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EXTRACELLULAR AND INTRACELLULAR CARBON DIOXIDE CONCENTRATION AS A FUNCTION OF TEMPERATURE IN THE TOAD *BUFO MARINUS*

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Summary

Previous studies of reptiles and amphibians have shown that changing the body temperature consistently produces transient changes in the respiratory exchange ratio (RE) and, hence, changes in whole-body CO_2 stores, and that the extracellular fluid compartment contributes to the temperature-related changes in CO_2 stores. The purpose of this study was to determine whether the intracellular fluid compartment contributes to the changes in CO_2 stores in undisturbed resting cane toads. Increasing body temperature from 10 to 30 °C temporarily elevated RE, and returning body temperature to 10 °C temporarily lowered RE. The estimated average change in whole-body CO_2 stores associated with the transient changes in RE was 1.0±0.8 mmol kg^{-1} (±S.D., N=6). Plasma [CO_2] and, thus, extracellular fluid [CO_2], were unaffected by the temperature change. Plasma calcium levels were also unaffected, so that bone CO_2 stores did not contribute to changes in whole-body CO_2 stores. Intracellular [CO_2] was determined for the lung, oesophagus, stomach, small intestine, liver, ventricle, red blood cells, skin and 14 skeletal muscles. [CO_2] was significantly lower (P<0.05) at higher temperature in 10 of these, and seven others, although not statistically significant (P>0.05), had mean values at least 0.5 mmol kg^{-1} lower at the higher temperature. The average change in intracellular [CO_2] for all tissues examined was -0.165 mmol kg^{-1} °C^{-1}. We conclude that, in cane toads, the temperature-related transients in RE result from intracellular CO_2 adjustments, that different tissues have unique intracellular CO_2/temperature relationships, and that a combination of respiratory and ion-exchange mechanisms is used to adjust pH as temperature changes.

Introduction

Robin (1962) reported that the plasma pH of freshwater turtles (*Pseudemys scripta elegans*) fell with increasing temperature. He also investigated acid–base changes in sealed samples of turtle blood, containing a constant CO_2 content, subjected to changes in

Key words: toad, *Bufo marinus*, temperature, oxygen consumption, carbon dioxide production, respiratory exchange ratio, acid–base balance, extracellular carbon dioxide content, intracellular carbon dioxide content.

temperature (a Rosenthal system). The *in vitro* $\Delta pH/\Delta t$ ratio was close to the *in vivo* value. He concluded that the passive physico-chemical changes occurring *in vitro* were responsible for the *in vivo* adjustments (Robin *et al.* 1969). Working on bullfrogs (*Rana catesbeiana*), Reeves (1972) also concluded that blood behaved *in vivo* like a Rosenthal constant-CO_2 system. In addition, Reeves applied this simple model to the intracellular compartment (Reeves, 1972, 1976*b*; Malan *et al.* 1976). A number of investigators, notably Rahn and his coworkers, have published data on reptiles and amphibians that support the concept of an *in vivo* Rosenthal system in which, as temperature increases, pH falls, CO_2 tension rises and the total CO_2 content of the animal is unchanged (Reeves, 1977). They concluded that temperature-related acid–base adjustments in ectotherms occur nearly instantaneously and that net protein charge is preserved over a wide range of body temperatures (Reeves, 1977).

Other investigators, however, have serious reservations about applying the Rosenthal system to animals *in vivo*. For example, collation of extracellular $\Delta pH/\Delta t$ values in reptiles, amphibians and fish by Heisler (1986) yielded an average $\Delta pH/\Delta t$ of -0.011 pH units $^{\circ}C^{-1}$, which is below that of a Rosenthal system (-0.0147 pH units $^{\circ}C^{-1}$; Rosenthal, 1948). Heisler reached a similar conclusion for the intracellular compartment (see also Cameron, 1989). Furthermore, a primary requirement of the Rosenthal system is a constant CO_2 content, and thus constant HCO_3^- content; i.e. there should be no ion exchange between tissue compartments and between the animal and the environment. While this is clearly not true of fish (Heisler, 1984), the situation for reptiles and amphibians still remains unclear. For several reasons, studies reporting CO_2 content in these animals are frequently open to criticism. The use of 'grab-and-stab' sampling techniques (Robin, 1962; Reeves, 1972; Malan *et al.* 1976) can cause the experimental animal to struggle, particularly at the higher temperatures, with consequent lactic acid production and titration (Heisler, 1986). In addition, $[HCO_3^-]$ has frequently not been measured directly but calculated using the Henderson–Hasselbalch equation. The selection of suitable values for pK' and α_{CO_2} can have a large effect upon calculated $[HCO_3^-]$ (Nicol *et al.* 1983). For example, Howell and Rahn (1976) showed that plasma $[HCO_3^-]$ of snapping turtles, calculated using a pK'_1 value from Reeves (1976*a*), was unaffected by temperature, but fell by about 0.76 mmol l^{-1} $^{\circ}C^{-1}$ when calculated using a pK'_1 value from Severinghaus (1965). Finally, almost all studies of temperature/CO_2 relationships in reptiles and amphibians are limited to measurements of the extracellular fluid (i.e. plasma). However, intracellular acid–base regulation, at least in amphibians, is relatively independent of extracellular acid–base regulation (Boutilier *et al.* 1987). Consequently, a constant CO_2 content in plasma does not necessarily mean that intracellular CO_2 content is independent of temperature.

A different picture concerning CO_2 content in air-breathing ectotherms has been presented (Stinner and Wardle, 1988). Whole-body CO_2 stores, estimated from changes in the respiratory exchange ratio (RE) in a snake (*Coluber constrictor*) and a turtle (*Chrysemys scripta*), were shown to be inversely dependent upon temperature. In the snake, CO_2 stores fell by 4.5 mmol kg^{-1} when body temperature was increased from 5 to

30 °C. There were concomitant changes in whole-blood [CO_2] ([CO_2]$_{WB}$) of $-0.23\,mmol\,l^{-1}\,°C^{-1}$. The authors concluded that this drop in [CO_2]$_{WB}$ was not enough to account for the $4.5\,mmol\,kg^{-1}$ change in CO_2 stores. Hence, the intracellular fluid of other tissues also appeared to be involved. Increasing temperature has a similar effect on respiratory exchange ratio (RE) in bullsnakes (Stinner, 1982), European tortoises, European common frogs, spiny-tailed lizards (Kayser, 1940) and painted turtles (Hall, 1924).

The objective of the present study was to determine, in the cane toad *Bufo marinus*, whether net changes in whole-body CO_2 stores, estimated from RE measurements, result from intracellular [CO_2] adjustments. In this species there are only small changes in plasma [HCO_3^-] between 10 and 30 °C (Boutilier *et al.* 1987).

Materials and methods

Animals

Toads, *Bufo marinus* L. were purchased from Charles D. Sullivan Co. (Nashville, Tennessee) and flown to Germany or Ohio. They were housed in cages equipped with heat lamps and water was available at all times. Approximately once weekly, the toads were fed on mice and rat pups. The toads used in this study appeared to be in good health and were starved for at least 1 week prior to experimentation.

Metabolism studies

Net changes in whole-body CO_2 stores caused by a 20 °C change in body temperature were assessed from measurements of RE. O_2 consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) in undisturbed toads were determined in a sealed recirculating apparatus. Each toad was placed inside a 3 l acrylic chamber, housed in a cabinet (Precision Scientific, model 815) in which temperature could be controlled to within ±1 °C. The chamber was then sealed and connected to incurrent and excurrent air lines. Two pumps (R-1 Flow Control, Ametek, Pittsburg, Pennsylvania), in parallel, outside the cabinet, recirculated air at a combined flow rate of $2.5\,l\,min^{-1}$ through the chamber and tubing. One of the pumps circulated air through a Mylar balloon, water absorbent (Drierite), and O_2 and CO_2 analyzers (Ametek S-3A and CD-3A). The balloon compensated for the pressure changes that would have resulted from changing the temperature of the closed-circuit system (Stinner and Wardle, 1988). The fractions of O_2 and CO_2 in dry air were continuously recorded onto a Gould 2400S chart recorder. The metabolism apparatus was flushed with room air when the O_2 levels had fallen to about 20 % (O_2 level was never allowed to fall below 19.5 %) and/or the CO_2 levels had risen to 1.0 % (CO_2 level was never allowed to rise above 1.4 %). This protocol gave measurement periods of 2–4 h at 30 °C and up to 14 h at 10 °C. To extend the measurement periods to 10–12 h at 30 °C for control RE measurements, the metabolism apparatus was enlarged by adding a 10 l glass bottle to the incurrent air line.

After flushing and sealing the metabolism apparatus, approximately 10–15 min was

allowed for mixing before beginning a measurement period. O_2 uptake (V_{O_2}) for a measurement period was calculated from the equation:

$$V_{O_2} = \left[(V - V_W)\frac{0.79025}{F_{I_{N_2}}}\right]F_{I_{O_2}} - \left[(V - V_W)\frac{F_{I_{N_2}}}{F_{E_{N_2}}}\right]F_{E_{O_2}}, \quad (1)$$

where V is the volume of metabolism apparatus at the time it was sealed minus the volume of the animal, V_W is volume of water vapour in metabolism chamber at the time that the apparatus was sealed, 0.79025 is the fraction of N_2 in dry room air, $F_{I_{N_2}}$ and $F_{I_{O_2}}$ are the fractions of N_2 and O_2 in dry air at the beginning of a measurement period, and $F_{E_{N_2}}$ and $F_{E_{O_2}}$ are fractions of N_2 and O_2 in dry air at the end of a measurement period.

V was a combination of the volumes inside the temperature cabinet (animal chamber plus about 100 ml in tubing) and outside (about 100 ml in tubing, the Mylar balloon and the 10 l bottle, when used). The volumes of the empty animal chamber and bottle were determined by their water contents. The volume of the tubing was determined using Boyle's Law. The balloon was partially filled with a known volume of air, using a syringe, prior to sealing the metabolism apparatus. The volume of the animal (in cm^3) was assumed to be equal to its mass (in g).

V_W, the volume of water vapour, was calculated from $V(RH \times P_S/100 \times P_B)$, where RH is the relative humidity, P_S is the saturated vapour pressure of water and P_B is ambient barometric pressure. The relative humidities of room air and the animal chamber were measured with certified instant digital hygrometer/thermometers (Fisher Scientific). The dry gas volumes inside and outside the temperature cabinet were converted to STP. The temperatures of the room air and the animal chamber were monitored with the hygrometer/thermometer or with a Yellow Springs Instrument 42 SF telethermometer equipped with a YSI General Purpose Probe.

The ratios $0.79025/F_{I_{N_2}}$ and $F_{I_{N_2}}/F_{E_{N_2}}$ correct for volume changes when RE values differ from 1.0 (Depocas and Hart, 1957). $F_{I_{N_2}}$ and $F_{E_{N_2}}$ were calculated from $1-(F_{I_{O_2}}+F_{I_{CO_2}})$ and $1-(F_{E_{O_2}}+F_{E_{CO_2}})$ respectively.

CO_2 production during a measurement period was calculated from the equation:

$$V_{CO_2} = \left[(V - V_W)\frac{F_{I_{N_2}}}{F_{E_{N_2}}}\right]F_{E_{CO_2}} - \left[(V - V_W)\frac{0.79025}{F_{I_{N_2}}}\right]F_{I_{CO_2}}. \quad (2)$$

Metabolic rates ($\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$) are reported in μl STPD $g^{-1}\,h^{-1}$. RE was calculated from the ratio $\dot{V}_{CO_2}/\dot{V}_{O_2}$.

Initially, each toad was allowed to adjust to the experimental apparatus for 2–3 days at 10 °C. Metabolic rate was then measured continuously for 5 days. After the first day of measurements, the cabinet temperature was increased from 10 to 30 °C within 5–6 h and held constant for 48 h. Temperature was then lowered back to 10 °C within 5–6 h and kept stable for another 48 h. Prior to the start of the metabolism studies, measurements of body (cloacal) temperature had shown that it stabilized to within 1 °C of cabinet temperature. During the 7–8 days that a toad was inside the metabolism chamber, it was left undisturbed and not fed or given water. At the end of this time, the toads had lost about 8 % of their initial body mass, presumably mainly because of evaporative water loss, but

they still appeared to be in excellent condition. Calculations of $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ used the initial body mass, i.e. the masses of the fully hydrated toads. The estimated net change in CO_2 stores during each measurement period (see below) represented the difference between the measured $\dot{V}_{CO_2}$ and the predicted $\dot{V}_{CO_2}$, assuming no change in RE with changes in temperature (i.e. control RE$\times\dot{V}_{O_2}$; Stinner, 1982).

Surgery and experimental apparatus for blood and tissue studies

Prior to surgery, toads were chilled for 1–2 h in crushed ice, and 0.1 ml Lidocaine (2 %) was injected at the surgical site. For arterial blood samples from 24 toads, a 1 cm incision was made on the dorsal surface near mid-thigh and a PE 50 cannula, filled with heparinized saline (1000 i.u. ml^{-1}), was tied occlusively into the femoral artery and advanced upstream 1–2 cm. The skin incision was closed with one or two stitches and the cannula was anchored to the leg by means of two stitches and methylcyanoacrylate adhesive placed on the stitches. In a separate group of 57 toads, the brachial vein was occlusively cannulated to obtain mixed venous blood samples, to inject isotopes and to inject lethal doses of anaesthetic at the termination of the experiment. A small ventral incision was made through the skin immediately caudal to the pectoral muscle, approximately midway between the sternum and the shoulder joint. The brachial vein was partially cut and the PE 50 cannula was advanced 3 cm downstream into the sinus venosus. To aid in venous sampling, 2–3 side holes had previously been cut near the end of the cannula. The other end of the cannula was passed under the skin by means of a blunt needle and exteriorized on the toad's back. The incision was closed and the cannula anchored as described above.

Following surgery, the toads were left to recover for at least 48 h at room temperature. They appeared to be in excellent health and there was no observable deficit in limb motor performance.

After recovery, the toads were placed inside individual darkened chambers where body temperature could be regulated to within ±1 °C. The chambers measured approximately 100 cm×100 cm×100 cm and the toads were free to move about within them. Each chamber contained about 2 cm of water and a dry area. The chambers were flushed by means of Wösthoff pumps (Bochum, Germany) with a continuous stream (600 ml min^{-1}) of humidified air thermostatted to the chamber temperature. The trailing end of a cannula was passed through a small hole in the chamber wall so that blood could be sampled and injections made without disturbing the toad. Each toad was left for 48 h at a constant temperature before beginning studies of blood, extracellular fluid volume and [CO_2].

Blood studies

CO_2 concentration

For arterial blood, the femoral artery cannula was thoroughly flushed with the toad's blood and then blood was drawn directly into 80 μl heparinized capillary tubes. $[CO_2]_{WB}$ was immediately determined in duplicate on 20 μl samples using a Capnicon III CO_2 analyzer (Cameron Instrument Co., Port Aransas, Texas). The remaining arterial blood was sealed and spun for 5 min at 11 500 revs min^{-1} in a microcapillary centrifuge.

Haematocrit (Hct) was measured, and the $[CO_2]$ of plasma ($[CO_2]_{Pl}$) was then determined in duplicate. $[CO_2]$ of red blood cells ($[CO_2]_{RBC}$) was calculated from mean values of $[CO_2]_{WB}$, $[CO_2]_{Pl}$ and Hct. Mixed venous $[CO_2]_{Pl}$ was measured in duplicate on sealed centrifuged blood, freshly collected from brachial cannulae in the same way.

Lactate concentration

Approximately 200 μl of arterial blood was withdrawn, centrifuged at 14 000 revs min^{-1} for 5 min, and 100 μl of the plasma was immediately deproteinized by adding an equal volume of a 0.6 mol l^{-1} perchloric acid solution. The mixture was then sealed and frozen, for not more than 1 week, before analysis with an enzymatic test kit calibrated against lactate standards (Boehringer, Mannheim).

Toads used for blood CO_2 and lactate studies were sampled at 2–5 temperatures. All the toads appeared to be in good health at the end of these experiments.

Plasma calcium concentration

Four toads, equipped with femoral artery cannulae, were kept in the 1 m^3 darkened chambers at 27 °C for 48 h. Body temperature was then lowered to 9 °C over a 6 h period. The temperature was held at 9 °C until the completion of blood sampling. Approximately 70 μl of blood was drawn into a heparinized capillary tube immediately before the temperature was lowered, and then additional samples were taken at hourly intervals for 10 h. A final sample was drawn 25 h after the first sample. All blood samples were centrifuged immediately at 14 000 revs min^{-1} for 5 min and the plasma was frozen in sealed containers for future analysis. Calcium concentration was determined spectrophotometrically on thawed plasma using Sigma test kit 587, and a calibration curve was constructed from Sigma standards (no. 360-11).

Extracellular fluid volume

Twenty-nine toads, each equipped with a brachial vein cannula, were placed inside the 1 m^3 chambers at 10, 20 or 30 °C for 48 h. Approximately 74 kBq of [^{3}H]inulin followed by 1 ml of amphibian Ringer's solution were injected through the cannula. 10 h after injection, 1.0 ml of blood was taken for six plasma isotope analyses by liquid scintillation counting (Packard Instruments, model 2660). Immediately after blood sampling, the animals were killed by anaesthetic overdose and tissue samples (100–500 mg) were removed for determination of radioactivity. Five tissue samples were taken from the liver, six from the skin, four each from the pectoralis and gastrocnemius muscles, three from the ventricle and two each from the lungs, oesophagus, stomach and small intestine. The samples were immediately weighed and then dried to constant mass (1 week at 100 °C). The dried tissue samples were pressed into filter paper pills and burnt with a sample oxidizer (Packard Instruments, model 306, modified) for analysis of [^{3}H]inulin by liquid scintillation counting (Heisler, 1975). The ratio of the mean concentration of [^{3}H]inulin in the plasma to that in the tissue was taken to represent fractional extracellular fluid volume (g ECF g^{-1} tissue).

Tissue $[CO_2]$ and body composition

Twenty-eight cannulated toads (brachial vein) were housed in the chambers for 48 h at

a constant temperature (10.5, 26 or 29 °C). They were killed by anaesthetic overdose and two tissue samples weighing 180–220 mg were immediately cut from the oesophagus, stomach, small intestine, liver, ventricle and lung and from 14 skeletal muscles (triceps femoris, semimembranosus, gracilis major, sartorius, gastrocnemius, pectoralis, anconeus, extensor carpi ulnaris, longissimus dorsi, coccygeo-iliacus, iliolumbaris, rectus abdominis, oblique, which was a combination of internal and external oblique muscles, and the submaxillary), making a total of 40 samples. The large number of skeletal muscles was sampled because skeletal muscle accounts for one-quarter of the toad's body mass (see below). Each sample was briefly rinsed in saline and blotted dry on filter paper. It was then quickly placed inside a tared 2 ml serum bottle and sealed with a tared aluminium seal and a Teflon-lined silicone septum (Wheaton, Millville, New Jersey). 0.7 ml of 0.1 mol l^{-1} NaOH solution was injected through the septum. The bottle plus contents were then weighed and left overnight at room temperature. Previous work had shown that CO_2 absorption by the NaOH solution was complete within 10 h. The 0.1 mol l^{-1} NaOH solution was made freshly each month and stored in a siphon bottle under Ascarite to keep it CO_2-free. A syringe attached to the bottle allowed direct injection of the NaOH solution through the septa of the serum bottles without access to air. After overnight soaking, 40 μl samples were withdrawn using a Hamilton syringe and the [CO_2] was measured using the Capnicon III. The [CO_2] of each bottle was determined in duplicate and averaged. After each measurement, the extraction chamber of the Capnicon III was thoroughly rinsed with de-ionized water in order to minimize foaming within the chamber. Rinsing was necessary for maximum accuracy. Calibration of the Capnicon III was frequently checked between tissue measurements and it did not vary by more than ±2 %.

[CO_2] of the tissue ([CO_2]$_T$) was calculated as [(sample mass+NaOH mass)/sample mass]×Capnicon III reading. This calculation assumes that the bottle contents, i.e. tissue pieces and NaOH solution, are homogeneous with respect to CO_2. To test this assumption, eight skeletal muscle samples were soaked overnight as previously described. [CO_2] was then measured before and after thorough homogenization under CO_2-free N_2. The calculated [CO_2]$_T$ of the homogenized samples averaged only 3 % lower than before grinding. Hence, the assumption appeared reasonable.

Intracellular [CO_2] was calculated for each tissue sample using the following equation:

$$[CO_2]_{IC} = [CO_2]_T - \frac{([CO_2]_{EC} \times F_{ECFV})}{F_{ICFV}}, \quad (3)$$

where F_{ECFV} and F_{ICFV} are the fractions of extracellular fluid volume and intracellular fluid volume, IC denotes intracellular, T denotes whole tissue and EC denotes extracellular fluid. [CO_2]$_{EC}$ was taken to be the same as [CO_2] in plasma (24.0 mmol l^{-1}, Table 1), which was assumed to have a relative density of 1.0. Mean values of F_{ECFV} are given in Table 2. In estimating F_{ECFV} at 26 °C, a linear relationship between F_{ECFV} and temperature between 10 and 30 °C was assumed. Finally, the average of the F_{ECFV} for gastrocnemius and pectoralis were used for calculating [CO_2]$_{IC}$ of the other skeletal muscles.

Table 1. *Effect of temperature upon blood CO_2 concentration, haematocrit and lactate concentration in resting* Bufo marinus

	Temperature (°C)				
Variable	10	15	20	25	30
Systemic arterial plasma	23.7±2.8	22.2±3.5	24.6±3.1	22.9±1.7	24.1±2.6
$[CO_2]$ (mmol l^{-1})	(10)	(4)	(9)	(6)	(9)
Mixed venous plasma	24.5±2.3		25.2±3.3		24.9±1.6
$[CO_2]$ (mmol l^{-1})	(10)		(8)		(8)
Systemic arterial whole-blood	20.5±2.9	20.6±3.0	21.4±2.3*,**	19.7±1.8	20.4±2.4
$[CO_2]$ (mmol l^{-1})	(20)	(6)	(18)	(6)	(20)
Systemic arterial red blood cell	11.1±2.1	10.7±3.6	10.0±1.7	10.0±1.5	9.7±2.6*
$[CO_2]$ (mmol l^{-1})	(10)	(5)	(8)	(6)	(9)
Systemic arterial haematocrit (%)	18.6±3.0	19.5±7.1	20.0±4.2	21.9±5.0	22.4±3.4*
	(16)	(7)	(14)	(7)	(15)
Systemic arterial plasma	0.33±0.07		0.41±0.15		0.60±0.25*
[lactate] (mmol l^{-1})	(9)		(9)		(8)

Values are reported as means ± S.D., numbers in parentheses are the number of animals sampled.

Comparisons of values were performed using paired *t*-tests and significance was assumed when $P<0.05$; * indicates values that are significantly different from those at 10 °C; ** indicates values that are significantly different from those at 30 °C.

Note the decrease in red blood cell CO_2 concentration as temperatures increase from 10 to 30 °C.

In addition to removing tissue samples for CO_2 analysis, 12 toads were carefully dissected and the percentage masses of skeletal muscle, ventricle, digestive system, lungs, skin, bone, egg sac and urine were determined.

Results

Metabolism

$\dot{V}_{O_2}$ in resting toads at 10 and 30 °C averaged 9.3±0.6 and 55.5±5.2 μl STPD g^{-1} h^{-1} (±S.D., N=6, mean mass=409±77 g, range=345–552 g). Before increasing the temperature, control RE averaged 0.70±0.04 at 10 °C (Fig. 1). Raising the temperature to 30 °C temporarily increased RE, which then returned to control levels within a few hours. Control RE at 30 °C averaged 0.72±0.03. Lowering the temperature again to 10 °C depressed RE, but after approximately 12 h it had returned to near control levels (Fig. 1). When the temperature was increased, RE averaged 0.88±0.01 during the first 2.5–4 h measurement period. When the temperature was lowered, RE averaged 0.58±0.09 for the six toads during the first 1.5–3 h measurement period. These averages were significantly different from the control RE values ($P<0.02$, Student's *t*-test). The average change in CO_2 stores calculated from these transient changes in RE is 1.0±0.8 mmol kg^{-1}.

Two potential problems with the metabolism studies were the effects of CO_2 retention upon RE during each measurement period and of dehydration during the 7–8 days over which experiments were run. There was a modest decrease in body mass (8 %), presumably due to evaporation, which probably had very little effect upon CO_2 stores.

Table 2. *Effect of temperature upon extracellular fluid volume (g ECF g^{-1} tissue × 100) of selected organs in* Bufo marinus

Organ	Extracellular fluid volume (%)		
	10 °C (*N*=9)	20 °C (*N*=10)	30 °C (*N*=10)
Lung	42.1±7.6	56.1±9.5*	59.0±7.7*
Liver	33.0±4.8	46.4±13.2*,**	67.5±21.7*
Oesophagus	52.0±4.6	56.8±5.7	63.0±10.2*
Stomach	42.7±5.7	45.4±10.3	47.1±4.2
Small intestine	33.2±7.4	38.1±6.9**	46.2±7.2*
Ventricle	23.7±3.7	22.1±2.3	26.3±6.8
Gastrocnemius	11.1±2.15	13.15±2.6	15.3±4.1*
Pectoralis	14.2±1.5	18.0±3.8*	17.5±3.9*
Skin	48.8±9.6	51.5±8.0	58.1±7.9*

Symbols as in Table 1.

Note that there is a general increase in the percentage extracellular fluid volume with increased temperature.

B. marinus stores large quantities of dilute urine in its bladder that can be reabsorbed during dehydration to preserve plasma ion concentrations (Shoemaker, 1964) and acid–base balance (Boutilier *et al.* 1979*a*,*b*). In later studies, $[CO_2]_{Pl}$ was maintained at normal values even when toads were dehydrated to 73 % of their initial hydrated masses. With the reabsorption of bladder water there was some urine acidification, which represented less than a 0.1 mmol decrease in urine bicarbonate stores. CO_2 retention in the toads may have occurred as CO_2 accumulated inside the closed metabolism chambers. While this might be expected to lower RE values, our control results at 10 ˚C (0.70±0.04) and 30 ˚C (0.72±0.03) are close to that predicted for a fasting animal metabolizing fat (i.e. 0.71). *B. marinus* hyperventilates during exposure to hypercapnia, and this may have reduced CO_2 retention (Boutilier *et al.* 1979*a*). Furthermore, differences in CO_2 accumulation between measurement periods were minimized by consistently stopping the measurements at a chamber $[CO_2]$ of about 1 %.

Blood studies

Blood $[CO_2]$, Hct and lactate concentration in toads kept at constant temperature for 48 h are reported in Table 1. Arterial and mixed venous $[CO_2]_{Pl}$ did not vary significantly with temperature (P>0.05, paired t-test). Arterial $[CO_2]_{WB}$ was also stable, except that at 20 ˚C $[CO_2]_{WB}$ was about 1.0 mmol l^{-1} higher than at 10 or 30 ˚C. $[CO_2]_{RBC}$ was significantly higher at 10 ˚C than at 30 ˚C (P<0.01, paired t-test). Hct was positively correlated with temperature, rising from 18.6 % at 10 ˚C to 22.4 % at 30 ˚C. Linear regression yielded the relationship Hct(%)=16.47+0.20t(˚C) (P<0.01). The drop in $[CO_2]_{RBC}$, together with the rise in Hct as temperature rises from 10 to 30 ˚C, should have lowered $[CO_2]_{WB}$ by about 0.5 mmol l^{-1}. This is not evident in Table 1, possibly because the predicted change is too small to detect, or perhaps because of the small increase in $[CO_2]_{Pl}$.

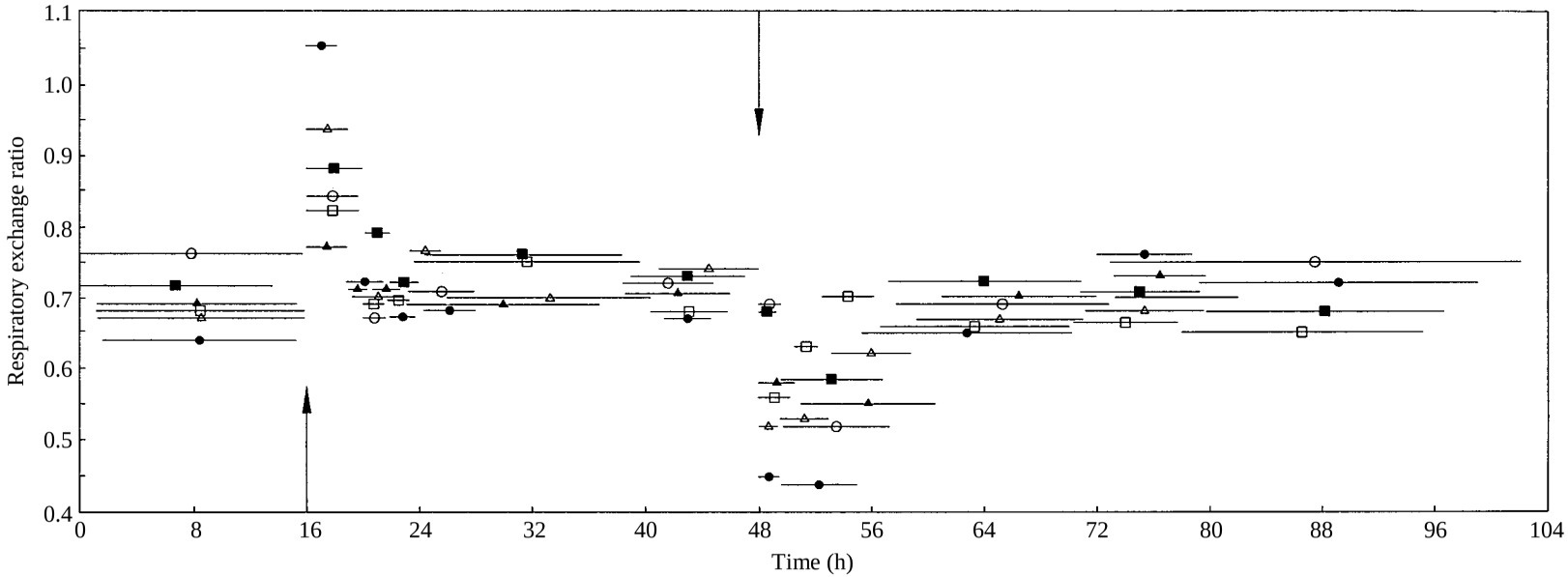

Fig. 1. Effect of changing temperature by 20 ˚C upon the respiratory exchange ratio in *Bufo marinus* (*N*=6). Different symbols represent individual toads and the lengths of the horizontal bars correspond to the duration of individual measurement periods. The arrow at 16 h marks the time at which ambient temperature was increased from 10 to 30 ˚C and the arrow at 48 h marks the time at which ambient temperature was lowered from 30 to 10 ˚C. The changes in air temperature took 5–6 h to complete and oxygen consumption reached stable values within 6–8 h, indicating that body temperature had stabilized by that time.

Arterial plasma lactate concentrations in *B. marinus* were well below 1.0 mmol l^{-1}, indicating that the toads were reasonably calm in the experimental apparatus. As temperature rose from 10 to 30 °C, lactate concentration rose by 0.27 mmol l^{-1}, which represented an 82 % increase.

Cooling four toads from 27 to 9 °C did not change plasma calcium ion levels. Mean calcium ion concentration for 42 plasma samples was 1.81±0.16 mmol l^{-1}.

Extracellular fluid volume

There were large differences in fractional extracellular fluid volumes between organs (Table 2). Skeletal muscle contained the smallest percentage ECFV (approximately 10–20 %), followed by the ventricle (about 20–25 %). Percentage ECFV was highest in the lungs, liver, gut and skin (roughly 30–70 %). In general, the percentage ECFV increased as temperature increased in all nine of the organs examined. In seven of these, the percentage ECFV at 30 °C was significantly higher than at 10 °C (P<0.05, Student's t-test).

Intracellular [CO_2]

Results given in Table 3 show that, with the exception of a few skeletal muscles (particularly the limb muscles), there is a decrease in mean $[CO_2]_{IC}$ with rising temperature. This is true for all of the systems examined, i.e. respiratory (lung), digestive (liver, gut), skeletal muscle and circulatory (ventricle; see also red blood cells). Thus, 10 tissues (including red blood cells) exhibited an inverse dependence of $[CO_2]$ on temperature. Furthermore, of the remaining 12 tissues, seven had mean $[CO_2]_{IC}$ values at least 0.5 mmol l^{-1} lower at the higher temperature, suggesting a $-\Delta[CO_2]_{IC}/\Delta t$ in these cells as well. None of the tissue cells, with the exception of skin, showed evidence of increasing $[CO_2]_{IC}$ with temperature.

A weakness of the skeletal muscle $[CO_2]_{IC}$ values in Table 3 is that F_{ECFV} was measured only in gastrocnemius and pectoralis muscles, and their averages were used to calculate $[CO_2]_{IC}$ in the other skeletal muscles. The error introduced by this assumption is probably not large because of the relatively small F_{ECFV} in skeletal muscle. Another potential source of error in the $[CO_2]_{IC}$ estimates is that F_{ECFV} was not measured at the same temperature as $[CO_2]_T$. The difference in temperatures was as much as 4 °C. The potential error is presumably greatest in those tissues with relatively large F_{ECFV} that were most affected by temperature (liver, oesphagus, lung and small intestine).

Body composition

The largest single organ system in the cane toad is skeletal muscle, which was one-quarter of the body mass on average (Table 4). Other large components are bone, skin, urine and egg sacs, which collectively made up 51 % of the body mass. The digestive system represented less than 5 % and the respiratory system (i.e. lungs without extrapulmonary airways) only 1 %. Other structures weighed together accounted for 9 % of the animal. These included the urogenital system, eyeballs, tongue and parotid glands. In addition, approximately 10 % of the animal was not weighed. This material was fluid from the gut (especially the contents of the large intestine), blood vessels, peritoneal space and lymphatics, and a small amount of fluid lost by evaporation during dissection.

Table 3. *Intracellular [CO_2] (mmol kg^{-1}) as a function of temperature in* Bufo marinus

	$[CO_2]_{IC}$ (mmol kg^{-1})		
Organ	10.5 °C	26 °C	29 °C
Lung	13.9±5.0 (7)	4.3±4.9* (7)	
Liver	12.1±4.8 (7)	3.6±4.9* (7)	
Oesophagus	9.5±8.4 (5)	6.4±5.5 (7)	
Stomach	7.7±5.5 (10)	4.2±3.0 (9)	
Small intestine	14.8±5.0 (8)	3.7±6.1* (8)	
Ventricle	8.9±2.4 (7)	5.6±2.3* (7)	
Triceps femoris	7.3±1.1 (7)	6.7±1.1 (9)	
Semimembranosus	7.1±1.6 (7)	7.0±1.1 (9)	
Gracilis major	8.1±1.9 (7)	7.8±1.3 (7)	
Sartorius	7.5±1.9 (7)	6.8±1.5 (7)	
Gastrocnemius	8.8±2.7 (10)	8.4±1.3 (10)	
Pectoralis	9.4±2.0 (10)	8.2±1.4 (10)	
Anconeus	8.0±1.4 (7)	8.0±0.9 (7)	
Extensor carpi ulnaris	7.2±0.9 (7)		6.3±0.7 (7)
Longissimus dorsi	5.2±2.0 (7)		3.2±2.0* (7)
Coccygeo-iliacus	5.3±1.3 (7)		3.9±1.4 (7)
Iliolumbaris	7.6±1.6 (7)		4.7±0.6* (7)
Oblique (external plus internal)	9.7±4.0 (11)	4.0±2.1* (7)	2.4±1.3* (5)
Rectus abdominis	7.8±1.9 (7)		4.7±1.1* (7)
Submaxillary	12.0±2.1 (7)		8.4±1.3* (7)
Skin	40.3±2.2 (5)	45.0±11.5 (4)	

Values given are means ± 1 S.D.; numbers in parentheses are the number of animals sampled. Comparisons of values were performed using Student's *t*-test; * indicates values that are significantly different ($P<0.05$) from those at 10.5 °C.

Intracellular [CO_2] was calculated by subtracting extracellular CO_2 content (24 mmol $l^{-1} \times F_{ECFV}$) from tissue CO_2 content.

See text for further details. Note that in most cases mean intracellular CO_2 concentration is lower at the higher temperature.

Discussion

The RE measurements and calculated $[CO_2]_{IC}$ values obtained in this study provide strong evidence that whole-body CO_2 stores in *B. marinus* are inversely dependent upon temperature. Hence, *B. marinus* does not behave according to a closed system with respect to CO_2 content (i.e. as a Rosenthal system), as was earlier suggested from studies limited to plasma acid–base status and temperature in this species (Baumgardner and Rahn, 1967; Howell *et al.* 1970; Reeves, 1972). Our findings support previous studies showing that whole-body CO_2 stores are inversely dependent upon temperature in air-breathing ectotherms (see Introduction). Furthermore, we found no evidence for changes in plasma calcium ion concentration and, hence, bone calcium carbonate, or $[CO_2]_{EC}$ adjustments. The effect of temperature upon $[CO_2]_{IC}$ has not been measured in other amphibians. However, Reeves (1972) measured $[CO_2]_T$ of liver, ventricle and skeletal muscle (a combination of sartorius and gastrocnemius) in the bullfrog, and did not find a

decrease with temperature. Differences between Reeves' study and ours may reflect species differences. In addition, possible changes in tissue F_{ECFV} were not accounted for by Reeves. In *B. marinus*, there was an increase in the F_{ECFV} of tissues with rising temperature (Table 2). The ECFV has a high $[CO_2]$ relative to that of the ICFV, so that the increase in F_{ECFV} partially masked a decrease in $[CO_2]_{IC}$ when we measured $[CO_2]_T$ in *B. marinus*.

One of the most striking findings is that there are large differences in the $[CO_2]_{IC}$/temperature relationships among tissues. For example, $[CO_2]_{IC}$ in the lung, liver, small intestine and oblique skeletal muscles decreased by 0.4–0.7 mmol kg^{-1} $°C^{-1}$, whereas in the semimembranosus and anconeus skeletal muscles $[CO_2]_{IC}$ did not change significantly with temperature. Differences in the $\Delta[CO_2]_{IC}/\Delta t$ slopes agree qualitatively with the presence of unique intracellular pH/temperature relationships in tissues of *B. marinus* and other ectotherms (Boutilier *et al.* 1987; Cameron, 1984; Heisler, 1986). The reason for the differences in acid–base/temperature relationships is not known, but may reflect differences in the composition of intracellular buffers (Cameron, 1989). Vertebrate skeletal muscle, for example, shows variation in both concentration and type of buffer within and among species (Castellini and Somero, 1981; Heisler, 1984; Abe *et al.* 1985), although the extent of the variation appears to be considerably less than originally thought (Pörtner, 1990). Histidine-related buffering within skeletal muscle occurs in the myofibrillar protein, in free histidine and in dipeptides such as carnosine and anserine. The pK′ values of the imidazole moieties in these compounds vary. Other skeletal muscle buffers include phosphate and taurine. Consequently, it is quite likely that average $\Delta pK'/\Delta t$ values could differ among tissues in *B. marinus*, as in other species.

Preservation of a constant protein charge (z-stat or alphastat), rather than a constant pH,

Table 4. *Organ mass, organ systems and urine as a percentage of body mass in the toad* Bufo marinus

Structure	Percentage of total body mass
Skeletal muscle	25.2±3.3 (11)
Ventricle	0.27±0.05 (11)
Stomach/oesophagus	0.91±0.26 (11)
Small intestine	0.37±0.29 (11)
Large intestine	0.26±0.07 (10)
Liver	3.1±1.0 (11)
Lung	0.96±0.42 (11)
Bone	12.5±3.1 (9)
Skin	10.1±1.8 (11)
Egg sac	10.6±4.7 (8)
Urine	17.7±9.3 (12)
Other (kidneys, gonads, eyeballs, tongue, parotid glands, etc.)	8.7±1.4 (9)

Values are means ± 1 S.D.; the number of animals dissected is shown in parentheses.

The percentages do not sum to 100% because of gut contents plus blood and peritoneal fluid losses and a small amount of fluid lost by evaporation during dissection.

has been central to formulating theories of acid–base regulation (see reviews by Cameron, 1989; Nattie, 1990). Heating increases the dissociation of weak acids (e.g. proteins) in accordance with the van't Hoff relationship. Hence, net negative protein charge should increase, which could alter enzyme activity, membrane transport and buffering. One solution to this problem, recognized by Stadie *et al.* (1925), is to select function-determining titratable groups with $\Delta pK'/\Delta t$ coefficients that match the $\Delta pH/\Delta t$ of water (approximately -0.016 pH units $^{\circ}C^{-1}$). Then, as an aqueous solution is heated, protein charge is preserved because ionization is prevented by the rise in $[H^+]$ associated with enhanced dissociation of water. In blood, histidine-imidazole moieties are the predominant titratable sites within plasma proteins and haemoglobin and, although their individual $\Delta pK'_{IM}/\Delta t$ ratios vary widely because of adjacent charged groups, the average value is close to -0.016 pH units $^{\circ}C^{-1}$. Hence, in a Rosenthal system, blood pH varies with temperature but protein net charge is preserved. At the same time, total CO_2 content, and hence $[HCO_3^-]$, is constant in the closed blood sample, but P_{CO_2} increases with heating because of the decreases in α_{CO_2}, pK'_1 and pH. Application of this relatively simple system to whole animals requires that extracellular and intracellular proteins have average $\Delta pK'/\Delta t$ values close to -0.016 pH units $^{\circ}C^{-1}$ and that ventilation is regulated to defend the appropriate temperature-dependent P_{CO_2}.

In *B. marinus*, as in other air-breathing ectotherms, P_{CO_2} increases with rising temperature, which clearly supports a decrease in pH. However, adjustment of P_{CO_2} alone cannot possibly preserve protein charge in all tissues where the buffering properties are diverse. It may be that in *B. marinus* the difference in P_{CO_2} alone at 10 °C as opposed to 30 °C is largely sufficient to maintain protein charge in those tissue compartments where $\Delta pK/\Delta t$ coefficients are around -0.016 pH units $^{\circ}C^{-1}$ (e.g. plasma and pectoral muscle; see Table 3 and Boutilier *et al.* 1987). Additional acidification would be required to maintain net protein charge in compartments where the average overall $\Delta pK/\Delta t$ exceeds -0.016 pH units $^{\circ}C^{-1}$. This could occur by a Na^+/H^+ exchanger, which would reduce CO_2 stores and transiently elevate RE with warming, as was observed in this study. Thus, heart muscle exhibits a $\Delta pH/\Delta t$ of -0.026 pH units $^{\circ}C^{-1}$ (Boutilier *et al.* 1987) and a $\Delta[CO_2]_{IC}/\Delta t$ of -0.2 mmol kg^{-1} $^{\circ}C^{-1}$ (Table 3). Ion exchange processes are known to be important in compensation of hypercapnia in *B. marinus* (Toews and Stiffler, 1990; Snyder and Nestler, 1991).

We conclude that *B. marinus* uses a combination of respiratory adjustments and relatively slow ion-exchange mechanisms to alter intracellular pH as temperature changes and that the relative importance of these two mechanisms appears to vary markedly among tissues. Furthermore, the changes in $[CO_2]_{IC}$ and hence $[HCO_3^-]_{IC}$ that occur with changes in body temperature suggest transfer of strong ions, such as Na^+ and Cl^-, between the extracellular and intracellular fluid compartments. Consequently, application of a Rosenthal system to the extracellular fluid compartment of *B. marinus* is not justified on the basis of the present data.

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