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Acid-base regulation in the toad *Bufo marinus* during environmental hypoxia

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Abstract. Gas exchange and the correlated changes in blood and tissue metabolic and acid-base status were investigated during long term exposure of the toad *Bufo marinus* to graded levels of hypoxia. During hypoxia, P_{CO_2} values in blood and tissues fell, leading to a transient alkalosis in the extracellular but not in the intracellular space. A reduction in blood perfusion of the skin during hypoxia may explain why P_{CO_2} was low in sartorius muscle under normoxia, but approached the P_{CO_2} values found in other tissues (gastrocnemius muscle, ventricle) under hypoxia. At P_{O_2} values below the critical P_{O_2}, lactate was formed and the decrease in total CO_2 was accelerated. Lactate levels in the plasma were higher than in the intracellular space of the skeletal muscles, a finding attributed to the pH-dependent distribution of lactic acid across the cell membrane. The comparison of metabolic proton quantities with those found in the extra- and intracellular acid-base status suggests that CO_2 release was accelerated by anaerobic proton formation. The alkalizing effect of decreasing P_{CO_2} in the skeletal musculature was compensated for by a release of base equivalents into the blood. The resulting alkalosis in the blood was probably compensated for by the release of base equivalents into the environment.

Acid-base balance, during hypoxia (toad); Amphibians, toad *Bufo marinus*; Hypoxia, tissue acid-base balance (toad)

Many amphibian species are well adapted to undergo periods of hypoxia during diving or burrowing. In a burrowing species, *Bufo marinus*, the critical P_{O_2} had been analysed during long-term exposure to different degrees of hypoxia (Pörtner *et al.*, 1991). This species tolerated hypoxia down to a critical P_{O_2} (Pc) of 30–37 Torr without transition to anaerobic energy production. In contrast to the traditional concept of the Pc, which focuses on the oxygen tension where oxygen supply becomes limiting and oxygen consumption falls, the critical P_{O_2} of *Bufo marinus* could be evaluated considering the essential metabolic transition from aerobic to anaerobic metabolism. This Pc was characterized by the onset of lactate accumulation, an increase in overall oxygen

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consumption and the reduction of oxygen provision by the blood in peripheral tissues like the hindlimb. Above this critical P_{O_2} a regulated aerobic rate of energy metabolism was maintained.

To maintain oxygen consumption over a wide range of P_{O_2} and to delay anaerobiosis, an increase in the ventilated gas volume is a defence strategy for metabolic homeostasis (see Shelton and Boutilier, 1982; Glass and Wood, 1983; Boutilier, 1988 for the ventilatory responses of lower vertebrates in hypoxia). For the same animals studied by Pörtner *et al.* (1991) the present paper reports how these changes in ventilatory behaviour and the onset of anaerobic metabolism affected the carbon dioxide stores and the extracellular and intracellular acid-base status.

For a complete picture of the changes in the CO_2 stores within the body, fluctuations of P_{CO_2} and bicarbonate should be known in both the extra- and intracellular body compartments. The present study includes an analysis of the changes in intracellular bicarbonate and P_{CO_2} levels during hypoxia in the toad. Recently, a procedure was developed to evaluate these parameters (Pörtner *et al.*, 1990).

Materials and methods

Animals. Toads (*Bufo marinus*, 200–500 g) were obtained from a commercial dealer (Sullivan Inc., Nashville, TN, U.S.A.). Upon arrival they were kept at room temperature and ambient light in moistened aquaria with a bottom layer of sand. They were provided with ample water and fed on beef liver on a weekly basis. The animals were used after a period of starvation of at least 24 h.

Experimental procedure. To exclude diurnal fluctuations in oxygen consumption, each animal was kept in a darkened chamber (vol. 2.97 L) containing 400 ml of dechlorinated tap water. Water was changed at regular intervals. Changes in the oxygen consumption and the release of CO_2 by *Bufo marinus* were measured under normoxia and after long term (12–14 h) exposure to various levels of hypoxia which were reached during a stepwise reduction in ambient P_{O_2}. For these analyses, Ametek Applied Electrochemistry (Pittsburgh, PA, U.S.A.) equipment was used: N-37M double-cell oxygen sensor and S-3AII oxygen analyser, P61B carbon dioxide cell and CD-3A carbon dioxide analyser, R-2 flow control. Oxygen uptake could be derived from differential readings whereas the measurement of carbon dioxide release required repeated changes between calibration and sampling gases at regular intervals. Calibration gases and gases withdrawn from the animal chambers were led through glass columns filled with drierite before being fed into the sensors. All gases were prepared from pure nitrogen, CO_2 and oxygen by gas mixing pumps (type 303/a-F, Wösthoff AG, Bochum, Germany) and saturated with water at 20 ± 0.1 °C. The room in which the experiments took place was controlled at 20 ± 1 °C.

In parallel, animals were cannulated (occlusively) in the ischiadic artery of the right leg and (non-occlusively) in the femoral vein of the left leg as outlined by Pörtner *et al.*

(1991). They were exposed to different degrees of hypoxia for the sampling of tissues and blood. In these experiments, the transition to one severely hypoxic oxygen tension ($P_{O_2} \leq 35$ Torr) only occurred after 12 h of preacclimatization at P_{O_2} = 75 Torr. Animals were exposed to the final P_{O_2} for 24 h except at 14 Torr, where the experiments were finished after 6 h since the animals had shown a gradual decrease in oxygen consumption. After blood was withdrawn anaerobically from both the vein and the artery, animals were anaesthetized for the sampling of tissues (gastrocnemius and sartorius muscle, kidney, ventricle). Tissues were freeze-clamped and stored under liquid N_2 until analysed (see Pörtner *et al.*, 1990, 1991).

Analysis of acid-base parameters. Blood samples were analysed for pH and P_{CO_2} using a Radiometer (Copenhagen) electrode chamber thermostatted to the incubation temperature of the animals (20 ± 0.1 °C). The electrodes were calibrated with precision phosphate buffers (pH, Radiometer), or calibration gases (CO_2-electrodes) prepared as described above. Plasma bicarbonate values were calculated using values of CO_2 solubility α and pK''' as calculated according to Heisler (1986). Haematocrit was analysed after centrifugation of blood samples in a Clay-Adams microfuge.

Intracellular pH and P_{CO_2} were evaluated using the homogenate technique described by Pörtner *et al.* (1990). In brief, tissue samples were ground under liquid nitrogen using a porcelain mortar and pestle. During the work with liquid nitrogen, contamination of the muscle powder with condensing CO_2 was avoided by minimizing the exposure to air of tissue samples and the liquid nitrogen used. In an 0.5 ml Eppendorf tube, capped and filled to the top in order to exclude air bubbles, tissue powder (100–150 mg) was mixed with icecold medium containing potassium fluoride (130 mmol · L^{-1}) and nitrilotriacetic acid (5 mmol · L^{-1}, Sigma, St. Louis, MO, U.S.A.), pH 7. After brief centrifugation (5–15 s) pH and total CO_2 were measured in the supernatant using a capillary pH electrode (see above) and a gas chromatographic method for CO_2 analysis (for details see Pörtner *et al.*, 1990).

Ammonium and lactate levels were measured according to Bergmeyer (1984) after extraction of the tissue powder and the plasma in ice-cold perchloric acid according to Beis and Newsholme (1975).

Calculations. Oxygen consumption and carbon dioxide release were calculated according to Withers (1977). For the evaluation of intracellular values (Ci) the measured tissue C_{CO_2} levels (C, μmol · (g wet weight)$^{-1}$) were corrected for the contamination by extracellular CO_2 levels (Ce, eq. (1)) using values for the fractions of tissue water F_{H_2O} in the tissue or extracellular water Q in tissue water (F_{H_2O} = 0.779, 0.786, 0.808; Q = 0.145, 0.202, 0.257 for gastrocnemius, sartorius and ventricle muscles, respectively) provided by Boutilier *et al.* (1987) and taking the effect of a Donnan factor (1.05) into account (Pörtner *et al.*, 1990).

$$Ci = (C/F_{H_2O} - Q \cdot Ce)/(1 - Q) \quad (mmol \cdot L^{-1}) \tag{1}$$

The resulting intracellular CO_2 content is unlikely to include carbamate formed by intracellular proteins (Gros and Dodgson, 1988). Based on the analysis of mean intracellular pH in the homogenates and on the evaluation of appropriate values for pK''' and CO_2 solubility α (Heisler, 1986), intracellular P_{CO_2} values (Pi_{CO_2}) were then calculated for gastrocnemius and sartorius muscles (Pörtner *et al.*, 1990):

$$P_{CO_2} = C_{CO_2}/(10^{pH - pK'''} \cdot \alpha + \alpha) \quad \text{(Torr)} \tag{2}$$

A preliminary calculation of P_{CO_2} was performed for the ventricle, using a DMO-based pHi value in eq. (2) which was selected 0.22 units higher than homogenate pH. Such a difference between homogenate- and DMO-based values of intracellular pH must be considered in tissues containing high fractions of mitochondria. In these organs pHi analysis based on weak acid (DMO) distribution leads to overestimated pHi values. However, since CO_2 is a weak acid as well, these values are required for P_{CO_2} calculations (equivalent to the calculation of undissociated acid levels in a modified Henderson-Hasselbalch equation, eq. (2), cf. Pörtner *et al.*, 1990).

The comparison of non-respiratory proton quantities in the acid-base status of the plasma and of gastrocnemius and ventricular muscles with metabolic proton production followed the rationale of Heisler (1986) and Pörtner (1987a,b). Non-respiratory proton quantities were calculated from changes (Δ) in pH and bicarbonate levels in each compartment:

$$\Delta H^+_{non\text{-}resp.} = -|\beta_{NB}| \cdot \Delta pH - \Delta[HCO_3^-] \quad (mmol \cdot L^{-1}) \tag{3}$$

Since changes in ammonia levels were insignificant, metabolic proton production was evaluated from intra- and extracellular phosphocreatine, lactate and ATP levels considering eq. (1) and the proton stoichiometries in anaerobic glycolysis, phosphagen degradation and adenylate catabolism (Pörtner, 1987a: equimolar proton production during lactate formation; pHi-dependent proton consumption or release during depletion of the high energy phosphates) based on metabolite values reported by Pörtner *et al.* (1991) for the animals used in the present study (cf. fig. 6 for lactate values). The buffer value of the blood (β_{NB} = 12.6 mmol · $pH^{-1} \cdot L^{-1}$) was calculated from haematocrit values measured under normoxia (19.9 ± 4.1%) using the equation of McDonald *et al.* (1980). The non-bicarbonate buffer value for gastrocnemius muscle cell water (β_{NB} = 29.8 mmol · $pH^{-1} \cdot L^{-1}$) was adopted from Pörtner (1990). Metabolite levels in the intracellular fluid compartments were calculated using eq. (1). Intracellular lactate levels expected from weak acid distribution were calculated based on plasma lactate levels and the pH gradient between intra- and extracellular fluids (eq. (4), $pK' = 3.73$).

$$Ci = Ce \cdot (10^{pHi - pK'} + 1)/(10^{pHe - pK'} + 1) \quad (mmol \cdot L^{-1}) \tag{4}$$

A comparison with the actual intracellular lactate concentrations should reveal whether the pH gradient influences the distribution of lactate between blood and tissues.

Differences were tested for significance at the 5% level by using Student's *t*-test for paired and unpaired samples.

Results

Figure 1 shows the oxygen consumption and the release of CO_2 by *Bufo marinus* during a stepwise reduction in ambient P_{O_2}. Since gas exchange was measured after long periods of preadaptation (12–14 h at each single P_{O_2}) CO_2 release more or less paralleled the oxygen consumption during normoxia and moderate hypoxia (P_{O_2} = 75 Torr) which resulted in a more or less constant respiratory exchange ratio (RE). At 21 Torr the large elevation in oxygen uptake was paralleled by a significant increase in CO_2 production leading to a respiratory exchange ratio significantly above the ratio at P_{O_2} = 75 Torr. At a P_{O_2} of 14 Torr no constant rate of oxygen consumption was observed. CO_2 production was transiently elevated, however, when the oxygen consumption had started to decrease steadily. Maximum RE values found under these conditions were 1.62 ± 0.22.

When the ambient P_{O_2} changed from 157 to 75 Torr, arterial P_{CO_2} and the venous P_{CO_2} of the hindlimb dropped by only 0.7 Torr (fig. 2). Bicarbonate levels in the plasma fell slightly, but insignificantly in both arterial and venous plasma and pH remained more or less constant. Only severe hypoxia ($P_{O_2} \leq 35$ Torr) caused a significant deple-

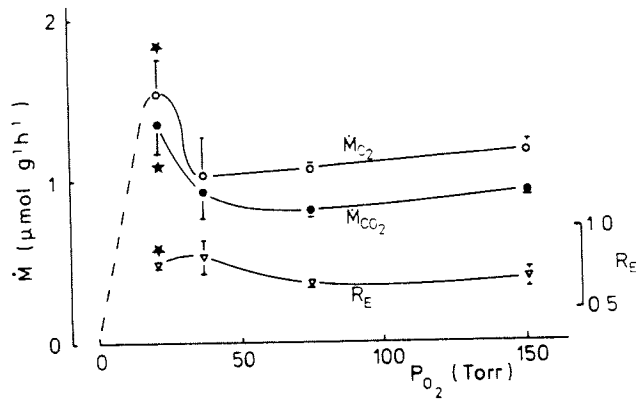

Fig. 1. Oxygen consumption ($\dot{M}_{O_2}$, adopted from Pörtner *et al.*, 1991) and CO_2 release ($\dot{M}_{CO_2}$) in *Bufo marinus* during a stepwise reduction in ambient P_{O_2}. The dashed line indicates the range of P_{O_2} where no steady state rate of oxygen uptake was maintained. The respiratory exchange ratio (RE) is given for those oxygen tensions where the rate of oxygen consumption was constant during the measurement period. ($\bar{x}$ ± SD, n = 4; * denotes a significant difference from values at 35 Torr or at 75 Torr, $P < 0.01$–0.02, paired samples).

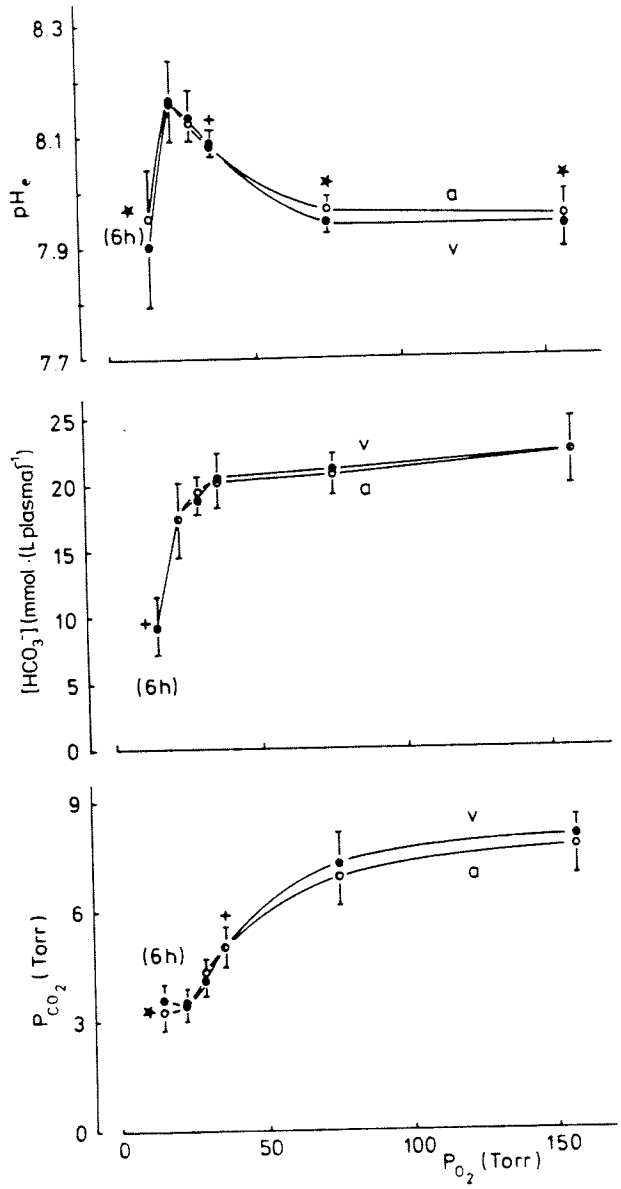

Fig. 2. Changes in the acid-base status of *Bufo marinus* during hypoxia. Arterial (a,○) and venous (v,●) plasma pH (pHe), bicarbonate levels and P_{CO_2} are depicted for animals subjected to long term (24 h, where not indicated) exposure to different degrees of hypoxia ($\bar{x} \pm$ SD; $n = 5$, except for incubation under $P_{O_2} = 14$ Torr, where $n = 4$; + denotes significant deviation from normoxic controls, $P < 0.01$, unpaired samples; * denotes significant difference between artery and vein, $P < 0.01$–0.05, paired samples).

tion of CO_2 stores, first indicated by the significant drop in P_{CO_2} at 35 Torr (fig. 2). At this P_{O_2} the drop in bicarbonate levels was still small, leading to a significant increase in plasma pH. During 24 h of incubation at P_{O_2} = 22 Torr, arterial and venous P_{CO_2} fell by more than half. Plasma bicarbonate reached values of 17.5 ± 2.7 mmol · L^{-1} in both artery and vein but this decrease was still not significant. Plasma pH reached a maximum value (8.16 ± 0.07 both arterial and venous) until the P_{O_2} reached 14 Torr when both pH and bicarbonate levels began to fall. The 6 h of incubation at this P_{O_2} also caused a rapid fall in plasma P_{CO_2} down to that seen after 24 h at 22 Torr. Hypoxia caused the haematocrit to increase significantly from 19.9 ± 4.1% under normoxia to 28.1 ± 6.9% at 29 Torr and to 30.0 ± 6.4% at 14 Torr.

The arteriovenous differences in pH, P_{CO_2} and bicarbonate levels were small at all oxygen tensions. At 157 and 75 Torr arterial pH was significantly elevated above the venous values. P_{CO_2} values were somewhat higher in venous than in arterial plasma. This difference was significant only after 6 h at P_{O_2} = 14 Torr (fig. 2).

A drop in intracellular P_{CO_2} also occurred during hypoxia (fig. 3). Similar Pi_{CO_2} values were found in the gastrocnemius and in the ventricle. In these tissues the significant drop in intracellular P_{CO_2} paralleled the change in blood P_{CO_2}. In the sartorius muscle, however, the P_{CO_2} was smaller than that found in the gastrocnemius and ventricle muscles (by 3 Torr). Owing to a high scatter of calculated P_{CO_2} values, the difference in P_{CO_2} was insignificant between sartorius and gastrocnemius muscles, but was significant between ventricular and sartorius muscles. (Non-directional experimental errors in the determination of tissue and plasma C_{CO_2}, and pH may have additive effects in the calculation procedure thus being responsible for the observed variability in Pi_{CO_2}, *cf.* Pörtner *et al.*, 1990). After 24 h of hypoxia at 22 Torr, the P_{CO_2} in the sartorius muscle was close to that seen in the gastrocnemius muscle and the ventricle. After 6 h at P_{O_2} = 14 Torr intracellular P_{CO_2} values were similar to those observed after 24 h at P_{O_2} = 22 Torr in all tissues.

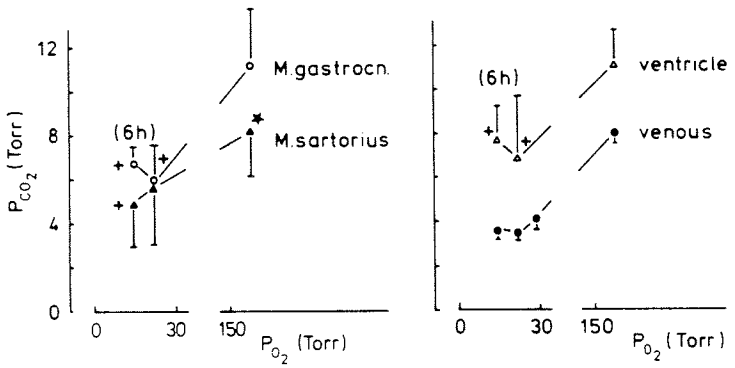

Fig. 3. Blood plasma and tissue (intracellular) P_{CO_2} values (*cf.* fig. 2) of the toad *Bufo marinus* exposed to different degrees of hypoxia ($\bar{x}$ ± SD, for values of *n* see fig. 2; + denotes significant difference from normoxic controls, $P < 0.01$–0.05, unpaired samples, * denotes significant difference from the intracellular P_{CO_2} of the ventricle, $P < 0.05$, paired samples).

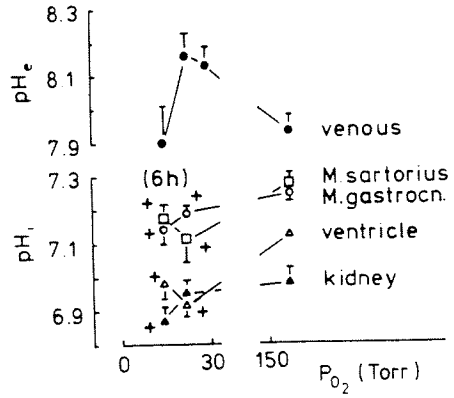

Fig. 4. Plasma (e: extracellular) and tissue (i: intracellular) pH values (*cf.* fig. 2) of the toad *Bufo marinus* exposed to different degrees of hypoxia ($\bar{x} \pm$ SD, for values of n see fig. 2; + denotes significant difference from normoxic controls, $P < 0.01$, unpaired samples).

The decrease in intracellular P_{CO_2} was not accompanied by an increase in intracellular pH (fig. 4). The pHi fell significantly in the tissues when the ambient P_{O_2} had reached values below 30 Torr. In the kidney this drop only occurred at a P_{O_2} of 14 Torr. Based on the analysis for gastrocnemius, sartorius and ventricular muscles, the fall in intracellular bicarbonate levels paralleled the decrease in venous plasma bicarbonate values until a P_{O_2} of 22 Torr was reached (fig. 5). At P_{O_2} = 14 Torr, the large drop in

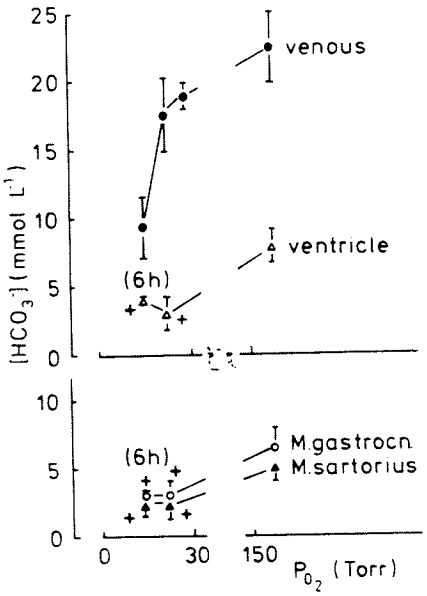

Fig. 5. Plasma and tissue (intracellular) bicarbonate levels (*cf.* fig. 2) of the toad *Bufo marinus* exposed to different degrees of hypoxia ($\bar{x} \pm$ SD, for values of n see fig. 2; + denotes significant difference from normoxic controls, $P < 0.01$–0.02, unpaired samples).

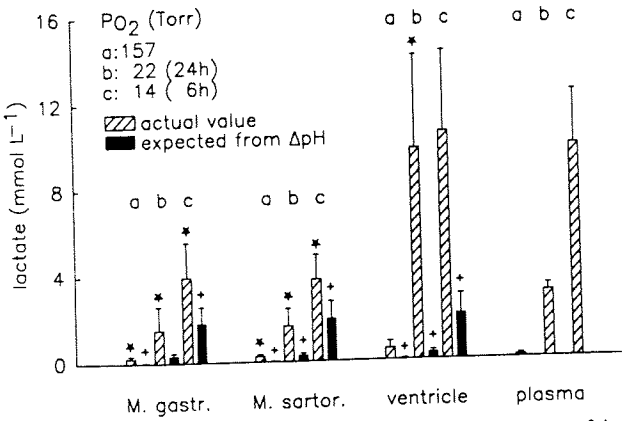

Fig. 6. Lactate concentrations in the cell water of various tissues and in the plasma of the toad *Bufo marinus* exposed to different degrees of hypoxia. The intracellular lactate levels (actual values) have been calculated based on measurements in tissue and plasma (*cf.* Pörtner *et al.* 1991) and are compared to those expected from the pH gradient (ΔpH) between intra- and extracellular compartments ($\bar{x} \pm$ SD, for values of n see fig. 2; * denotes significant difference from plasma concentrations, + identifies intracellular levels expected from ΔpH which are significantly below the actual values, $P < 0.05$, paired samples).

both extracellular pH and bicarbonate values appeared not to be related to a decline in the respective intracellular parameters. There was no significant change in the levels of ammonia in the plasma, the skeletal muscles, the ventricle and the kidney during hypoxia. Normoxic values of 0.31 ± 0.14 mmol · L^{-1} (plasma) or 0.24 ± 0.04 (gastrocnemius muscle), 0.24 ± 0.01 (ventricle) and 0.99 ± 0.18 μmol · (g fresh wt.)$^{-1}$ (kidney) were recorded.

The comparison of intra- and extracellular lactate levels under hypoxia revealed concentrations in the skeletal muscles below those found in the plasma. Lactate levels in arterial and venous plasma were identical. In the ventricle, however, cellular lactate concentrations reached values close to those in the plasma (fig. 6). In all cases intracellular concentrations expected from a weak acid distribution of lactic acid remained below those actually found. This discrepancy was large, especially in the ventricle.

Discussion

Lactate distribution and pH. A previous study by Jackson and Heisler (1983) addressed the question of whether low intra- and high extracellular lactate levels in anoxic turtles could be explained by the pH-dependent transmembrane distribution of undissociated lactic acid. The authors concluded that a permeation coefficient at least 10^4-fold higher for lactic acid than for lactate would be required and considered this to be most unlikely. However, recent studies on frog muscle, cardiac tissue and skeletal muscle membrane vesicles provided evidence for a pH-dependent lactate carrier (Mason and Thomas, 1988; De Hemptinne *et al.*, 1983; Roth and Brooks, 1990). The proposed mechanisms

of either an exchange of lactate$^-$ for OH^- anions or a symport of lactate$^-$ and H^+ would be equivalent to the facilitated movement of undissociated lactic acid and would represent an apparent increase in membrane permeability for this species. In accordance with low intra- and high extracellular pH values, this would explain low intra- and high extracellular lactate levels found during long-term hypoxia. However, the intracellular lactate levels actually found were somewhat higher than expected from a pH-dependent distribution in the skeletal muscles and even higher in the ventricle. This discrepancy could decrease if interstitial pH, which should be effective at the site of lactate exchange, was lower than plasma pH (*cf.* Pörtner *et al.*, 1990). Part of the discrepancy may also be related to the different rates of lactate formation by anaerobic glycolysis. In the resting skeletal muscles the rate of lactate production was small, so that lactate was closer to an equilibrium distribution according to pH. In the working ventricle the rate of lactate formation was much higher so that the intracellular concentration was far above that expected from equilibrium distribution. As a corollary, the pH gradient and the concentration gradient for lactate would be major driving forces causing an accelerated release of lactate into the plasma by use of a pH-dependent carrier mechanism. These considerations would support the conclusion that a large fraction of lactate seen in the plasma originated from the ventricle and other working aerobic organs which experienced anaerobiosis during maintenance of function in hypoxia (*cf.* Pörtner *et al.*, 1991).

High extra- and low intracellular lactate levels would suggest that osmotic water uptake by the intracellular space did not occur. This was actually found during an analysis of fluid compartments in anoxic turtles (Jackson and Heisler, 1983). Since such water movements can be excluded as unlikely and since the water content of toad erythrocytes does not respond to catecholamines the observed increase in haematocrit would rather indicate that erythrocyte recruitment occurred from the spleen (*cf.* Tufts *et al.*, 1987), possibly as a response to an increase in plasma catecholamine levels below the Pc (*cf.* Pörtner *et al.*, 1991).

CO_2 release and changes in P_{CO_2}. The oxygen consumption of *Bufo marinus* was regulated down to the critical P_{O_2}, below which oxygen uptake first increased before it finally fell (Pörtner *et al.*, 1991). At low oxygen tensions these changes were linked to an increased release of CO_2 (fig. 1). It can be assumed that, under normoxia and moderate hypoxia (P_{O_2} = 75 Torr), the respiratory exchange ratio (RE) was close to the respiratory quotient (RQ) of the animals. This RQ (0.76–0.79) reflects a mixed composition of the animal's food (beef liver) and suggests that, although being starved for at least 24 h, the animals were not relying on the body's carbohydrate stores. A large increase in RE to a value above the RQ is typical for the short term response to extreme hypoxia and is in accordance with a rapid unloading of CO_2 stores. High values of up to 6 have been recorded during short term measurements (Ultsch and Anderson, 1988). In the present and in a very recent study by Wood and Malvin (1991), however, hypoxic RE values still remained elevated at a level slightly but significantly above the RQ even during a stepwise reduction in P_{O_2} and a long term incubation at each oxygen tension.

Values as high as 1.6 (no 'steady state' value, therefore not shown in fig. 1) were detected when oxygen consumption decreased steadily during the final transition to asphyxia.

There are very few reports of venous blood gas parameters measured after non-obstructive cannulation in freely moving amphibians (*e.g.* Toews *et al.*, 1971). To evaluate the meaning of these values requires a consideration of the amphibian circulatory system (*e.g.* Burggren, 1988). Arterial and venous P_{CO_2} were very similar under all experimental conditions applied. This observation would either suggest that CO_2 production by the hindleg was too small to cause a large elevation in venous P_{CO_2} or, alternatively, that blood withdrawn from the femoral vein not only contained venous blood of higher P_{CO_2} from the tissues but also blood of low P_{CO_2} from the skin. In contrast to other vertebrates, the degree of systemic and pulmocutaneous blood supply to the skin and the use of the skin as a gas exchange organ (see Burggren, 1988) could, generally, decrease the measured gradients in arterial and venous parameters in the amphibians. The measured venous P_{CO_2} values may, as a result, be somewhat lower than the actual venous P_{CO_2} of the investigated tissues. This may be true even for the gastrocnemius muscle, although the femoral vein drains the leg. However, even if gas exchange via the skin influenced the venous parameters, this influence did still not completely eliminate the respiratory influence of the tissue. An arteriovenous P_{O_2} and oxygenation difference was reported by Pörtner *et al.* (1991). A potential difference in plasma P_{CO_2} (fig. 2) could be minimized by the associated Haldane effect in the erythrocytes.

The increase in ventilatory effort for the maintenance of oxygen consumption led to a significant drop in both arterial and venous P_{CO_2} even at P_{O_2} levels above the Pc. During long term incubation the changes in extracellular P_{CO_2} and in the intracellular P_{CO_2} of gastrocnemius and ventricular muscles were similar (fig. 3). Thus, changes in blood P_{CO_2} reflected the changes in P_{CO_2} for both tissues. Further investigations are required, however, to evaluate the P_{CO_2} gradient between intracellular fluid and venous plasma. This can be more accurately studied in animal species other than amphibians.

Only in sartorius muscle, during normoxia, was the intracellular P_{CO_2} lower than in the other tissues. This may be due to the specific location of the sartorius muscle which forms a thin band close to the skin on the ventral side of the thigh. Skin perfusion, especially of ventral areas, is high under normoxia (Boutilier *et al.*, 1986) and may contribute to reduce P_{CO_2} in peripheral areas of the body. However, mean intracellular P_{CO_2} of the sartorius muscle under hypoxia was close to the intracellular P_{CO_2} of the gastrocnemius and the ventricle. In the experiments, the animals were sitting in water with both the water and ambient air hypoxic. Aquatic hypoxia has been shown to reduce the blood flow to the skin, possibly in order to protect the body oxygen stores against oxygen loss to hypoxic water (Boutilier *et al.*, 1986). This mechanism could easily explain how hypoxic exposure caused a relative P_{CO_2} increase in sartorius muscle.

Gas exchange and acid-base regulation. The drop in blood and tissue P_{CO_2} should cause a respiratory alkalosis, if bicarbonate levels remain elevated. Accordingly, hypoxia in *Bufo marinus* led to an initial respiratory alkalosis in the plasma (fig. 2, *cf.* Boutilier and

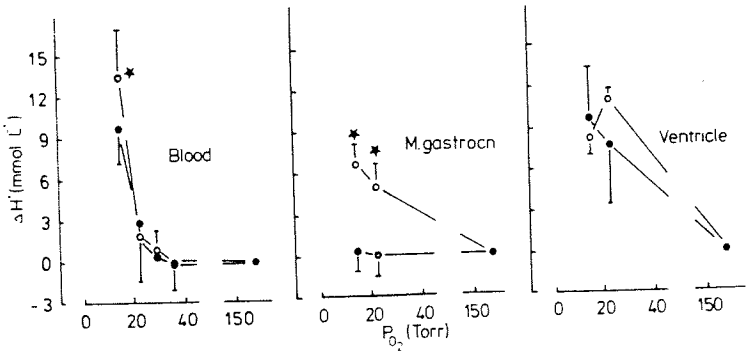

Fig. 7. Comparison of non-respiratory proton loads (○) of the intracellular compartments of various tissues and the (venous) blood with the proton quantities expected from metabolism (●) (based on Pörtner, 1987a, Pörtner *et al.*, 1991). For the blood, the latter quantity was calculated assuming that the accumulated lactate results from the dissociation of lactic acid in the blood ($\bar{x} \pm$ SD, for values of n see fig. 2; * denotes significant difference from the metabolic proton load, $P < 0.01$–0.02, paired samples).

Toews, 1977). To evaluate whether the changes in the acid-base status were influenced by non-respiratory processes (*i.e.* by metabolic proton turnover or by ion exchange processes), the origin of the observed pH change was investigated in the blood (fig. 7). The analysis reveals that non-respiratory protons began to accumulate at P_{O_2} values below 35 Torr. Proton quantities expected from the dissociation of plasma lactic acid (fig. 6) and the amount of non-respiratory protons were almost equal down to 22 Torr. However, non-respiratory protons exceeded lactate levels after 6 h at a P_{O_2} of 14 Torr in accordance with a compensation of the alkalosis via ion exchange mechanisms (see below).

A similar analysis (fig. 7) was possible for the gastrocnemius muscle. Consideration of the difference in intra- and extracellular P_{CO_2} was important for the accurate calculation of non-respiratory proton quantities in the tissues (eq. (3)). If this difference had been ignored and cellular bicarbonate levels had been calculated based on venous P_{CO_2}, their contribution to cellular buffering would have been underestimated. The results reveal a large discrepancy between non-respiratory proton quantities in the intracellular acid-base status and metabolic proton formation. The non-respiratory protons exceeded the metabolic proton formation by about 10 times. As a corollary, base equivalents were probably released from the skeletal muscle tissues into the blood, possibly to compensate for the decrease of P_{CO_2} in the gastrocnemius and/or the excessive proton formation by other tissues. Such a regulatory decrease in pHi could facilitate the pH-driven release of lactate (lactic acid) from the muscle tissue (see above). This pattern of a pHi decrease during extracellular alkalosis could be characteristic for hypoxic exposure in air, whereas long term anaerobiosis in turtles (submerged in water) caused a transient hypercapnia and a more or less similar decrease in pH in all considered body compartments (Jackson and Heisler, 1983).

The same analysis was used for the ventricle which exhibited high rates of lactate accumulation (see fig. 7). The analysis was preliminary in that, based on recent work

about the influence of organic phosphate degradation on the measurement of tissue buffer values (Pörtner, 1990), identical non-bicarbonate buffer values were assumed for the ventricle and for the gastrocnemius muscle. Even with a difference in buffer values the resulting error must be small, since the major proton fraction in the calculation (eq. (3)) resulted from the depletion of cellular bicarbonate. Both non-respiratory and metabolic proton quantities were probably close in this organ. Obviously, lactate formed in excess of the actual intracellular levels and the associated protons would have been released from the ventricle into the plasma in close to equimolar amounts (see above).

The release of base equivalents from the skeletal muscles did not cause an extracellular base excess under hypoxia and did not compensate for the observed excess of non-respiratory protons in the plasma seen after 6 h at a P_{O_2} of 14 Torr (fig. 7). For pH maintenance, the reduction in P_{CO_2} by increased ventilation and the net release of bicarbonate from intracellular sources would require the concomitant release of base equivalents into the environment (see Toews and Boutilier, 1986 for a discussion of potential sites and mechanisms in amphibians). The extracellular alkalosis indicates that the removal of base was delayed above the critical P_{O_2} and did not completely compensate for the decrease in P_{CO_2}.

During transition to severe hypoxia below the critical P_{O_2}, the large drop in plasma bicarbonate levels and the observation that bicarbonate was increasingly titrated by metabolic acid formation, indicate that the release of CO_2 via respiration was accelerated. Despite a stepwise reduction of ambient P_{O_2} during long term incubations the RE transiently reached a value of 1.6 during transition to asphyxia. Metabolic acid formation most likely also caused the even higher RE values (up to 6) in the short term experiments of Ultsch and Anderson (1988) on gas exchange in hypoxic turtles.

In conclusion, the critical P_{O_2} (Pc) in *Bufo marinus* is not only characterized by an increase in oxygen uptake, a reduction in oxygen delivery to peripheral tissues via the blood and the onset of an anaerobic energy production (Pörtner *et al.*, 1991). The onset of anaerobiosis also leads to an increased release of CO_2 which is mediated by the titration of bicarbonate stores and occurs via gas exchange. The loss of base equivalents and the onset of metabolic acidification causes the development of an uncompensated non-respiratory acidosis in the intracellular compartments. These processes may contribute to limit the resistance of the animals to long-term exposure to extreme hypoxia.

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References

Beis, I. and E.A. Newsholme (1975). The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. *Biochem. J.* 152: 23–32.

Bergmeyer, H.U. (1984). Methods of Enzymatic Analysis. 3rd ed., Vols. I–XII. Weinheim: Verlag Chemie.

Boutilier, R.G. and D.P. Toews (1977). The effect of progressive hypoxia on respiration in the toad *Bufo marinus*. *J. Exp. Biol.* 68: 99–107.

Boutilier, R.G., M.L. Glass and N. Heisler (1986). The relative distribution of pulmocutaneous blood flow in *Rana catesbeiana*: effects of pulmonary or cutaneous hypoxia. *J. Exp. Biol.* 126: 33–39.

Boutilier, R.G., M.L. Glass and N. Heisler (1987). Blood gases, and extracellular/intracellular acid-base status as a function of temperature in the anuran amphibians *Xenopus laevis* and *Bufo marinus*. *J. Exp. Biol.* 130: 13–25.

Boutilier, R.G. (1988). Control of arrhythmic breathing in bimodal breathers: amphibia. *Can. J. Zool.* 66: 6–19.

Burggren, W.W. (1988). Role of the central circulation in regulation of cutaneous gas exchange. *Am. Zool.* 28: 985–998.

De Hemptinne, A., R. Marannes and B. Vanheel (1983). Influences of organic acids on intracellular pH. *Am. J. Physiol.* 254: C178–C183.

Glass, M.L. and S.C. Wood (1983). Gas exchange and control of breathing in reptiles. *Physiol. Rev.* 63: 323–355.

Gros, G. and S.J. Dodgson (1988). Velocity of CO_2 exchange in muscle and liver. *Annu. Rev. Physiol.* 50: 669–694.

Heisler, N. (1986). Buffering and transmembrane ion transfer processes. In: Acid-Base Regulation in Animals, edited by N. Heisler. Amsterdam: Elsevier, pp. 3–47.

Jackson, D.C. and N. Heisler (1983). Intracellular and extracellular acid-base and electrolyte status of submerged anoxic turtles at 3 °C. *Respir. Physiol.* 53: 187–201.

Mason, M.J. and R.C. Thomas (1988). A microelectrode study of the mechanisms of L-lactate entry into and release from frog sartorius muscle. *J. Physiol.* 400: 459–479.

McDonald, D.G., R.G. Boutilier and D.P. Toews (1980). The effects of enforced activity on ventilation, circulation and blood acid-base balance in the semi-terrestrial anuran, *Bufo marinus*. *J. Exp. Biol.* 84: 273–287.

Pörtner, H.O. (1987a). Contributions of anaerobic metabolism to pH regulation in animal tissues: theory. *J. Exp. Biol.* 131: 69–87.

Pörtner, H.O. (1987b). Anaerobic metabolism and changes in the acid-base status: quantitative inter-relationships and pH regulation in the marine worm *Sipunculus nudus*. *J. Exp. Biol.* 131: 89–105.

Pörtner, H.O. (1990). Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* 81: 275–288.

Pörtner, H.O., R.G. Boutilier, Y. Tang and D.P. Toews (1990). Determination of intracellular pH after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* 81: 255–274.

Pörtner, H.O., L.M. MacLatchy and D.P. Toews (1991). Metabolic responses of the toad *Bufo marinus* to environmental hypoxia: an analysis of the critical P_{O_2}. *Physiol. Zool.* in press.

Roth, D.A. and G.A. Brooks (1990). Lactate and pyruvate transport is dominated by a pH gradient-sensitive carrier in rat skeletal muscle sarcolemmal vesicles. *Arch. Biochem. Biophys.* 279: 386–394.

Shelton, G. and R.G. Boutilier (1982). Apnoea in amphibians and reptiles. *J. Exp. Biol.* 100: 245–273.

Toews, D.P., G. Shelton and D.J. Randall (1971). Gas tensions in the lungs and major blood vessels of the urodele amphibian, *Amphiuma tridactylum*. *J. Exp. Biol.* 55: 47–61.

Toews, D.P. and R.G. Boutilier (1986). Acid-base regulation in the amphibia. In: Acid-Base Regulation in Animals, edited by N. Heisler. Amsterdam: Elsevier, pp. 265–308.

Tufts, B.L., D.C. Mense and D.J. Randall (1987). The effects of forced activity on circulating catecholamines and pH and water content of erythrocytes in the toad. *J. Exp. Biol.* 128: 411–418.

Ultsch, G.R. and J.F. Anderson (1988). Gas exchange during hypoxia and hypercarbia of terrestrial turtles: a comparison of a fossorial species (*Gopherus polyphemus*) with a sympatric nonfossorial species (*Terrapene carolina*). *Physiol. Zool.* 61: 142–152.

Withers, P.C. (1977). Measurement of $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$, and evaporative water loss with a flow-through mask. *J. Appl. Physiol.* 42: 120–123.

Wood, S.C. and G.M. Malvin (1991). Physiological significance of behavioral hypothermia in hypoxic toads, *Bufo marinus*. *J. Exp. Biol.* in press.