeability ratio (pNH_3/pNH_4^+) of the cell membrane to the non-ionized and ionized forms of ammonia. At the extreme asymptotes of this relationship, represented by teleost fish and mammals (see Fig. 3), the permeability ratios must be less than 20 and greater than 300, respectively, based on the measured distribution ratios, pHi−pHe gradients and membrane potentials in the two classes.

We therefore hypothesized that a relative reduction in cell membrane permeability to NH_4^+ (i.e. increase in pNH_3/pNH_4^+) may have accompanied the evolutionary transition from ammoniotelism to ureotelism, for transmembrane electrical gradients do not appear to have changed greatly. Amphibians are intermediate forms exhibiting a wide variation in the degree of ammonio-/ureotelism amongst different species (Munro, 1953; Cragg *et al.* 1961; Balinsky, 1970). Virtually nothing is known about ICF/ECF ammonia or urea distributions in this class, or their possible relationships with the pHi−pHe gradient. Our goal was to test this hypothesis by examining Tamm, H^+ and urea distributions *in vivo* between ECF and ICF of muscle, to see whether such a transition could be detected. Five amphibian species covering the range from largely ammoniotelic to largely ureotelic were examined. In addition, we tested whether the pNH_3/pNH_4^+ ratio might be sensitive to physiological adjustment, by subjecting the most ammoniotelic species (*Xenopus laevis*) to a treatment (saline exposure) reported to convert its metabolism from ammoniagenesis to ureagenesis (Balinsky *et al.* 1961; Janssens, 1964; Janssens & Cohen, 1968; McBean & Goldstein, 1970*a*,*b*).

Materials and methods

Experimental animals

Adult bullfrogs (*Rana catesbeiana*; 453 ± 23 g, $N = 9$), adult marine toads (*Bufo marinus*; 236 ± 40 g, $N = 13$), adult South African clawed toads (*Xenopus laevis*; 42 ± 4 g, $N = 29$), neotenic mudpuppies (*Necturus maculosus*; 58 ± 4 g, $N = 11$) and larval tiger salamanders (*Ambystoma tigrinum*; 98 ± 5 g, $N = 12$) were obtained from commercial suppliers (Charles D. Sullivan Co. Inc, Nashville, TN, USA; Carolina Biological Supply, NC, USA; Ward's Natural Sciences Ltd, Mississauga, Ontario, Canada). *Xenopus*, *Necturus* and *Ambystoma* were held in

dechlorinated Hamilton tapwater (Na^+, 0·6; Cl^-, 0·8; Ca^{2+}, 1·8; Mg^{2+}, 0·5, K^+, 0·04 mequiv l^{-1}; titration alkalinity to pH 4·0, 1·9 mequiv l^{-1}; total hardness as $CaCO_3$, 140 mg l^{-1}; pH 8·0; temperature, 7–15°C). *Rana* and *Bufo* were held in terraria allowing voluntary access to a pool of the same tapwater. The animals were starved for several weeks to eliminate any effect of feeding history on nitrogenous excretion (Balinsky, 1970). Approximately 1 week prior to experimentation, the animals were transferred to the experimental temperature (20 ± 1°C) in the laboratory.

Xenopus subjected to the salt-stress treatment were transferred sequentially at 2-day intervals to 100, 200, 300 and finally 400 mosmol kg^{-1} solutions of NaCl in dechlorinated tapwater at 20°C. They were then kept at 400 mosmol kg^{-1} for 4–6 days prior to experimentation.

Nitrogenous waste excretion measurements

Amphibians were placed in 10 times their volume of constantly aerated tapwater (or 400 mosmol kg^{-1} water) in individual containers. For *Necturus* and *Ambystoma*, these were fish flux boxes of the style described by McDonald & Rogano (1986), for *Rana* and *Bufo*, 10 l plastic buckets, and for *Xenopus*, 1 l plastic beakers. Thus the *Necturus* and *Ambystoma* were submerged, the *Xenopus* floating or diving, and the *Rana* and *Bufo* sitting in several centimetres of water with most of their upper bodies exposed. The first 0·5 h of the flux period was ignored to avoid any complicating effects of urination caused by handling. Water samples taken over the next 5 h yielded ammonia and urea excretion rates which were linear with time for *Necturus*, *Ambystoma* and *Xenopus*. Excretion rates were irregular over short intervals in *Rana* and *Bufo*, probably reflecting intermittent urination; average flux rates over 48 h were therefore determined. Additional information on relative ammonia and urea excretions in these two species was obtained by collecting bladder urine at the end of the experiment.

Ammonia, urea and H^+ distribution measurements

Several days after completion of the flux measurements, the animals were anaesthetized by immersion in MS-222 (0·05 % for *Xenopus* and *Necturus*, 0·10 % for the other species) for implantation of indwelling arterial catheters. In the anurans, the femoral artery of one leg was occlusively cannulated as described by Boutilier *et al.* (1979; *Rana*, *Bufo*) and Boutilier (1984; *Xenopus*). In both *Necturus* and *Ambystoma*, the right second branchial artery was occlusively cannulated as outlined by Stiffler *et al.* (1983). The animals were then allowed to recover for 24–48 h in their individual containers. Post-operational survival was close to 100 % in all groups except the salt-stressed *Xenopus*, where only four of 12 animals recovered.

To determine pHi in muscle, each animal was infused with 7 μCi kg^{-1} of the weak acid [^{14}C]DMO (5,5-dimethyl-2,4-oxazolidinedione; NEN, specific activity 50 mCi $mmol^{-1}$) and 28 μCi kg^{-1} of the ECF marker [^{3}H]PEG-4000 (polyethylene glycol, M_r = 4000; Amersham, specific activity 8 mCi $mmol^{-1}$). [^{3}H]PEG-4000 w

employed as the ECF marker in this study in preference to other commonly used compounds such as [^{3}H]mannitol or [^{3}H]inulin. In our experience, PEG is a conservative label, yielding slightly lower estimates of ECF volume (ECFV) than mannitol, and generally comparable to those of inulin. At the same time, however, it is extremely stable, thereby avoiding the problems of radioautolysis which necessitate cleaning inulin by column chromatography before use. [^{14}C]DMO and [^{3}H]PEG-4000 were each carried in 1 ml kg^{-1} of physiological saline, followed by a wash-in of equal volume. The water in the containers was then renewed. After an 8–10 h equilibration period, a blood sample of 450 μl was withdrawn *via* the arterial catheter into a gas-tight Hamilton syringe for determination of extracellular acid–base status, plasma Tamm, urea and water content, and [^{14}C]DMO and [^{3}H]PEG-4000 radioactivities. Plasma was immediately separated by centrifugation at 9000 *g*; a portion was frozen in liquid N_2 and stored at −70 °C for later analysis of Tamm and urea.

Less than 5 min after blood withdrawal, the animal was quickly killed by double pithing (*Rana*, *Bufo*) or decapitation (*Xenopus*, *Necturus*, *Ambystoma*). Skeletal muscle samples were excised and a thin slice of tissue immediately frozen in freeze-clamp tongs cooled in liquid N_2. The frozen tissue was stored at −70 °C for later determination of intracellular Tamm and urea levels, and additional tissue samples were dried separately for analysis of water content and digested for [^{14}C]DMO and [^{3}H]PEG-4000 analysis. In the anurans, the muscle was taken from the triceps femoris and gracilis complexes of the thigh, contralateral to the cannulated limb. In *Necturus* and *Ambystoma*, the dorsalis trunci muscle approximately half-way down the body was utilized. The entire excision and freeze-clamping procedure took approximately 1·5 min.

Analytical techniques and calculations

Arterial pHe was determined with a Radiometer microelectrode assembly (E5021) and acid–base analyser (PHM71) maintained at 20 °C. True plasma total CO_2 concentration was measured with a Corning 965 CO_2 analyser using plasma obtained by centrifugation (5000 *g* for 2 min) in sealed hematocrit tubes. Plasma P_{CO_2} and HCO_3^- were calculated by standard manipulations of the Henderson–Hasselbalch equation using appropriate values of pK′ and αCO_2 (Severinghaus, 1965). Plasma protein and water content were determined by refractometry (American Optical TS meter). Muscle water content was determined by drying to a constant weight at 85 °C.

Ammonia concentrations (Tamm) in water were determined by the salicylate–hypochlorite method of Verdouw *et al.* (1978). Levels of Tamm in plasma and muscle were determined by the L-glutamic dehydrogenase/NAD method of Kun & Kearney (1971), after deproteinization and homogenization in 8 % $HClO_4$, as detailed by Wright *et al.* (1988). Urea levels in water, plasma and the $HClO_4$ extracts of muscle tissue were measured by the diacetyl monoxime method of Crocker (1967), using Sigma prepackaged reagents.

[^{14}C]DMO and [^{3}H]PEG-4000 radioactivities were measured by digesting

duplicate plasma (100 μl) and muscle samples (50–150 mg) in 2 ml of NCS tissue solubilizer (Amersham) at 40 °C. The digests were neutralized with 60 μl of glacial acetic acid and 10 ml of fluor (OCS; Amersham) was added. After overnight storage in the dark to eliminate chemiluminescence, the samples were counted on an LKB 1217 liquid scintillation counter programmed for dual-label quench correction by the external standard ratio method, as described by Kobayashi & Maudsley (1974).

The equations used for calculation of ECFV, ICFV, pHi, $Tamm_e$, $Tamm_i$, and NH_4^+, NH_3 and P_{NH_3} levels in the two compartments were identical to those of Wright *et al.* (1988), using [^{3}H]PEG-4000 radioactivities in substitution for [^{3}H]mannitol radioactivities as the ECF marker. Appropriate values for the pK of DMO were taken from Malan *et al.* (1976), and for the pK of ammonia and αNH_3 from Cameron & Heisler (1983). Extracellular and intracellular urea levels were calculated as for $Tamm_e$ and $Tamm_i$ (i.e. as concentrations per ml of ECF or ICF water, respectively).

Data are presented as means ± 1 S.E.M. (N). Regression lines were fitted by the method of least squares and the significance of the simple correlation coefficient (r) evaluated. Student's unpaired two-tailed t-test, with appropriate transformations to normalize variances where necessary, was used to assess the significance ($P \leq 0.05$) of differences between mean values.

Results

Nitrogenous waste excretion rates

The five amphibian species studied in fresh water spanned the range from largely ureotelic (Bufo; only 10% of N-excretion as ammonia-N) to largely ammoniotelic (*Xenopus*; 79% as ammonia-N), as assessed by their excretion rates to the external water (Fig. 1A). Absolute flux rates on a mass-specific basis (Fig. 1B) were greatest in the larval *Ambystoma*, and higher in the smaller adult species (*Xenopus*, *Necturus*) than in the larger ones (*Bufo*, *Rana*). Within each species, the relative proportions as ammonia-N and urea-N were quite uniform amongst individuals, with the exception of *Rana*. Here, individual values averaged over 48 h ranged from 9% to 91% ammoniotelism, with a mean of 41%. Analysis of urine composition in the bladders of *Bufo* ($N = 9$) and *Rana* ($N = 7$) revealed four- to fivefold higher levels of both ammonia-N (1.65 ± 0.21 mmol l^{-1} *versus* 8.50 ± 1.16 mmol l^{-1}) and urea-N (20.51 ± 3.30 mmol l^{-1} *versus* 85.73 ± 12.50 mmol l^{-1}) in the latter. The mean ammonia-N/urea-N ratio in *Bufo* urine (8%) agreed closely with that determined from measured excretion rates (10%), whereas that in *Rana* urine (10%) was very different from the mean flux value (41%).

When *Xenopus*, the most ammoniotelic of the five species in fresh water, was subjected to 400 mosmol kg^{-1} salt stress, the ammoniotelism dropped from 79% to 29% (Fig. 1A). This was achieved by a 10-fold stimulation of urea output, with no change in ammonia flux (Fig. 1B). On an absolute basis, the total nitroge

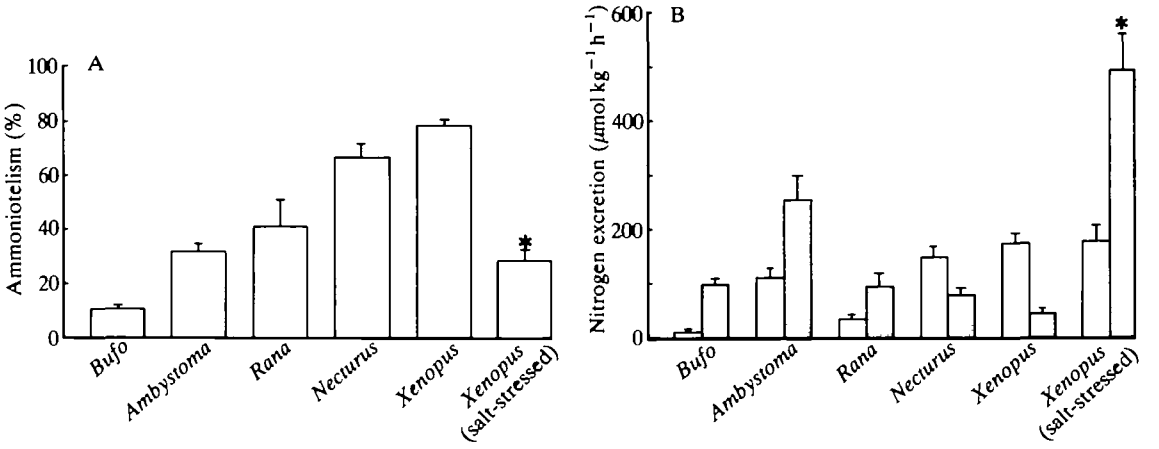

Fig. 1. (A) The percentages of total nitrogen excretion (ammonia-N + urea-N) in the form of ammonia (% ammoniotelism) in five different amphibian species in fresh water, and in *Xenopus* subjected to salt stress. (B) Absolute nitrogen excretion rates as ammonia-N (open columns) and urea-N (shaded columns) in these same animals. Means ± 1 S.E.M. *Bufo* ($N = 8$), *Ambystoma* ($N = 12$), *Rana* ($N = 10$), *Necturus* ($N = 7$), freshwater *Xenopus* ($N = 17$), salt-stressed *Xenopus* ($N = 12$). * indicates significant difference ($P \leq 0{\cdot}05$) from corresponding value for freshwater *Xenopus*.

excretion rates of salt-stressed *Xenopus* were the highest recorded in any of the species.

Extracellular and intracellular acid–base status

Despite large differences in Pa_{CO_2} and HCO_3^- levels amongst species, most regulated pHa close to 7·8 at 20°C (Table 1). The one exception was *Necturus* where mean pHa was 7·66. Pa_{CO_2} and HCO_3^- levels were lowest in this obligate bimodal water-breather (gills, skin), higher in the trimodal *Ambystoma* (gills, skin and lungs), and higher still in bimodal *Bufo*, *Xenopus* and *Rana* (lungs, skin). There was no obvious relationship between these blood gas characteristics and the degree of ammoniotelism *versus* ureotelism. Adaptation of *Xenopus* to 400 mosmol kg^{-1} NaCl induced a profound metabolic acidosis (pHa ≈ 7·2) as shown by a 60% loss of plasma HCO_3^-. Pa_{CO_2} was also significantly elevated, but this probably reflected the observation that the salt-stressed animals tended to remain under water during sampling, in contrast to the freshwater *Xenopus*.

Intracellular pH of skeletal muscle was regulated as tightly as extracellular pH within species, but exhibited greater variation amongst species (Table 1). As a result, the pHi−pHe gradient ranged from 0·40 in *Necturus* to 0·71 in *Xenopus* and *Rana*. Interestingly, the pHi of skeletal muscle in salt-stressed *Xenopus* remained unchanged in the face of the dramatic extracellular acidosis; thus the pHi−pHe gradient was reduced to a value not significantly different from zero.

The distribution of fluid volume between ECF and ICF, plasma protein levels

Table 1. *Arterial (extracellular) and skeletal muscle (intracellular) acid–base status in five different amphibian species in fresh water, and in* Xenopus *subjected to salt stress*

	Arterial plasma (ECF)			Muscle (ICF)	
	Pa_{CO_2} (kPa)	$[HCO_3^-]$ (mmol l^{-1})	pHa	pHi	pHi–pHe gradient
Bufo ($N = 12$)	1·34 ± 0·13	24·58 ± 0·99	7·884 ± 0·030	7·274 ± 0·046	0·610 ± 0·030
Ambystoma ($N = 6$)	1·12 ± 0·13	17·42 ± 0·73	7·818 ± 0·032	7·349 ± 0·015	0·469 ± 0·031
Rana ($N = 9$)	2·28 ± 0·33	35·28 ± 2·45	7·831 ± 0·036	7·117 ± 0·036	0·714 ± 0·034
Necturus ($N = 11$)	0·59 ± 0·03	6·38 ± 0·28	7·661 ± 0·008	7·259 ± 0·011	0·402 ± 0·012
Xenopus ($N = 14$)	1·72 ± 0·10	23·61 ± 0·96	7·758 ± 0·028	7·044 ± 0·026	0·714 ± 0·025
Xenopus (salt stressed) ($N = 4$)	2·46* ± 0·38	8·76* ± 1·67	7·209* ± 0·136	7·125 ± 0·071	0·075* ± 0·127

Values are means ± 1 S.E.M.
* Significantly different ($P \leq 0·05$) from corresponding value for *Xenopus* in fresh water.

and hematocrits in the five species are summarized in Table 2. In general, total muscle water content and the extracellular fraction tended to be greater, and plasma protein levels lower, in the more aquatic animals (*Necturus*, *Ambystoma*) than in the more terrestrial ones (*Rana*, *Bufo*). Note, however, that there was a significant redistribution of water from ICF to ECF, and an associated fall in plasma protein concentration, when *Xenopus* were transferred from fresh water to 400 mosmol kg^{-1} NaCl (Table 2).

Extracellular and intracellular ammonia and urea levels

Plasma $Tamm_e$ concentrations in animals in fresh water were generally low, ranging from 49 μmol l^{-1} in the more terrestrial *Rana* to 147 μmol l^{-1} in the obligate water-breather *Necturus* (Table 3). $Tamm_i$ levels in the ICF of muscle were approximately one order of magnitude greater, yielding $Tamm_i/Tamm_e$ ratios of 9·1 (*Bufo*) to 16·7 (*Xenopus*). In every case, this distribution ratio was much higher than that predictable from the measured pHi–pHe gradient alone, yet much lower than that predictable from a reasonable estimate of the membrane potential (see Discussion). There was a strong positive correlation between the percentage ammoniotelism and the $Tamm_i/Tamm_e$ distribution ratio in different species (Fig. 2A). Both $Tamm_e$ and $Tamm_i$ increased significantly in *Xenopus*

Table 2. *Muscle fluid volume distribution, plasma protein concentrations and hematocrits in five different amphibian species in fresh water, and in* Xenopus *subjected to salt stress*

	ECFV ($ml\,g^{-1}$)	ICFV ($ml\,g^{-1}$)	Total H_2O ($ml\,g^{-1}$)	Plasma protein ($g\,100\,ml^{-1}$)	Hematocrit (%)
Bufo ($N=12$)	0·124 ± 0·005	0·680 ± 0·007	0·804 ± 0·007	3·0 ± 0·3	32·0 ± 2·0
Ambystoma ($N=6$)	0·149 ± 0·022	0·677 ± 0·019	0·826 ± 0·004	1·6 ± 0·2	16·5 ± 4·3
Rana ($N=9$)	0·076 ± 0·005	0·723 ± 0·005	0·799 ± 0·003	3·6 ± 0·2	30·3 ± 2·9
Necturus ($N=11$)	0·187 ± 0·007	0·660 ± 0·007	0·847 ± 0·002	1·2 ± 0·1	24·3 ± 1·3
Xenopus ($N=14$)	0·154 ± 0·015	0·626 ± 0·011	0·780 ± 0·005	3·6 ± 0·2	23·7 ± 2·3
Xenopus (salt-stressed) ($N=4$)	0·264* ± 0·062	0·496* ±0·062	0·760 ±0·003	2·4* ± 0·5	25·9 ± 8·1

Values are means ± 1 S.E.M.
* Significantly different ($P \leq 0{\cdot}05$) from corresponding value for *Xenopus* in fresh water.

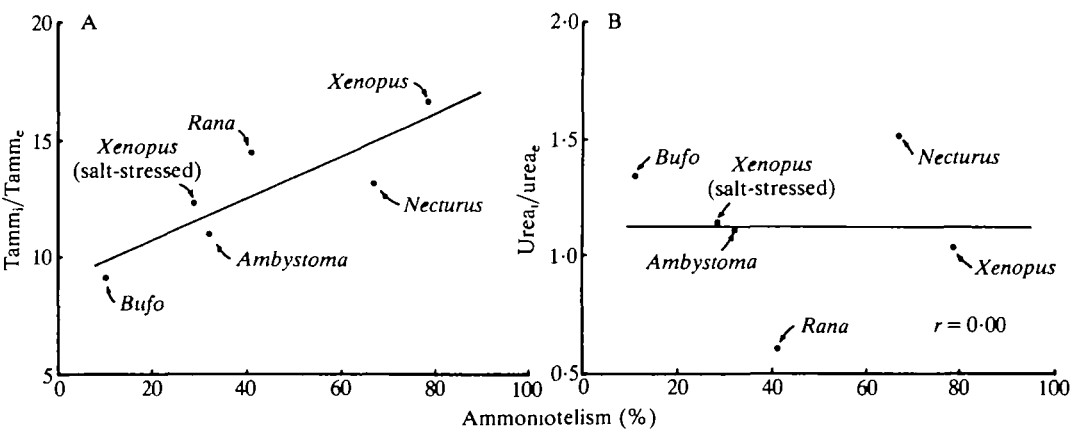

Fig. 2. The relationship between the mean distribution ratio for ammonia between intracellular and extracellular fluids of muscle ($Tamm_i/Tamm_e$) and the percentage ammoniotelism in five different amphibian species in fresh water, and in *Xenopus* subjected to salt stress. The fitted regression line is: $y = 0{\cdot}0899x + 8{\cdot}95$ ($r = 0{\cdot}87$; $P \leq 0{\cdot}05$). (B) The lack of relationship between the mean distribution ratio for urea between intracellular and extracellular fluids of muscle ($urea_i/urea_e$) and the percentage ammoniotelism in these same animals ($r = 0{\cdot}00$; $P > 0{\cdot}5$).

Table 3. *Total ammonia concentrations (Tamm) in arterial blood plasma (extracellular fluid) and skeletal muscle intracellular fluid, and their mean ratio, in five different amphibian species in fresh water, and in* Xenopus *subjected to salt stress*

	Plasma ECF $Tamm_e$ ($\mu mol\,l^{-1}$)	Muscle ICF $Tamm_i$ ($\mu mol\,l^{-1}$)	Ratio $Tamm_i/Tamm_e$
Bufo ($N = 12$)	111·2 ± 7·5	1015·2 ± 176·5	9·13
Ambystoma ($N = 6$)	114·6 ± 11·9	1253·9 ± 277·7	10·95
Rana ($N = 9$)	49·4 ± 5·4	720·9 ± 145·0	14·60
Necturus ($N = 11$)	147·3 ± 19·4	1934·2 ± 103·9	13·13
Xenopus ($N = 14$)	113·1 ± 20·6	1889·6 ± 373·9	16·71
Xenopus (salt-stressed) ($N = 4$)	369·1* ± 103·0	4549·3* ± 1409·8	12·33

Values are means ± 1 S.E.M.
* Significantly different ($P \leq 0{\cdot}05$) from corresponding value for *Xenopus* in fresh water.

subjected to salt stress, while the mean distribution ratio fell (Table 3; Fig. 2A), in accord with the switch to ureotelism (Fig. 1).

Plasma urea levels (see Table 4) were considerably higher than plasma ammonia levels (Table 3), the difference ranging from 12-fold in *Necturus* to 146-fold in *Ambystoma* on a nitrogen basis. There was much greater variation in urea levels than in ammonia levels amongst different species. The species with the highest absolute rate of urea output in fresh water (*Ambystoma*; Fig. 1) had by far the highest plasma concentration. In contrast to ammonia, intracellular urea concentrations were very similar to extracellular ones, yielding distribution ratios close to 1·0 (Table 4). Both extracellular and intracellular urea levels increased to a much greater extent (Table 4) than did $Tamm_e$ and $Tamm_i$ (Table 3) in salt-stressed *Xenopus*, but the $urea_i/urea_e$ distribution ratio remained unchanged (Table 4). There was no correlation between the urea distribution ratio and the percentage ammoniotelism in different species (Fig. 2B).

Discussion

Comparison with previous studies

No previous investigation has measured the same combination of parameters in this range of amphibian species, but some have been determined separately in earlier studies. The present data for arterial acid–base status (Table 1) are in close agreement with previous measurements at comparable temperature on *Buf*

Table 4. *Urea concentrations in arterial blood plasma (extracellular fluid) and skeletal muscle intracellular fluid, and their mean ratio, in five different amphibian species in fresh water, and in* Xenopus *subjected to salt stress*

	Plasma ECF urea ($mmol\,l^{-1}$)	Muscle ICF urea ($mmol\,l^{-1}$)	$urea_i/urea_e$
Bufo ($N = 12$)	3·14 ± 0·28	4·20 ± 0·51	1·34
Ambystoma ($N = 6$)	8·37 ± 0·99	9·26 ± 1·09	1·11
Rana ($N = 9$)	2·88 ± 0·85	1·73 ± 1·25	0·60
Necturus ($N = 11$)	0·89 ± 0·30	1·35 ± 0·29	1·52
Xenopus ($N = 14$)	2·15 ± 0·35	2·22 ± 0·32	1·03
Xenopus (salt-stressed) ($N = 4$)	17·27* ± 6·03	19·62* ± 6·10	1·14

Values are means ± 1 S.E.M.
* Significantly different ($P \leq 0{\cdot}05$) from corresponding value for *Xenopus* in fresh water.

marinus (Boutilier *et al.* 1979, 1987; Toews & Heisler, 1982), *Ambystoma tigrinum* (Stiffler *et al.* 1983), *Rana catesbeiana* (Malan *et al.* 1976; Lindinger *et al.* 1987), *Necturus maculosus* (Stiffler *et al.* 1983) and *Xenopus laevis* in fresh water (Boutilier, 1984; Boutilier *et al.* 1987). Earlier data on intracellular acid–base status are scant, but the present pHi values in skeletal muscle (Table 1) agree well with those reported by Malan *et al.* (1976) in *Rana catesbeiana* and by Boutilier *et al.* (1987) in *Bufo marinus* and *Xenopus laevis*. The pHi values of Toews & Heisler (1982) for *Bufo* muscle were somewhat lower.

Absolute rates of nitrogen excretion measured here (Fig. 1), and the associated percentage ammoniotelism, are in good agreement with previous reports on *Xenopus laevis* (Munro, 1953; Cragg *et al.* 1961; Janssens, 1964; Balinsky *et al.* 1967*a*,*b*; McBean & Goldstein, 1970*a*,*b*) and *Rana catesbeiana* (Smith, 1929; Yoshimura *et al.* 1961). Particularly notable is the fact that Smith (1929) found similarly high inter-individual variability in percentage ammoniotelism in *Rana catesbeiana*. This variability presumably reflects differing contributions from urinary excretion, where urea is dominant, and transcutaneous excretion, where ammonia is dominant (Lindinger & McDonald, 1986). The present data for *Necturus maculosus* (Fig. 1) are similar to those of Fanelli & Goldstein (1964) for this same species, though the percentage ammoniotelism (67%) is somewhat lower than the 90% reported by these authors. For the present data on *Ambystoma tigrinum* and *Bufo marinus* (Fig. 1), the closest available comparisons

are with earlier measurements of Munro (1953) and Cragg *et al.* (1961) on *Ambystoma mexicanum* and *Bufo bufo*; again agreement is good.

Many of the above-mentioned studies measured plasma ammonia and/or urea concentrations, though the animals were in various feeding states, a factor which is known to influence the blood levels of nitrogenous wastes (Balinsky, 1970). In general, the present plasma data from starved animals (Tables 3, 4) were similar to, but somewhat lower than, those reported previously. However, the only earlier information on tissue levels or ICF/ECF distribution ratios for Tamm or urea were a few isolated observations on *Xenopus laevis*, taken without benefit of freeze-clamping and/or ECF/ICF space estimates (Balinsky *et al.* 1961, 1967*b*; Janssens, 1964, 1972; Unsworth & Crook, 1967). Although highly variable, these data suggest $Tamm_i/Tamm_e$ ratios of 6–14 and $urea_i/urea_e$ ratios of 0·5–2·0, in reasonable agreement with the present observations (Fig. 2; Tables 3, 4).

Ammonia distribution and the evolution of ureotelism

The distribution ratios for urea between ICF and ECF were uniformly close to 1·0 in the five species surveyed and unaffected by the degree of ammoniotelism *versus* ureotelism (Table 4; Fig. 2B). This finding agrees with the original work of Conway & Kane (1934) on the distribution of this neutral molecule in frog sartorius muscle. They demonstrated that urea reaches a passive diffusive equilibrium with plasma levels throughout the tissue water compartment. Thus, the very different distribution of Tamm (Table 3; Fig. 2) between ICF and ECF is not a general property of nitrogenous wastes, but rather peculiar to ammonia.

Fig. 3 illustrates the theoretical relationship between $Tamm_i/Tamm_e$ and the relative permeability of the muscle cell membranes to NH_3 and NH_4^+ (pNH_3/pNH_4^+), as generated by equation 3. For the purposes of this analysis, nominal, fixed values of pHi−pHe of 0·5 units and transmembrane potential (E_M) of −90 mV, chosen for amphibians (Katz, 1966), have been employed. Although these appear to be broadly representative of vertebrates in general, values in individual species may vary somewhat, so the relationship should be viewed as a general rather than exact one. At typical values (about 30) of $Tamm_i/Tamm_e$ for teleost fish (Wright *et al.* 1988; Wright & Wood, 1988), the pNH_3/pNH_4^+ ratio is relatively low, so distribution is entirely determined by the membrane potential (actually closer to −80 mV than −90 mV in teleost fish). Note, however, that absolute pNH_3 could still be up to 20-fold greater than absolute pNH_4^+ (Table 5). At typical values (about 3) of $Tamm_i/Tamm_e$ for ureotelic mammals (Stabenau *et al.* 1959; Visek, 1968; Meyer *et al.* 1980; Mutch & Banister, 1983), the pNH_3/pNH_4^+ ratio is much higher (>300; Fig. 3; Table 5), so distribution is entirely determined by the pHi−pHe gradient.

The typical values (9·1–16·7) of $Tamm_i/Tamm_e$ measured for amphibians in the present study (Table 3) lie midway between those for ammoniotelic teleost fish and ureotelic mammals (Fig. 3). The equilibrium distributions are therefore influenced by both the membrane potential and the pHi−pHe gradient, rather

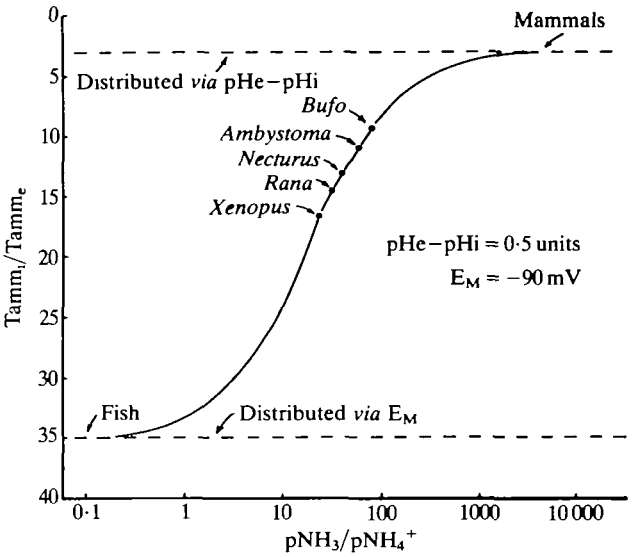

Fig. 3. The relationship between the distribution of ammonia at equilibrium between intracellular and extracellular compartments of muscle ($Tamm_i/Tamm_e$) and the relative permeability (pNH_3/pNH_4^+) of the cell membrane to NH_3 and NH_4^+ (see equation 3). A membrane potential (E_M) of −90 mV and a pHi−pHe gradient of 0·5 units have been assumed. Measured $Tamm_i/Tamm_e$ values from the present study for five different amphibian species in fresh water have been plotted on this general relationship, as well as the ranges measured in previous studies for teleost fish and mammals. See text for additional details.

Table 5. *The relative permeability (pNH_3/pNH_4^+) of the muscle cell membranes to NH_3 and NH_4^+ in five different amphibian species in fresh water, and in* Xenopus *subjected to salt stress*

	pNH_3/pNH_4^+
Bufo	73·8
Ambystoma	36·8
Rana	45·1
Necturus	31·7
Xenopus	39·4
Xenopus (salt-stressed)	43·4
Fish	<20
Mammals	>300

The estimates of pNH_3/pNH_4^+ were calculated from equation 3 using the measured values of $Tamm_i/Tamm_e$ from Table 3 and the measured values of pHi and pHe from Table 1 and an assumed E_M of −90 mV.

Ranges for teleost fish and mammals are included for comparison.

than being an exclusive function of either. These data, therefore, support the original hypothesis of an evolutionary transition in ammonia distribution accompanying the progression from ammoniotelism to ureotelism. Indeed, there is a direct correlation between the distribution ratio and the percentage ammoniotelism in the five species surveyed (Fig. 2A). If a uniform E_M of −90 mV (Katz, 1966) is assumed in each species, then the relative permeability of the muscle cell membranes to NH_3 and NH_4^+ (pNH_3/pNH_4^+) can be calculated from equation 3, using these measured $Tamm_i/Tamm_e$ ratios. This analysis yields values of pNH_3/pNH_4^+ in the range 30–70, clearly different from those of both teleost fish and mammals (Table 5). Although there is a general tendency for the more ureotelic species to have the higher values, as predicted by theory, this is not as clearcut as the trend in $Tamm_i/Tamm_e$ (Fig. 2A). In other words, the calculated pNH_3/pNH_4^+ ratios do not fall in the same rank order as the species' relative percentage ammoniotelism, as suggested by Fig. 3. This is due to the large differences in pHi−pHe gradients amongst the various species (Table 1). It is possible that the relationship would improve if the real values for E_M were known for each species; alternatively, of course, it is equally possible that the relationship might deteriorate.

In consequence of the fact that there is a significant cell membrane permeability to NH_4^+ in amphibians, and therefore a higher intracellular Tamm than predicted by the pHi−pHe gradient, substantial standing diffusion gradients for P_{NH_3} exist between ICF and ECF (Fig. 4). However, in the absence of ammonia production or consumption within the cell, this is an equilibrium situation so there is no net loss of Tamm, despite the resulting NH_3 diffusion from ICF to ECF. As quickly as NH_3 diffuses out of the cell along its P_{NH_3} gradient, NH_4^+ diffuses into the cell along its electrical gradient. As NH_4^+ enters, it immediately dissociates (reaction half time <50 ms, Stumm & Morgan, 1981) to NH_3 and H^+, because H^+ is maintained out of electrochemical equilibrium by active H^+ extrusion from the cell (Roos & Boron, 1981). If this were not the case, then at $E_M = -90$ mV and pHe = 7·8, pHi would be about 6·2 rather than the measured values close to 7·2. Thus, hydrogen ions are shuttled actively and NH_3/NH_4^+ molecules are shuttled passively in a futile cycle from ICF to ECF to ICF. The net effect is simply much higher intracellular Tamm levels than would occur if pNH_3/pNH_4^+ were higher and Tamm were distributed according to the pHi−pHe gradient.

Whether this situation is advantageous under steady-state conditions is unclear, though two possible benefits are a metabolic role or a buffering role for the higher intracellular Tamm. Elevated intracellular NH_3 production during exercise (Meyer *et al.* 1980; Dobson & Hochachka, 1987) or hypoxia (Lindinger *et al.* 1987) will undoubtedly minimize glycolytic acidosis. Both NH_3 and NH_4^+ will pass out across muscle cell membranes, the latter serving to raise pHi. Similar movements of NH_3 and NH_4^+ may occur across gills, skin and kidney. With the evolution to ureotelism, glutamine replaces ammonia as the major nitrogen carrier between tissues (Mommsen & Walsh, 1989), and much less ammonia is moved across excretory organs. The accompanying increase in cell membrane pNH_3/pNH_4^+

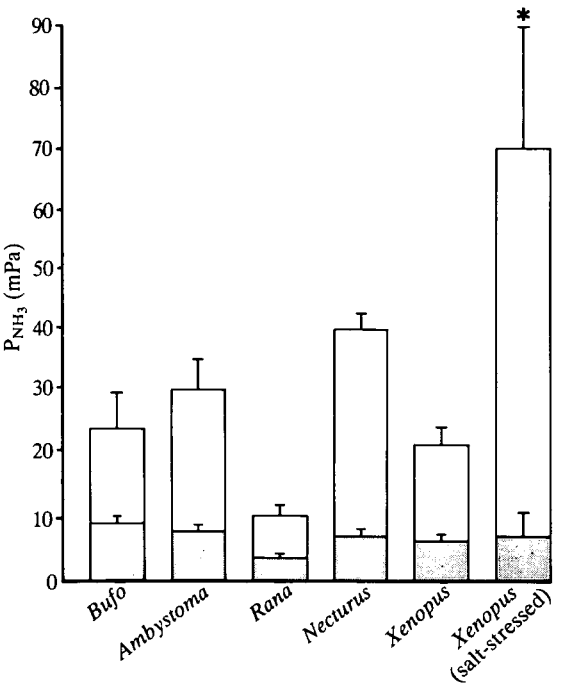

Fig. 4. P_{NH_3} level in muscle (open bars) and blood plasma (shaded bars) of five different amphibian species in fresh water, and in *Xenopus* subjected to salt stress. Note the large standing P_{NH_3} gradients from ICF to ECF. Means ± 1 S.E.M. *Bufo* ($N = 12$), *Ambystoma* ($N = 6$), *Rana* ($N = 9$), *Necturus* ($N = 11$), freshwater *Xenopus* ($N = 14$), salt-stressed *Xenopus* ($N = 4$). * indicates significant difference ($P \leq 0{\cdot}05$) from corresponding value for freshwater *Xenopus*. (10 mPa = 75 μTorr.)

(Figs 2A, 4; Table 5) makes sense, as NH_4^+ permeability is no longer required, and the resulting fall in $Tamm_i/Tamm_e$ considerably reduces the body store of ammonia.

A change in the simple diffusive permeability of the lipid bilayers to NH_4^+ would be the simplest explanation, but it is by no means the only one. Wright *et al.* (1988) have reviewed the many pathways by which NH_4^+ may traverse cell membranes, including simple diffusion, movement through cation-selective channels, substitution for K^+ in such channels or in selective K^+ transport mechanisms, or substitution for either Na^+ or H^+ in Na^+/H^+ exchange. Any or all of these mechanisms could be altered coincident with the evolutionary change from ammoniotelism to ureotelism. It seems possible that the higher plasma NH_4^+ levels in ammoniotelic animals might result in a greater incidence of NH_4^+ transport across cell membranes. However, we are aware of no quantitative data on the relative incidence of any of these processes in different vertebrate classes, or even in different amphibian species. There is clearly a need for research in this area.

Ammonia distribution and the induction of ureotelism in Xenopus

Exposure to saline conditions (400 mosmol kg^{-1} NaCl) induced a switch to ureotelism as the major pathway of nitrogen excretion in *Xenopus* (Fig. 1), in accord with many previous studies (e.g. Balinsky *et al.* 1961; Janssens, 1964; Janssens & Cohen, 1968; McBean & Goldstein, 1970*a*,*b*). It has been argued that the main adaptive significance of this response is the accumulation of a less toxic nitrogenous endproduct in a situation where water is unavailable to form the voluminous urine which normally flushes out the more toxic ammonia (Balinsky, 1970). However, in the present study, ammonia-N excretion did not decrease during salt stress, yet urea-N output increased 10-fold (Fig. 1B). Furthermore, ammonia-N and urea-N concentrations both increased in plasma and tissues, though the latter to a much greater extent (see Tables 3, 4). Similar results were obtained by McBean & Goldstein (1970*a*,*b*), who also noted that urine flow continued at 30 % of normal levels under a slightly lower salt stress (300 mosmol kg^{-1}). We therefore favour their explanation that the elevated urea serves primarily as an osmotic filler, thereby reducing the need for inorganic ion accumulation, and ensuring that some water remains available to sustain a minimal urine production. Nevertheless, the greatly elevated amino acid catabolism presumably associated with this elevated urea production and excretion must place an enormous metabolic load on the animal.

Salt stress also induced a marked metabolic acidosis, lowering plasma HCO_3^- by 60 % (Table 1). Although acid–base status has not been monitored in the many previous studies on saline-exposed *Xenopus*, a similar phenomenon was reported by Katz (1981) in the euryhaline toad, *Bufo viridis*. Katz attributed the response to a blockade of H^+ extrusion through the Na^+/H^+ exchange mechanism in the skin. Alternative possibilities include a differential invasion of the extracellular fluids by the strong anion Cl^- (relative to Na^+), as occurs in stenohaline teleost fish during saline exposure (Wilkes & McMahon, 1986), or HCO_3^- removal *via* accelerated ureagenesis (Atkinson & Bourke, 1987). The theory behind the latter possibility remains controversial (e.g. Halperin *et al.* 1986; Walser, 1986). Whatever the cause, this acidosis was not transferred to the intracellular fluids of muscle (Table 1), indicating the primacy of cellular acid–base regulation.

The observed fall in the $Tamm_i/Tamm_e$ distribution ratio associated with salt stress in *Xenopus* (Table 3; Fig. 2A) suggests that the pNH_3/pNH_4^+ permeability ratio changed in the predicted fashion with increasing ureotelism (i.e. that it is sensitive to physiological adjustment within an animal). However, upon closer analysis, this becomes uncertain. The $Tamm_i/Tamm_e$ ratio represents an equilibrium resulting from the combined influences of the transmembrane pH and electrical gradients (see equation 3). During salt stress, the remarkable abolition of the pHi–pHe gradient (Table 1) was sufficient to account for the fall in $Tamm_i/Tamm_e$ without significant change in the calculated pNH_3/pNH_4^+ ratio (Table 5). Inasmuch as the ammonia-N output rate remained unchanged (Fig. 1B), this result is not surprising, for the requirement for ammonia movement across cell membranes was not reduced. Thus, the present data provide n

evidence that the pNH_3/pNH_4^+ ratio is under physiological control, only under evolutionary control with the transition from ammoniotelism to ureotelism. However, it may be worth assessing this same question in amphibians as they undergo metamorphosis, for at this time there are absolute reductions in ammonia-N output as well as increases in urea-N excretion (Munro, 1953; Balinsky *et al.* 1967*a*,*b*; Balinsky, 1970).

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