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EFFECT OF TEMPERATURE ON pH AND ELECTROLYTE CONCENTRATION IN AIR-BREATHING ECTOTHERMS

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Summary

The aim of this study was to determine the effects of temperature upon pH, protein charge and acid–base-relevant ion exchange in air-breathing ectotherms. Plasma and skeletal muscles in cane toads (*Bufo marinus*) and bullfrogs (*Rana catesbeiana*) were examined at 30, 20 and 10 °C. In addition, skeletal muscle ion concentrations were examined in black racer snakes (*Coluber constrictor*) at 30 and 10 °C. Cooling the amphibians produced a reduction in most of the plasma ion concentrations (Na^+, K^+, Ca^{2+}, Cl^-, SO_4^{2-}) and in protein concentration because of increased hydration. Between 30 and 10 °C, total plasma osmolality fell by 14 % in the toads and by 5 % in the frogs. Plasma protein charge, calculated using the principle of electroneutrality, was unaffected by temperature, except possibly for the toads at 10 °C. The *in vivo* skeletal muscle $\Delta pHi/\Delta T$ ratio, where pHi is intracellular pH and T is temperature, between 30 and 20 °C averaged $-0.014\,°C^{-1}$ in the toads and $-0.019\,°C^{-1}$ in the frogs. Between 20 and 10 °C, there was no change in pHi in the toads and a $-0.005\,°C^{-1}$ change in the frogs. The *in vitro* skeletal muscle $\Delta pHi/\Delta T$ averaged $-0.011\,°C^{-1}$ in both toads and frogs. In all three species, skeletal muscle inulin space declined with cooling. Intracellular ion concentrations were calculated by subtracting extracellular fluid ion concentrations from whole-muscle ion concentrations. In general, temperature had a large effect upon intracellular ion concentrations (Na^+, K^+, Cl^-) and intracellular CO_2 levels. The relevance of the changes in intracellular ion concentration to skeletal muscle acid–base status and protein charge and the possible mechanisms producing the adjustments in intracellular ion concentration are discussed. It is concluded that ion-exchange mechanisms make an important contribution to adjusting pH with changes in temperature.

Key words: *Bufo marinus*, *Rana catesbeiana*, *Coluber constrictor*, reptile, amphibian, temperature, acid–base physiology, pH, protein charge, electrolyte, skeletal muscle, blood.

Introduction

Studies of acid–base status as a function of temperature in ectotherms have been of interest since the seminal work by Stadie et al. (1925) and Austin et al. (1927). These early studies established that, in sealed blood samples, the principal factor determining the change in plasma pH (pH_{pl}) with changing temperature ($\Delta pH_{pl}/\Delta T$) is the change in ionization constant of the plasma proteins ($\Delta pK_{pro}/\Delta T$). In addition, from the buffer equation of Van Slyke, it was recognized that as long as an animal maintained a constant $pH_{pl}-pK_{pro}$ with changes in body temperature, then protein charge was conserved. Austin et al. (1927) sampled blood from alligators at 9 and 35 °C and concluded that the animals regulated pH *in vivo* to maintain a constant plasma protein charge. Subsequent studies extended this early work to other reptiles, amphibians, fish and invertebrates (for reviews, see Heisler, 1986b; Reeves, 1977). Reeves (1972) focused attention upon the imidazole of histidine as the principal ionizing group of proteins.

The same model of constant protein charge and variable pH was first applied to the intracellular compartment by Malan et al. (1976). They concluded that intracellular pH (pHi) was regulated to preserve a constant $pHi-pK_{pro}$ over a wide temperature range just as in the extracellular fluid. By preserving protein charge over a wide temperature range, ectotherms supposedly prevent changes in enzyme activity as well as shifts in ions and water. However, as more studies of individual species became available, it was clear that there are considerable differences in the magnitude of the $\Delta pH/\Delta T$ relationship among species, between tissues within an individual animal and over the temperature range of an animal (Whiteley et al., 1995; Lutz et al., 1989; Rocha and Branco, 1998; Butler and Day, 1993; Lehoux and Guderley, 1997; Boutilier et al., 1987; Stinner and Wardle, 1988). While some recent studies conclude that protein (i.e. imidazole) charge is independent of temperature (Van Dijk et al., 1997; Hitzig et al., 1994; Rodeau, 1984; Graber et al., 1992; Gaillard and Malan, 1985; Johnson et al., 1993; Douse and Mitchell, 1991;

Ultsch and Jackson, 1996), no unifying theory of acid–base regulation in animals has yet been formulated.

Another concept, in addition to regulating pH to maintain a constant protein charge, emerging from the studies of temperature and acid–base status in ectotherms is that water-breathers use primarily ion-exchange mechanisms to adjust pH whereas air-breathers use ventilation to adjust pH (Reeves, 1977; Cameron, 1984). The low solubility of oxygen in water relative to air imposes constraints on reductions in ventilation in water-breathers. In air-breathers, the decrease in pH that occurs with an increase in temperature is associated with substantial reductions in minute ventilation relative to metabolic rate ($\dot{V}_{min}/\dot{V}_{CO_2}$). This allows for the rise in P_{CO_2}, which is caused by passive temperature-induced decreases in the solubility coefficient and pK of the CO_2/HCO_3^- buffer system, with virtually no change in CO_2 content. In water-breathers such as fish, however, $\dot{V}_{min}/\dot{V}_{CO_2}$ is relatively independent of temperature so that, with transepithelial and transmembrane ion exchange, P_{CO_2} is nearly constant and $[HCO_3^-]$ varies inversely with temperature. The putative advantage of using $\dot{V}_{min}/\dot{V}_{CO_2}$ to adjust pH in air-breathers is that it can occur almost instantaneously with the change in body temperature, whereas ion-exchange mechanisms take many hours to reach a new steady-state pH. Hence, it would appear that air-breathers are better able to avoid disruption of pH-dependent biochemical homeostasis, including enzyme activity, with changes in temperature.

However, an exception to this view of air-breathers rapidly adjusting acid–base status with changes in body temperature is the black racer snake *Coluber constrictor* (Stinner et al., 1996; Stinner and Wardle, 1988). When the snakes were cooled from 30 to 10 °C and from 30 to 5 °C, a steady-state plasma pH was not reached for approximately 35 h and 60 h respectively. There were also gradual increases in $[CO_2]_{pl}$. In a more recent study, Stinner et al. (1998) concluded that these slow acid–base changes resulted from a gradual decline in $\dot{V}_{min}/\dot{V}_{CO_2}$ with concomitant CO_2 retention. CO_2 retention also occurred in skeletal muscle, and the $\Delta pHi/\Delta T$ ratio of skeletal muscle was consequently much lower than the estimated $\Delta pK_{pro}/\Delta T$ ratio. Stinner et al. (1998) found no evidence for strong ion shifts, and hence ion-exchange mechanisms, adjusting pH_{pl} with the change in temperature in the snakes. The authors also concluded that acid–base-relevant ion exchange did not occur in the muscle cells, but this was based upon measurements of strong ion levels in whole muscle rather than in intracellular fluid. The possibility of temperature affecting skeletal muscle extracellular fluid space, as observed in the cane toad *Bufo marinus* (Stinner et al., 1994), weakened this conclusion.

In their study of *B. marinus* and the African clawed frog *Xenopis laevis*, Boutilier et al. (1987) reported large differences in the $\Delta pHi/\Delta T$ relationship among skeletal muscles and concluded that ion-exchange mechanisms must be operating. However, they did not examine muscle cell strong ion concentrations. It is possible that their findings resulted from differences in passive $\Delta pK_{pro}/\Delta T$ resulting from different protein compositions among skeletal muscles (Cameron, 1989). The purpose of the present study was to determine acid–base-relevant intracellular ion concentrations and protein charge as a function of temperature in *C. constrictor*, *B. marinus* and the bullfrog *R. catesbeiana*. We used the principle of electroneutrality (the sum of the cations in mequivalents equals the sum of the anions in mequivalents) to estimate plasma protein charge and compared this with the *in vivo* and *in vitro* $\Delta pH_{pl}/\Delta T$ relationships. We also examined skeletal muscle intracellular ion concentrations and their relationship to the *in vivo* and *in vitro* $\Delta pHi/\Delta T$ ratios. The two amphibian species played prominent roles in the development of the commonly held view that air-breathing ectotherms maintain a constant protein charge over a wide temperature range by passive shifts in the buffer equilibria and active adjustment of $\dot{V}_{min}/\dot{V}_{CO_2}$ (Reeves, 1972, 1977).

Materials and methods

Animals

Cane toads (*Bufo marinus*) (mean mass 177.1±61.6 g, *N*=181), bullfrogs (*Rana catesbeiana*) (mean mass 307.4±97.9 g, *N*=136) and black racer snakes (*Coluber constrictor*) (mean mass 212.9±56.7 g, *N*=20) were purchased from commercial suppliers. They were housed in the animal facility at The University of Akron, where room temperature was maintained at 25–30 °C. The cages for the cane toads and black racer snakes contained light bulbs at one end that provided basking sites at 35–40 °C. The lights were set to a 12 h:12 h L:D cycle. Approximately once weekly, the animals were fed on small rats. The animals were starved for 1 week prior to experimentation to avoid acid–base and electrolyte changes associated with the digestion and absorption of food (Coulson and Hernandez, 1964).

Surgery, experimental apparatus and blood analyses

The sciatic artery of cane toads and bullfrogs was occlusively cannulated (PE 50). The toads were anesthetized by exposure in a sealed chamber to cotton soaked in isoflurane. The bullfrogs were anesthetized in a 1 % solution of MS-222 buffered to a pH 7.3–7.4 by addition of 25 ml l^{-1} of 0.5 mol l^{-1} Na_2HPO_4. After a post-operative recovery period of at least 24 h, the toads and frogs were placed inside individual chambers within a darkened Precision (model 815) temperature-controlled cabinet (±0.5 °C). The individual animal chambers measured 32 cm×22 cm×17 cm and were made of an opaque plastic. Each chamber contained 1–1.5 l of water and was tipped at an angle to provide a dry area.

Pumps inside the cabinets continuously circulated water-saturated cabinet air through the animal chambers. At 20 and 30 °C, the chamber water was siphoned off and replaced with fresh temperature-equilibrated tapwater at least once every 48 h. For blood sampling purposes, the trailing end of the cannula was passed through a hole in the chamber lid and tied to a stick which prevented the end of the cannula from being pulled inside the chamber. The cannulas were flushed once every 2–3 days with heparinized (20 i.u. ml^{-1}) amphibian

Ringer's solution (2.15 mmol l^{-1} KCl, 2.0 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 68.1 mmol l^{-1} NaCl, 3.89 mmol l^{-1} Na_2HPO_4, 15.0 mmol l^{-1} $NaHCO_3$, 1.2 mmol l^{-1} $MgSO_4 \cdot 7H_2O$).

Prior to opening the temperature cabinet door for blood sampling, the laboratory lights were turned off to minimize disturbance to the animals. For pH measurements, blood was sampled directly into heparinized microcapillary hematocrit tubes, which were then quickly immersed in iced water. pH was measured using temperature-equilibrated Radiometer BMS-3 electrodes, as described previously (Stinner et al., 1998). The blood samples were centrifuged at 15 000 ***g*** for 5 min in a microcapillary centrifuge, and each plasma sample was then measured at 10 and 30 °C for determination of the *in vitro* $\Delta pH_{pl}/\Delta T$ relationship. For ion and water analyses, 0.5 ml of blood was collected and immediately centrifuged at 2000 ***g*** for 5 min in an Eppendorf microcentrifuge. The plasma was sealed in a microcentrifuge tube and frozen until analysis. $[Na^+]$, $[K^+]$, $[Cl^-]$, $[Ca^{2+}]$, $[Mg^{2+}]$, [inorganic phosphate], [protein], $[SO_4^{2-}]$ and osmolality were determined as described previously (Stinner et al., 1998). Water content was determined by drying weighed plasma samples (100–200 µl) overnight at 60 °C. Animals were sampled for blood at one temperature only. Animals were kept for at least 48 h at 30 °C, 72 h at 20 °C and 96 h at 10 °C. None was kept for more than 1 week in the experimental apparatus before blood sampling.

Skeletal muscle

The experimental arrangement and temperature exposure times were the same as described above. Animals were quickly decapitated, and $[CO_2]$ was then determined on muscle samples weighing 0.18–0.22 g, after overnight soaking in 0.1 mol l^{-1} NaOH using a Capni-con 3a CO_2 analyzer, as described previously (Stinner et al., 1994, 1998). Each muscle was sampled twice, and each sample was measured in duplicate for $[CO_2]$. To minimize the time between decapitation and sealing the samples in vials, no more than five muscles were sampled from each animal. Muscle $[CO_2]$ values for *B. marinus* at 10 °C were taken from an earlier study (Stinner et al., 1994). For $[Na^+]$, $[K^+]$ and $[Cl^-]$ determinations, additional muscle samples weighing approximately 0.25 g were harvested and soaked in 4 ml of 0.1 mol l^{-1} HNO_3 for 24 h with gentle shaking (Boutilier et al., 1986). Each muscle was sampled twice. $[Na^+]$ and $[K^+]$ were measured in duplicate using flame photometry (Coleman model 51; Bacharach, Inc., Pittsburgh, PA, USA). $[Cl^-]$ was measured (4–6 replicates) using a Digital chloridometer (Buchler Instruments, Lenexa, KS, USA). For water contents, duplicate muscle samples weighing 0.2–0.4 g were dried overnight to a constant mass at 60 °C. In those cases where previously cannulated animals were used, care was taken to sample muscles from the uncannulated leg.

Skeletal muscle pHi was determined using the homogenate technique of Pörtner et al. (1990). Details of this procedure in our laboratory have been described previously by Stinner et al. (1998). Prior to muscle sampling, the toads were anesthetized by exposure to cotton soaked in isoflurane after siphoning off the chamber water and turning the air pump off. The bullfrogs were anesthetized by siphoning off the chamber water and replacing it with 1 l of temperature-equilibrated 1 % MS-222 solution buffered to pH 7.3–7.4. Anesthesia took 15–20 min. No more than five muscles were sampled from each animal. Each muscle was sampled twice, and each sample was used to prepare two tubes of muscle powder plus inhibitor solution. For animals held at 10 °C, the supernatant pH of one tube was measured at 10 °C, and the supernatant pH of the other tube was measured at 30 °C. The difference in pH was used to determine the passive component of the temperature-dependent adjustment of pHi (i.e. $\Delta pK_{pro}/\Delta T$). For animals held at 20 and 30 °C, both samples taken from the same ground muscle were measured at the animal's body temperature and the values were averaged.

Extracellular fluid volume was determined using [^{3}H]inulin. The sciatic artery of the toads and frogs was occlusively cannulated. A posterior branch of the dorsal aorta of the black racer snakes was occlusively cannulated as described previously (Stinner et al., 1996). After a recovery period of at least 24 h, followed by exposure to 10, 20 or 30 °C (see above), 148 kBq 100 g^{-1} of [^{3}H]inulin followed by approximately 0.5 ml of amphibian Ringer's solution or reptilian Ringer's solution (3.8 mmol l^{-1} KCl, 2.9 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 128.0 mmol l^{-1} NaCl, 1.55 mmol l^{-1} Na_2HPO_4, 24.0 mmol l^{-1} $NaHCO_3$, 1.2 mmol l^{-1} $MgSO_4 \cdot 7H_2O$) were injected through the cannula. After 12–15 h, the toads and snakes were anesthetized with cotton soaked in isoflurane and the bullfrogs were anesthetized with MS-222 (see above). An incision was made through the ventral body wall, and 1.0 ml of blood was taken by heart puncture for six plasma sample (approximately 0.1 g) isotope analyses by liquid scintillation counting (Beckman model LS-6500, Beckman Instruments, Fullerton, CA, USA). Immediately after blood collection, five samples (approximately 0.2 g) from each skeletal muscle were placed inside scintillation vials and dissolved in 2 ml of Solvable at 60 °C, with occasional swirling, for approximately 4 h (Packard Instrument Co., Meriden, CT, USA). The dissolved muscle samples were allowed to cool to room temperature, and 10 ml of Ultima Gold (Packard Instrument Co.) was added for determination of radioactivity in the liquid scintillation counter. Inulin space and plasma concentrations of ions and water were used to correct whole-muscle ion and water concentrations to intracellular values (Stinner et al., 1994).

Results are presented as means ± S.D. (*N*).

Results

The body temperatures of the cane toads and bullfrogs, measured with a Schulteis quick-reading thermometer inserted into the cloaca, were 10.1±0.8 °C (*N*=44), 20.0±0.8 °C (*N*=62) and 30.2±1.0 °C (*N*=57). The body temperature of the black racer snakes was assumed to be the same as the cabinet temperature.

Plasma

In vitro $\Delta pH_{pl}/\Delta T$ measured in 10 *B. marinus* was

$-0.014\pm0.002\,°C^{-1}$. The *in vitro* $\Delta pH_{pl}/\Delta T$ coefficient varied with plasma protein concentration according to the linear regression $\Delta pH_{pl}/\Delta T=-7.95\times10^{-3}-2.43\times10^{-4}$[protein], where protein concentration is in $g\,l^{-1}$ ($P<0.01$). Cooling caused dilution of the plasma in the cane toads (Table 1). Although not significantly different, water content was lowest at 30 °C, and plasma osmolality fell significantly by about 7 % for each 10 °C drop in temperature. This dilution is evident for most of the individual ions (Na^+, K^+, Ca^{2+}, Cl^-, SO_4^{2-}) and for protein. Concentrations of three ions did not decrease with a reduction in temperature, Mg^{2+}, HCO_3^- and phosphate. Plasma $[HCO_3^-]$ was estimated by subtracting dissolved CO_2 (i.e. αP_{CO_2}) from total $[CO_2]$. Total $[CO_2]$ and P_{CO_2} were taken from Stinner et al. (1994) and Howell et al. (1970), respectively, and α was calculated using the equation of Boutilier (Heisler, 1986a). Protein charge was calculated from the principle of electroneutrality as (the sum of the concentrations of Na^+, K^+, $2\times Ca^{2+}$ and $2\times Mg^{2+}$) minus (the sum of the concentrations of Cl^-, lactate, $2\times SO_4^{2-}$, $2\times HPO_4^{2-}$ and $H_2PO_4^-$). [Lactate] was taken from Stinner et al. (1994). $[HPO_4^{2-}]$ and $[H_2PO_4^-]$ were estimated from the Henderson–Hasselbalch equation, using pH values from Howell et al. (1970) and a pK of 6.791 at 30 °C (Siggaard-Anderson, 1974). The van't Hoff equation, $pK-6.791=(\Delta H°/4.576)[(1/T)-(1/310)]$, was used to correct pK to 30, 20 and 10 °C, where $\Delta H°$ (the enthalpy change) is $982\,cal\,mol^{-1}$ (Edsall and Wyman, 1958). Plasma protein charge was the same ($-0.31\,mequiv\,g^{-1}$ protein) at 30 and 20 °C, but was higher ($-0.37\,mequiv\,g^{-1}$ protein) at 10 °C. This increase in protein charge was the result of a 7 % decrease in protein concentration between 20 and 10 °C, while the nonprotein anion gap (defined as the sum of the cations in mequivalents minus the sum of the nonprotein anions in mequivalents) remained unchanged. Simple dilution of the plasma should have decreased the nonprotein anion gap. However, the decline in the nonprotein anion concentration was relatively large (10.7 %), exceeding the decrease in cation concentration, suggesting a net active removal of anions (e.g. Cl^-) from the extracellular fluid.

In the bullfrogs, there was little or no difference in plasma ion concentrations and osmolality at 30 and 20 °C (Table 2). However, the values were approximately 5 % lower at 10 °C, indicative of some dilution by water, although $[Mg^{2+}]$ and [phosphate] were significantly higher at 10 °C. $[HCO_3^-]$ was calculated, as described above, using total $[CO_2]$ and P_{CO_2} taken from Mackenzie and Jackson (1978). Plasma protein charge, calculated as described above using a lactate

Table 1. *Systemic arterial plasma electrolyte levels and water content in the cane toad* Bufo marinus

Variable	30 °C	20 °C	10 °C
$[Na^+]$ ($mmol\,l^{-1}$)	113.7±6.8‡ (28)	107.9±6.8 (15)	98.3±7.0*,‡ (29)
$[K^+]$ ($mmol\,l^{-1}$)	2.9±0.2‡ (15)	2.4±0.4 (15)	2.3±0.5* (15)
$[Ca^{2+}]$ ($mmol\,l^{-1}$)	2.0±0.4 (15)	2.0±0.4 (14)	1.7±0.4* (15)
$[Mg^{2+}]$ ($mmol\,l^{-1}$)	0.6±0.2 (18)	0.6±0.2 (13)	0.6±0.1 (16)
$[Cl^-]$ ($mmol\,l^{-1}$)	80.8±5.2‡ (28)	76.1±4.9 (15)	66.1±5.7*,‡ (28)
$[HCO_3^-]$ ($mmol\,l^{-1}$)	23.1	24.1	23.6
[Phosphate] ($mmol\,l^{-1}$)	0.9±0.1 (15)	1.2±0.6 (13)	1.0±0.2 (15)
$[SO_4^{2-}]$ ($mmol\,l^{-1}$)	2.8±0.8‡ (12)	1.9±0.7 (12)	1.6±0.7* (15)
[Protein] ($g\,l^{-1}$)	31.7±7.0 (28)	28.5±6.9 (15)	26.4±8.3* (29)
Protein charge ($mequiv\,g^{-1}$)	–0.31	–0.30	–0.37
Osmolality ($mmol\,kg^{-1}$)	253.8±12.0‡ (25)	234.3±10.2 (13)	219.4±8.7*,‡ (26)
H_2O (%)	95.9±1.0 (15)	96.7±1.5 (10)	96.6±1.3 (18)

Values are means ± S.D.; number in parentheses is the numbers of animals used.

* indicates a value significantly different ($P<0.05$) from that at 30 °C; ‡ indicates a value significantly different ($P<0.05$) from that at 20 °C.

Comparisons between values were performed using Student's *t*-test.

$[HCO_3^-]$ was calculated by subtracting the dissolved $[CO_2]$, i.e. αP_{CO_2} from the total $[CO_2]$.

Protein charge was calculated by subtracting the sum of the anions (in mequiv) from the sum of the cations (in mequiv).

See text for additional explanation.

Table 2. *Systemic arterial plasma electrolyte levels in the bullfrog* Rana catesbeiana

Variable	30 °C	20 °C	10 °C
$[Na^+]$ ($mmol\,l^{-1}$)	99.7±5.0	100.2±5.1	95.6±4.2*,‡
$[K^+]$ ($mmol\,l^{-1}$)	2.5±0.3	2.5±0.6	1.7±0.2*,‡
$[Ca^{2+}]$ ($mmol\,l^{-1}$)	2.0±0.4	1.9±0.4	1.7±0.3*
$[Mg^{2+}]$ ($mmol\,l^{-1}$)	0.7±0.1	0.7±0.1	0.8±0.1*
$[Cl^-]$ ($mmol\,l^{-1}$)	58.5±4.9	58.5±6.5	56.5±5.0
$[HCO_3^-]$ ($mmol\,l^{-1}$)	25.9	27.1	24.6
[Phosphate] ($mmol\,l^{-1}$)	1.3±0.3	1.3±0.2	1.6±0.3*,‡
$[SO_4^{2-}]$ ($mmol\,l^{-1}$)	0.9±0.2‡	0.7±0.2	0.6±0.2*
[Protein] ($g\,l^{-1}$)	36.8±10.6	35.4±9.8	33.6±5.7
Protein charge ($mequiv\,g^{-1}$)	–0.48	–0.49	–0.49
Osmolality ($mmol\,kg^{-1}$)	214.6±7.5	211.0±7.1	202.9±9.1*,‡

Values are means ± S.D.

Symbols and statistical treatment are as in Table 1.

15 animals were sampled at 30 °C; 13 at 20 °C and 14 at 10 °C.

$[HCO_3^-]$ and protein charge were calculated as for Table 1.

Table 3. *Skeletal muscle pH and* in vitro *ΔpH/Δ*T *in the cane toad* Bufo marinus

Muscle	pH 30 °C	pH 20 °C	pH 10 °C	*In vitro* ΔpH/Δ*T* (°C^{-1})
Submaxillary	6.973±0.097‡ (13)	7.143±0.083 (10)	7.242±0.079*,‡ (16)	–0.011±0.003 (8)
Obliques	6.993±0.114‡ (16)	7.235±0.073 (9)	7.170±0.133* (17)	–0.008±0.003 (10)
Rectus abdominis	6.988±0.092‡ (10)	7.237±0.051 (10)	7.140±0.091* (14)	–0.012±0.003 (8)
Gastrocnemius	7.112±0.064‡ (21)	7.205±0.041 (9)	7.193±0.107* (20)	–0.012±0.003 (9)
Triceps femoris	7.142±0.017‡ (10)	7.222±0.048 (10)	7.225±0.033* (10)	–0.010±0.002 (9)
Gracilis major	7.102±0.065‡ (11)	7.179±0.070 (8)	7.193±0.087* (10)	–0.011±0.002 (8)
Semimembranosus	7.102±0.057‡ (10)	7.186±0.039 (10)	7.209±0.061* (10)	–0.010±0.003 (9)
Sartorius	7.039±0.080‡ (10)	7.193±0.066 (11)	7.122±0.128 (9)	–0.010±0.001 (4)
Mean ± S.D.	7.056±0.066‡	7.200±0.032	7.197±0.055*	–0.011±0.001

Values are reported as means ± S.D.; the number in parentheses is the number of animals used. Symbols and statistical treatment are as in Table 1.

In vitro ΔpH was determined from pH values at 30 and 10 °C.

Table 4. *Skeletal muscle pH and* in vitro *ΔpH/Δ*T *in the bullfrog* Rana catesbeiana

Muscle	pH 30 °C	pH 20 °C	pH 10 °C	*In vitro* ΔpH/Δ*T* (°C^{-1})
Submaxillary			7.274±0.074	–0.014±0.005
Obliques			7.325±0.085	–0.010±0.003
Rectus abdominis			7.362±0.056	–0.012±0.002
Gastrocnemius	7.075±0.077‡	7.277±0.022	7.355±0.051*,‡	–0.011±0.003
Triceps femoris	7.092±0.068‡	7.292±0.034	7.318±0.031*	–0.011±0.003
Gracilis major	7.078±0.070‡	7.274±0.029	7.286±0.045*	–0.010±0.001
Semimembranosus	7.095±0.052‡	7.284±0.013	7.337±0.051*,‡	–0.011±0.003
Sartorius	7.084±0.087‡	7.254±0.027	7.309±0.077*	–0.011±0.003
Mean ± S.D.	7.085±0.009‡	7.276±0.014	7.321±0.031*,‡	–0.011±0.001

Values are reported as means ± S.D. (*N*=8).

Symbols and statistical treatment are as in Table 1.

In vitro ΔpH was determined from pH values at 30 and 10 °C.

concentration of 0.8 mmol l^{-1} (Lindinger and McDonald, 1986), was unaffected by temperature.

Skeletal muscle

Skeletal muscle cell pH (pHi) in the cane toads and bullfrogs followed a similar pattern with changes in temperature. Between 30 and 20 °C, there was a relatively large increase in pHi (–0.014 °C^{-1} in the cane toad and –0.019 °C^{-1} in the bullfrog). Between 20 and 10 °C, the pH values did not change significantly in the cane toad and only increased by approximately 0.005 °C^{-1} in the bullfrog. There were no significant differences in the *in vitro* ΔpHi/Δ*T* ratios among the muscles, which averaged –0.011 °C^{-1} between 30 and 10 °C (Tables 3, 4).

In the toads and frogs, inulin space was largest in the submaxillary, followed by the obliques and rectus abdominis, then the sartorius, and finally the triceps femoris, gracilis major, semimembranosus and the gastrocnemius muscle, for which values were all similar (Tables 5, 6). In general, inulin space was larger in the cane toads than in the bullfrogs, especially in the hindlimb muscles where the inulin space was approximately twice as great in the toads. For all three species, there was a general trend for inulin space to decrease with decreasing temperature (Tables 5–7).

For both the toads and frogs, intracellular [Na^+] and [Cl^-] were highest in the submaxillary, and lowest in the triceps femoris, gracilis major, semimembranosus and sartorius muscles (Tables 8, 9). Intracellular [K^+] followed the reverse order. Despite these differences in intracellular ion concentrations, all the muscles within each species followed the same general pattern with respect to temperature. Cooling the cane toads from 30 to 20 °C resulted in increases of $[Na^+]_i$, $[Cl^-]_i$ and $[CO_2]_i$ that averaged 12.9, 8.2 and 4.2 mmol l^{-1} respectively. $[K^+]_i$ decreased by 11.1 mmol l^{-1}. Cooling the toads from 20 to 10 °C decreased $[Na^+]_i$, $[Cl^-]_i$, $[CO_2]_i$ and $[K^+]_i$ by an average of 8.3, 1.0, 1.7 and 3.5 mmol l^{-1} respectively. Cooling the toads increased the water content of the cells. In the bullfrogs, cooling from 30 to 20 °C produced

Table 5. *Skeletal muscle inulin space in the cane toad* Bufo marinus

	Inulin space (%)		
Muscle	30 °C (*N*=10)	20 °C (*N*=10)	10 °C (*N*=9)
Submaxillary	31.0±4.0	29.4±3.9	24.1±4.0*,‡
Obliques	30.3±13.4	21.6±5.6	22.6±12.1
Rectus abdominis	23.5±7.3‡	17.5±4.2	16.2±3.4*
Triceps femoris	16.2±8.4	11.8±3.7	11.4±3.9
Gracilis major	16.2±7.2	12.3±3.2	11.6±3.6
Semimembranosus	15.4±6.8	11.8±2.8	10.3±3.6
Sartorius	18.4±8.8	15.4±5.4	14.9±5.9

Values are means ± S.D.
Symbols and statistical treatment are as in Table 1.
Inulin space was measured as (g g^{-1} tissue)×100.

an increase in $[Na^+]_i$, $[CO_2]_i$ and $[K^+]_i$ of 5.1 1.0 and 5.3 mmol l^{-1} respectively. $[Cl^-]_i$ was not affected. In response to cooling from 20 to 10 °C, $[Na^+]_i$, $[CO_2]_i$ and $[K^+]_i$ decreased by 6.2, 1.0 and 10.7 mmol l^{-1} respectively. $[Cl^-]_i$ was little affected. In the black racer snakes, cooling from 30 to 10 °C produced large reductions in $[Na^+]_i$ (10.6 mmol l^{-1}) and $[Cl^-]_i$ (10.7 mmol l^{-1}), a 2.8 mmol l^{-1} increase in $[CO_2]_i$ and no change in $[K^+]_i$ (Table 10).

Discussion

The results of this study document for the first time a strong thermal dependence of acid–base-relevant ion concentration in air-breathing ectotherms. In the cane toad, there is a large reduction in plasma electrolyte concentration with cooling, which largely reflects changes in hydration. It is known that some frogs take up water when transferred from room temperature to 2–5 °C, resulting in a weight gain of approximately 5 % and dilution of electrolytes in the plasma and gastrocnemius muscle (Weathers, 1975; Miller et al., 1968). This water retention is thought to be associated with a reduction in kidney function and a change in the set point for osmoregulation. In the present study, plasma protein concentration in *B. marinus* fell by 10.1 % between 30 and 20 °C and by 7.4 % between 20 and 10 °C, which is in excellent agreement with the reductions in hematocrit observed previously (Stinner et al., 1994). Hydration did not, however, lower the $[HCO_3^-]_{pl}$, which presumably would have produced an acidosis. Simple dilution of $[HCO_3^-]_{pl}$ between 30 and 10 °C would have reduced $[HCO_3^-]_{pl}$ by approximately 4 mmol l^{-1}. The generation of HCO_3^- by the toad was probably associated with an equivalent transfer of Na^+ into the extracellular fluid since dilution of Na^+ between 30 and 10 °C should have reduced its concentration by approximately 4 mmol l^{-1} below that actually measured. In the bullfrog, water retention in the plasma was not evident between 30 and 20 °C, but between 20 and 10 °C there was a modest 5 % reduction in osmolality. With the possible exception of K^+, the thermal dependence of intracellular ion concentrations cannot be explained by changes in water content in the toads and frogs (see below).

Table 6. *Skeletal muscle inulin space in the bullfrog* Rana catesbeiana

	Inulin space (%)		
Muscle	30 °C (*N*=10)	20 °C (*N*=9)	10 °C (*N*=11)
Submaxillary	26.7±2.4	28.4±3.3	27.3±3.6*
Obliques	17.3±2.0	16.0±2.3	14.1±1.6*,‡
Rectus abdominis	17.4±2.8	17.0±3.7	15.0±2.0*
Triceps femoris	6.5±0.4‡	5.6±1.3	5.4±0.4*
Gracilis major	7.5±1.0	6.7±1.4	6.0±0.9*
Semimembranosus	7.1±1.1	6.1±1.2	5.7±0.4*
Sartorius	10.4±2.2	9.7±1.4	8.0±1.0*,‡
Gatrocnemius	6.3±0.8‡	5.1±1.0	5.0±0.5*

Values are means ± S.D.
Symbols and statistical treatment are as in Table 1.
Inulin space was measured as (g g^{-1} tissue)×100.

Table 7. *Skeletal muscle inulin space in the black racer snake* Coluber constrictor

	Inulin space (%)	
Muscle	30 °C	10 °C
Transversospinalis	12.8±2.9	10.0±1.8*
Longissimus dorsi	13.3±3.2	11.1±1.9
Iliolumbaris	13.3±3.9	11.2±2.0

Values are means ± S.D. (*N*=10).
*Indicates a value significantly different (*P*<0.05) from that at 30 °C (Student's *t*-test).
Inulin space was measured as (g g^{-1} tissue)×100.

In *B. marinus*, the *in vivo* $\Delta pH_{pl}/\Delta T$ ratio is the same as we measured for the *in vitro* $\Delta pH/\Delta T$ ratio (–0.014 °C^{-1}; Howell et al., 1970; Boutilier et al., 1987). Our results show that the *in vivo* $\Delta pH/\Delta T$ relationship was produced not only by the passive $\Delta pK/\Delta T$ of the plasma proteins but also by ion exchange (e.g. $NaHCO_3$) offsetting a potential dilution acidosis. Since the *in vivo* $\Delta pHpl/\Delta T$ is close to that for the $\Delta pK_{pro}/\Delta T$, this suggests that plasma protein charge is independent of temperature (Stadie et al., 1925; Reeves, 1972). Estimated plasma protein charge in the toad is the same at 30 and 20 °C, but tends to be higher at 10 °C (Table 1). The increase between 20 and 10 °C may have resulted from a net removal of anions (e.g. Cl^-) from the plasma, or could simply reflect the cumulative effect of measurement errors associated with the electrolyte determinations and variation in electrolyte concentration among individual animals. Plasma protein charge is independent of temperature in *R. catesbeiana* (Table 2). Presumably, the *in vitro* $\Delta pH_{pl}/\Delta T$ coefficient in the bullfrog is close to that in the cane toad (–0.014 °C^{-1}). However, the *in vivo* $\Delta pH_{pl}/\Delta T$ relationship in *R. catesbeiana* is uncertain. The studies by Howell et al. (1970), Mackenzie and Jackson (1978) and Rocha and Branco (1998) are in

Table 8. *Skeletal muscle intracellular electrolyte concentrations, total carbon dioxide concentration and water content in the cane toad* Bufo marinus

Muscle	Variable	Concentration		
		30 °C	20 °C	10 °C
Submaxillary	$[Na^+]$ (mmol kg^{-1})	20.3±14.8‡ (11)	36.1±13.5 (11)	24.7±8.3* (12)
	$[K^+]$ (mmol kg^{-1})	87.7±7.8‡ (11)	79.1±10.4 (11)	75.2±7.6‡ (12)
	$[Cl^-]$ (mmol kg^{-1})	11.4±11.6‡ (11)	17.8±8.7 (11)	19.0±8.8* (12)
	$[CO_2]$ (mmol kg^{-1})	6.5±0.9‡ (8)	10.6±1.4 (8)	10.1±2.5* (7)
	H_2O (%)	75.6±4.4 (14)		78.8±2.3* (10)
Obliques	$[Na^+]$ (mmol kg^{-1})	8.1±13.3‡ (26)	28.4±8.1 (11)	18.7±5.6*·‡ (13)
	$[K^+]$ (mmol kg^{-1})	96.3±4.7‡ (10)	73.8±7.2 (11)	78.8±6.7‡ (13)
	$[Cl^-]$ (mmol kg^{-1})	–4.6±4.3‡ (10)	12.7±6.6 (11)	9.7±4.5* (13)
	$[CO_2]$ (mmol kg^{-1})	2.8±2.4‡ (8)	6.8±1.9 (8)	8.0±4.5* (12)
	H_2O (%)	70.3±3.2‡ (10)	77.5±2.2 (11)	75.2±2.9*·‡ (12)
Rectus abdominis	$[Na^+]$ (mmol kg^{-1})	11.0±7.3‡ (10)	25.9±5.4 (11)	20.8±7.4‡ (12)
	$[K^+]$ (mmol kg^{-1})	92.7±7.1‡ (10)	82.7±4.7 (11)	78.7±6.0‡ (12)
	$[Cl^-]$ (mmol kg^{-1})	2.4±4.1‡ (10)	9.7±4.0 (11)	9.9±4.6 (12)
	$[CO_2]$ (mmol kg^{-1})	5.6±1.4‡ (8)	8.2±1.2 (8)	7.2±2.0 (7)
	H_2O (%)	74.2±1.8‡ (8)	76.5±1.5 (10)	77.5±2.1* (12)
Gastrocnemius	$[Na^+]$ (mmol kg^{-1})	9.7±4.6‡ (10)	16.2±3.8 (11)	11.3±5.0* (13)
	$[K^+]$ (mmol kg^{-1})	99.9±4.2‡ (10)	94.6±5.2 (11)	88.3±5.1*·‡ (13)
	$[Cl^-]$ (mmol kg^{-1})	–0.2±1.9‡ (10)	2.9±3.1 (11)	3.7±2.7‡ (13)
	$[CO_2]$ (mmol kg^{-1})	8.4±1.4‡ (8)	12.0±1.3 (8)	8.7±2.7‡ (10)
	H_2O (%)	74.4±1.1‡ (10)	75.9±1.4 (11)	75.8±1.7* (12)
Triceps femoris	$[Na^+]$ (mmol kg^{-1})	7.3±5.4‡ (11)	18.4±3.4 (11)	8.8±4.5* (10)
	$[K^+]$ (mmol kg^{-1})	105.0±7.1‡ (11)	94.4±3.7 (11)	89.9±7.7‡ (10)
	$[Cl^-]$ (mmol kg^{-1})	–4.0±1.9‡ (11)	4.4±2.8 (11)	3.0±1.7‡ (10)
	$[CO_2]$ (mmol kg^{-1})	7.2±1.3‡ (8)	12.2±2.0 (8)	7.6±1.1‡ (7)
	H_2O (%)	74.4±1.5 (11)	77.3±1.6 (11)	77.0±1.0* (10)
Gracilis major	$[Na^+]$ (mmol kg^{-1})	10.8±4.8‡ (10)	21.9±3.6 (11)	14.1±4.1* (10)
	$[K^+]$ (mmol kg^{-1})	102.6±6.3‡ (10)	90.6±5.0 (11)	86.5±7.9‡ (10)
	$[Cl^-]$ (mmol kg^{-1})	0.2±4.9‡ (10)	6.1±3.0 (11)	3.9±2.2* (10)
	$[CO_2]$ (mmol kg^{-1})	4.7±1.5‡ (8)	9.6±1.1 (8)	8.3±1.9* (7)
	H_2O (%)	74.7±2.1‡ (5)	78.1±1.8 (11)	77.4±2.1 (8)
Semimembranosus	$[Na^+]$ (mmol kg^{-1})	11.7±4.9‡ (11)	22.2±6.0 (11)	13.2±3.6* (10)
	$[K^+]$ (mmol kg^{-1})	99.1±7.1‡ (11)	90.3±6.9 (11)	83.5±6.4*·‡ (10)
	$[Cl^-]$ (mmol kg^{-1})	–1.3±3.3‡ (11)	7.0±4.8 (11)	4.6±2.9‡ (10)
	$[CO_2]$ (mmol kg^{-1})	6.2±1.2‡ (8)	11.0±1.2 (8)	7.6±1.6‡ (7)
	H_2O (%)	75.3±2.3‡ (16)	77.8±1.9 (10)	77.2±1.3* (10)
Sartorius	$[CO_2]$ (mmol kg^{-1})	3.5±1.1‡ (8)	7.9±1.4 (8)	7.1±2.0* (7)
Mean ± S.D.	$[Na^+]$ (mmol kg^{-1})	11.3±4.3‡	24.2±6.7	15.9±5.6*·‡
	$[K^+]$ (mmol kg^{-1})	97.6±5.9‡	86.5±8.1	83.0±5.6*·‡
	$[Cl^-]$ (mmol kg^{-1})	0.5±5.4‡	8.7±5.2	7.7±5.8*
	$[CO_2]$ (mmol kg^{-1})	5.6±1.9‡	9.8±2.0	8.1±1.0*·‡
	H_2O (%)	74.1±1.8‡	77.2±0.8	77.1±1.2*

Values (means ± S.D.) were calculated by subtracting extracellular values from whole-muscle values.
Symbols are as in Table 1.
Comparisons of mean values were performed using paired *t*-tests.
Inulin space for the gastrocnemius and plasma $[CO_2]$ were taken from Stinner et al. (1994).

reasonable agreement at 30 and 20 °C, but not at 10 °C. Between 20 and 10 °C, Howell et al. (1970) reported an *in vivo* $\Delta pH_{pl}/\Delta T$ ratio of –0.022 $°C^{-1}$, while Mackenzie and Jackson (1978) and Rocha and Branco (1998) report a value of –0.006 $°C^{-1}$. Unfortunately, the frequently cited $\Delta pH_{pl}/\Delta T$ values reported by Reeves (1972) and Malan et al. (1976) were from blood samples taken by heart puncture on pithed frogs. Thus, these values may be seriously in error because of

Table 9. *Skeletal muscle intracellular electrolyte concentrations and total carbon dioxide concentration in the bullfrog* Rana catesbeiana

Muscle	Electrolyte	Concentration (mmol kg^{-1}) 30 °C	20 °C	10 °C
Submaxillary	$[Na^+]$	24.9±4.6‡	32.3±4.7	27.1±10.1
	$[K^+]$	69.1±9.7	74.4±7.0	63.1±7.3‡
	$[Cl^-]$	14.8±6.1	16.5±3.7	14.4±9.6
	$[CO_2]$	7.9±2.4	8.3±1.4	7.9±1.5
Obliques	$[Na^+]$	19.2±4.7‡	25.9±2.2	23.4±7.7
	$[K^+]$	76.3±6.7	80.0±6.9	69.3±5.4*·‡
	$[Cl^-]$	8.6±4.8	8.6±2.8	10.2±5.6
	$[CO_2]$	5.5±2.5	6.7±2.4	6.2±0.8
Rectus abdominis	$[Na^+]$	18.7±3.2	21.3±2.5	20.5±8.9
	$[K^+]$	79.7±9.1‡	89.6±7.1	73.6±7.5‡
	$[Cl^-]$	7.5±4.2	5.1±2.7	7.6±7.5
	$[CO_2]$	6.4±1.1	7.7±2.2	6.7±0.9
Gastrocnemius	$[Na^+]$	16.9±3.1‡	22.3±1.1	13.5±4.1*·‡
	$[K^+]$	86.4±7.6	89.4±4.2	84.3±8.4
	$[Cl^-]$	4.0±1.3	3.9±0.9	3.4±1.5
	$[CO_2]$	7.6±0.9	8.6±0.7	6.9±0.9‡
Triceps femoris	$[Na^+]$	17.9±2.3‡	22.6±2.5	13.7±4.2*·‡
	$[K^+]$	86.5±6.1‡	92.2±4.6	82.2±5.3‡
	$[Cl^-]$	4.4±1.1	4.1±1.8	3.4±1.3‡
	$[CO_2]$	7.7±1.3	8.1±0.7	6.8±0.7‡
Gracilis major	$[Na^+]$	18.9±4.3‡	24.3±2.1	15.1±5.1‡
	$[K^+]$	86.2±7.8	92.1±4.4	79.3±6.1*·‡
	$[Cl^-]$	4.4±2.1	4.0±1.2	3.7±1.9
	$[CO_2]$	5.8±0.5‡	7.0±0.5	5.8±0.8‡
Semimembranosus	$[Na^+]$	18.2±4.3	22.9±2.9	14.2±4.5‡
	$[K^+]$	87.4±8.1	90.7±4.2	84.4±5.4‡
	$[Cl^-]$	4.7±1.3	4.4±1.0	3.4±1.6*
	$[CO_2]$‡	6.3±0.8‡	7.5±1.0	6.0±1.1‡
Sartorius	$[Na^+]$	17.2±3.7‡	21.1±1.7	15.8±4.1‡
	$[K^+]$	88.4±5.0‡	93.9±3.1	80.2±6.3*·‡
	$[Cl^-]$	5.5±2.6	4.4±1.5	5.5±2.7
	$[CO_2]$	5.2±1.1‡	6.6±0.7	6.2±1.2
Mean ± S.D.	$[Na^+]$	19.0±2.5‡	24.1±3.7	17.9±5.1‡
	$[K^+]$	82.5±6.9‡	87.8±6.9	77.1±7.7*
	$[Cl^-]$	6.7±3.7	6.4±4.4	6.5±4.1
	$[CO_2]$	6.6±1.1‡	7.6±0.7	6.6±0.7‡

Values (means ± S.D.) were calculated by subtracting extracellular values from whole-muscle values.

Symbols are as in Table 1.

Comparisons of mean values were performed using paired *t*-tests.

Plasma $[CO_2]$ was taken from Mackenzie and Jackson (1978).

N=10 for electrolyte concentration and *N*=7 for CO_2 concentration.

Table 10. *Skeletal muscle intracellular electrolyte concentrations and total carbon dioxide concentration in the black racer snake* Coluber constrictor

Variable	Concentration (mmol kg^{-1}) 30 °C	10 °C
$[Na^+]$	34.5	23.9*
$[K^+]$	88.5	89.1
$[Cl^-]$	25.1	14.4*
$[CO_2]$	9.0	11.8*

Values were calculated by subtracting extracellular [electrolyte] and $[CO_2]$ from whole-muscle [electrolyte] and $[CO_2]$.

Symbols and statistical treatment are as in Table 1.

Plasma values and whole-muscle values were taken from Stinner et al., (1998), and a mean value for inulin space was taken from the present study.

struggling by the animals and sampling varying proportions of venous and arterial blood from the heart. Between 30 and 10 °C, plasma protein charge decreased by 21 % in *C. constrictor* (Stinner et al., 1998).

In *B. marinus* and *R. catesbeiana*, most of the *in vivo* skeletal muscle ΔpHi occurred between 30 and 20 °C and there was little change in pHi between 20 and 10 °C (Tables 3, 4). Hence, the *in vivo* $\Delta pHi/\Delta T$ did not result simply from the passive physicochemical response of the intracellular buffers, as occurs in the muscle homogenate. Active processes, e.g. adjustment in P_{CO_2} and/or ion exchange, must have been involved. Boutilier et al. (1987) also concluded that temperature-induced acid–base-relevant ion exchange occurs in the skeletal muscle of *B. marinus* because they also found nonlinear $\Delta pHi/\Delta T$ relationships. The lack of agreement between the *in vivo* and *in vitro* results strongly suggests that muscle protein charge is affected by temperature. For example, the low $\Delta pHi/\Delta T$ between 20 and 10 °C in both species suggests acidification of the intracellular proteins.

The pHi values listed in Table 3 for cane toads at 20 °C are close to those reported by Pörtner et al. (1990) and Boutilier et al. (1987), but our pHi values at 30 and 10 °C are at least 0.2 and 0.1 pH units lower, respectively, than those of Boutilier et al. (1987). In addition, Malan et al. (1976) reported much lower pHi values in *R. catesbeiana*, e.g. only 6.8 at 30 °C compared with our value of 7.085 (Table 4), and a linear $\Delta pHi/\Delta T$ relationship between 30 and 10 °C. We cannot explain the differences in reported pHi values. No systematic study, other than at 20 °C, has compared the DMO (5,5-dimethyl-2,4-oxazolidinedione) and homogenate techniques. Pörtner et al. (1990) discuss possible reasons for expecting different pHi values between the DMO and tissue homogenate methods. As discussed below, the $\Delta pHi/\Delta T$ relationships in Tables 3 and 4 are consistent with adjustments in muscle ion concentrations reported in Tables 8 and 9.

The results of the present study do not support the commonly held view that air-breathing ectotherms use only ventilatory regulation and passive physicochemical processes to adjust pH with changes in temperature. Tables 8 and 9 document large changes in $[Na^+]_i$, $[K^+]_i$, $[Cl^-]_i$ and $[CO_2]_i$ in the skeletal muscles of toads and frogs. Between 30 and 20 °C, $[Na^+]_i$ and $[CO_2]_i$ increase, and between 20 and 10 °C $[Na^+]_i$

and $[CO_2]_i$ decrease. While adjustments in P_{CO_2} (ventilatory regulation) with changes in temperature undoubtedly affect the $\Delta pHi/\Delta T$ coefficients, it is evident that there are important changes in $[NaHCO_3]$ that also contribute to the adjustment of pHi. For example, the decrease in $[NaHCO_3]$ between 20 and 10 °C helps to explain why the *in vivo* $\Delta pHi/\Delta T$ ratio is much lower than the *in vitro* $\Delta pHi/\Delta T$ ratio.

The large changes in intracellular ion concentrations observed in toads cooled from 30 to 20 °C are consistent with a general inhibition of ion pumps within the sarcolemma or possibly a general increase in permeability to ions across the sarcolemma. $[Na^+]_i$, $[Cl^-]_i$ and $[CO_2]_i$ increased and $[K^+]_i$ decreased, suggesting movement of these ions down their respective concentration gradients between the extracellular fluid and the sarcoplasm. The large inward movement of Na^+ was probably accompanied by some outward transfer of H^+. Na^+/H^+ exchangers are known to exist in the plasma membrane of frog skeletal muscle cells (Abercrombie et al., 1983). No evidence has been found for K^+/H^+ exchangers in amphibian skeletal muscle (Amorena et al., 1990). Between 20 and 10 °C, $[K^+]_i$ decreased by approximately 4 %, possibly reflecting dilution by water. $[Na^+]_i$, $[Cl^-]_i$ and $[CO_2]_i$ also declined, which cannot, of course, be explained by a general inhibition of ion pumps or increase in membrane permeability. To maintain electroneutrality, the large loss of Na^+ was probably accompanied by H^+ entering the cells and titrating protein, phosphate and bicarbonate buffers. In *R. catesbeiana*, between 30 and 20 °C, there was an increase in $[Na^+]_i$ and $[K^+]_i$ with little change in $[Cl^-]_i$. To maintain electroneutrality, there would have been a reciprocal shift of H^+ out of the cell, raising pHi, intracellular net negative protein charge and $[HCO_3^-]i$. Between 20 and 10 °C, the opposite occurred, $[Na^+]_i$ and $[K^+]_i$ decreased with little change in $[Cl^-]_i$, so $[H^+]_i$ would have increased. In the snakes, acidification of skeletal muscle was accompanied by decreases in $[Na]_i$ and, to a lesser extent, $[Cl^-]_i$ (Table 10). Because cells contain many kinds of ions, including organic compounds, it is virtually impossible to calculate intracellular protein charge from the principle of electroneutrality as was done in this study for plasma proteins. Despite this, the large intracellular ion concentration changes and the $\Delta pHi/\Delta T$ data relative to the *in vitro* $\Delta pH/\Delta T$ coefficients strongly suggest that skeletal muscle protein charge is not constant between 30 and 10 °C.

The bullfrog whole skeletal muscle $[CO_2]$ values obtained in the present study are approximately 4 mmol l^{-1} lower than previously published by Stinner et al. (1998) and Reeves (1972), despite using similar techniques. These large differences in $[CO_2]$ may reflect seasonal variation. Rocha and Branco (1998) reported that arterial P_{CO_2} is twice as high in winter-acclimatized as in summer- and spring-acclimatized *R. catesbeiana*, but pH was not affected, indicating increased $[HCO_3^-]_{pl}$ levels during winter. The data of Reeves (1972) were collected in March–June, the data of Stinner et al. (1998) were collected in October–April, while the muscle CO_2 results in the present report were obtained during July and August.

In summary, the results of this study show that, in cane toads and bullfrogs, plasma protein charge is independent of temperature between 30 and 10 °C, with the possible exception of cane toads at 10 °C. Increased hydration with cooling in the toads did not lower their $[HCO_3^-]_{pl}$ because of a compensatory increase in $[NaHCO_3]_{pl}$. In contrast to plasma, skeletal muscle protein charge in the cane toads and bullfrogs is temperature-dependent. Large adjustments in intracellular ion concentrations took place when the temperature was changed. While differences in hydration, membrane permeability to ions and Q_{10} effects upon ion pump activity may all be involved, $NaHCO_3$ transfer across the sarcolemma played a significant role in the adjustment of pHi. Large changes in skeletal muscle intracellular ion concentrations also occurred in the black racer snakes, possibly in response to temperature-induced changes in $\dot{V}_{min}/\dot{V}_{CO_2}$ and carbonic acid levels. We conclude that air-breathing ectotherms utilize ion-exchange mechanisms, in addition to passive physicochemical adjustments of the buffers and active ventilatory regulation, to adjust their pH in response to changes in body temperature and that skeletal muscle protein charge is not independent of temperature.

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