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ACID–BASE REGULATION AND BLOOD GASES IN THE ANURAN AMPHIBIAN, *BUFO MARINUS*, DURING ENVIRONMENTAL HYPERCAPNIA

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SUMMARY

Specimens of *Bufo marinus* were exposed to aerial and aquatic hypercapnia (5 % CO_2) in a closed, water recirculation system to evaluate mechanisms involved in the compensation of a respiratory acidosis in these animals. Arterial P_{CO_2} was elevated from about 9 mmHg (1 mmHg = 133·3 Pa) to 35 (1 h) and 37 mmHg (2 h), and gradually approached about 40 mmHg (24 h of hypercapnia). The typical hypercapnia-induced reduction in plasma pH from about 7·9 to below 7·4 was partially offset, at least during the first hours of hypercapnia, by a reduction in the inspired/arterial P_{CO_2} difference, presumably brought about by pulmonary hyperventilation. The predominant contributor to extracellular pH compensation, however, was a net gain of bicarbonate from the environment, mainly facilitated by ammonia excretion. Bicarbonate originating from the environment was accumulated in the body fluids, increasing the plasma concentration from the control of about 9 to 36 mmol l^{-1} after 24 h. Extracellular pH was compensated to only about 30 % of the shift expected at constant bicarbonate level and, according to the steady reduction of pH, non-bicarbonate buffering of CO_2 also contributed significantly to the elevation of bicarbonate. This relatively poor pH compensation (compared with fishes) could not be improved either by direct administration of bicarbonate into the bloodstream or by increased environmental ion concentrations. It is concluded that the availability of bicarbonate is not a limiting factor for pH compensation during hypercapnia, and that the inability of *Bufo* to accumulate bicarbonate to concentrations sufficient for better hypercapnia compensation is based on a constitutional 'bicarbonate threshold' of the resorbing and retaining structures for acid–base-relevant ions.

INTRODUCTION

Amphibians often encounter environmental hypercapnia as a result of CO_2 uptake from the water surrounding their highly permeable skin and/or rudimentary gills

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(Ultsch, 1976; Heisler, Forcht, Ultsch & Anderson, 1982), but may also become hypercapnic as a result of various innate activities such as diving, exercise or burrowing (Boutilier, Randall, Shelton & Toews, 1979*a*,*b*,*c*; Boutilier, McDonald & Toews, 1980; Boutilier & Toews, 1981; Shelton & Boutilier, 1982; Boutilier & Shelton, 1986). Chronic experimental exposure to elevated environmental CO_2 levels generally causes a pronounced fall of arterial plasma pH, which in some species is countered by plasma bicarbonate elevation. Plasma pH compensation in amphibians is far from complete (Toews & Boutilier, 1986) and is certainly lower than in most fish species (Heisler, 1980, 1986*b*). Indeed, in some amphibians, this type of acid–base regulation is completely lacking (Boutilier, 1981; Heisler *et al.* 1982; Stiffler, Tufts & Toews, 1983). The relative compensation of plasma pH typically ranges from 0 to 35 % during chronic hypercapnia in amphibians (Toews & Boutilier, 1986), with the one known exception being the Amazonian apodan, *Typhlonectes compressicauda*, capable of almost 80 % compensation (Toews & Macintyre, 1978).

Much of the available information on pH compensation during hypercapnia in amphibians has been collected in the semi-terrestrial toad, *Bufo marinus*. The degree of extracellular pH compensation during chronic exposure to an environment of 5 % CO_2 is comparatively low (17–30 %; Boutilier *et al.* 1979*a*), whereas pH in several intracellular body fluid compartments is restored to a much higher degree (Toews & Heisler, 1982). Although these data suggest that pH in intracellular body compartments is better protected against disturbances than the extracellular pH, little information is available as to the source of the intracellular and extracellular bicarbonate increases or on the potential of bicarbonate-equivalent ion transfer processes between the environment and various fluid compartments of the animal.

The urinary bladder plays a key role in generating some of the bicarbonate required for pH compensation, and there is also evidence that mobilization of internal calcareous deposits may contribute to an elevation of the total bicarbonate pool during chronic CO_2 exposure (Tufts & Toews, 1985). Quantification of the contribution of individual mechanisms to the overall compensation is made difficult because of the lack of relevant data. In all of the earlier studies of chronic CO_2 exposure in *Bufo marinus*, the animals were bathed throughout the experiment in a volume of water approximating to their body mass (about 250–500 ml). As stated by Toews & Heisler (1982) this water must not only have received the excreted end products of nitrogen metabolism, but elimination of H^+ to or uptake of bicarbonate ions from the environment may have been limited by the availability of adequate amounts of counterions in the water, a factor well-known as being limiting in fish (Heisler, 1986*b*).

The aim of the present study was to quantify the contribution of transepithelial ion transfer processes to pH compensation, and to evaluate the extent to which the limitation imposed by a small environmental water pool accounts for the much lower degree of extracellular pH compensation in *Bufo* than generally observed in fishes. This study was also intended to test the suggestion that comparatively incomplete

extracellular pH compensation may be related to a bicarbonate 'threshold' (Heisler, 1986*c*) beyond which any further elevation of the bicarbonate concentration is impossible. In addition, we tested the compatibility of arterial blood P_{O_2} and O_2 saturation data during hypercapnia with model predictions of O_2 transport in animals with cardiovascular shunts (Wood, 1982).

MATERIALS AND METHODS

Animals and preparations

Specimens of adult *Bufo marinus* (325–568 g, both sexes) were obtained from Charles D. Sullivan Co. Inc. (Nashville, TN, USA). The animals were held in large terraria at 20 °C with free access to water and dry areas for at least 1 month before the experiments. They were treated against internal parasite infestation and infections, and fed regularly on beef liver and mice.

Approximately 48 h prior to an experiment, the animals were anaesthetized by immersion in a 0·75 % solution of MS-222 (buffered to pH 7 with $NaHCO_3$) and intubated intratracheally for ventilation during surgical placement of an indwelling femoral artery cannula (Boutilier *et al.* 1979*a*). After surgery the animals were washed with fresh water and artificially ventilated until they regained consciousness.

Experimental apparatus

After recovery from anaesthesia, the animal was placed in a 2·7-litre water recirculation system (Fig. 1). Water was circulated at 1·5 l min^{-1} at 20 ± 0·5 °C. The experimental chamber was supplied with humidified gases at 20 ± 0·05 °C produced by a gas mixing pump (Wösthoff, Bochum, FRG) at a rate of 300 ml min^{-1}. A second gas mixing pump delivered gases to the fritted glass bottom of the aeration column. Oxygen concentration was maintained at 20 % during normocapnic and hypercapnic conditions.

Water from the recirculation system was delivered continuously by a roller pump (P_1, Fig. 1) to a 'delta-bicarbonate' monitoring system (Heisler, 1978, 1984). The water was fed through a series of three glass columns through the sintered glass bottoms of which a gas mixture of 1 % CO_2 in nitrogen was bubbled. The gas was provided by a Wösthoff gas mixing pump specially selected for long-term stability. The entire system was maintained at 30 ± 0·05 °C. A temperature different from that of the animal was deliberately chosen to improve electrode stability and response (see Heisler, 1988*a*). The water was fed over an electrode chain consisting of a pH glass electrode and a double electrolyte bridge Ag/AgCl reference with sleeve diaphragm (Ingold, Frankfurt, FRG) before being returned to the aeration column. The drift of the electrode chain was experimentally determined to be <0·001 pH unit per 24 h. Electrodes were connected to the recording system by way of high-impedance isolation amplifiers (Model 87, Knick, Berlin, FRG) to avoid ground loops and electromagnetic disturbances. The system was grounded by a platinum electrode placed in the water close to the sensing electrode. Changes in the pH of the water

thus standardized for temperature and P_{CO_2} will be the result of changes in bicarbonate concentration. The system was calibrated in absolute terms by the addition of precisely known amounts of HCl and $NaHCO_3$ standards.

The ammonia concentration ($[NH_4^+]$) of the water was automatically determined at certain intervals (see below) by means of an ammonia gas electrode (HNU Systems Inc., Newton, MA, USA), and a set of roller pumps, solenoid valves, and a microcomputer assembly. By activating the appropriate pumps and solenoid valves either NH_4Cl standards or water samples from the recirculation system were introduced into the thermostatted ($30 \pm 0{\cdot}05\,°C$) electrode chamber (2 ml) and mixed with a magnetic stirrer with about 2% of $10\,mol\,l^{-1}$ NaOH. Stirring was maintained when the pumps (P_2 and P_3, Fig. 1) were stopped after appropriate chamber flushing. Alkalinization of the solution to a pH higher than 12 converted ammonium to physically dissolved ammonia, which readily diffused past the hydrophobic membrane, affecting the pH of the inner electrode solution. The output from this single unit pH electrode was connected to an Orion Ionalyser (Orion Research, model 901), which was automatically calibrated and set by the microprocessor with two standards bracketing the ammonium concentrations in the recirculation system. The data output was recorded by means of a chart recorder, and the actual concentration values printed out after stabilization of the electrode reading. Data were thus automatically collected with an accuracy of $>0{\cdot}01\,mmol\,l^{-1}\,NH_4^+$ for periods of several days. The alkalinized water samples were discarded.

The amounts of bicarbonate-equivalent ions transferred between animal and environmental water were calculated as detailed before (Heisler, 1978, 1984, 1986*a*).

Procedure

After placing the toad in the experimental chamber, the recirculation system was flushed with fresh water for approximately 12 h. Thereafter, the system was filled with an artificial medium prepared by adding sodium bicarbonate and choline chloride to Göttingen tapwater, bringing the concentrations of Na^+ and Cl^- to $3{\cdot}5\,mmol\,l^{-1}$ and the $[HCO_3^-]$ to $2{\cdot}5\,mmol\,l^{-1}$. This ambient water was recirculated and completely exchanged with fresh water of the same ion concentration every 12 h. During an initial 24 h period control measurements of bicarbonate and ammonia release were monitored and the experiment was continued only when the data indicated that the animals had reached steady-state conditions. Two blood samples were withdrawn from each animal towards the end of this control period and analysed for blood gases and pH. The gas supply to the aerial and aquatic portions of the chamber was then switched from air to a gas mixture of 5% CO_2, 20% O_2 and 75% N_2. This time was defined as time zero for all subsequent measurements. Blood samples (220 μl) and water samples (for ammonia determination) were taken at 15 and 30 min, 1, 2, 4, 8 and 24 h. An additional water sample was taken at 16 h. The bicarbonate concentration of the ambient water was monitored continuously. At the end of this second period the toads (hypercapnia maintained) were infused intra-arterially (at $0{\cdot}35\,ml\,min^{-1}$ by a roller pump) with $4{\cdot}5\,mmol\,kg^{-1}$ body mass of

$NaHCO_3$ solution in about 8–10 min. Blood and water samples were analysed at the end of the infusion period (new time zero). Blood samples were analysed 15 and 30 min 1, 2, 4 and 24 h post-infusion and water samples at 1, 2, 3, 4 and 24 h post-infusion.

Analyses

Blood samples were analysed at the same temperature as the toad for pH, P_{CO_2} and P_{O_2} using appropriate microelectrodes (Radiometer, BMS 3). The oxygen and carbon dioxide electrodes were calibrated with humidified gas mixtures ($20 \pm 0{\cdot}05$ °C) produced by a Wösthoff pump, with each measurement being referenced to a gas mixture close to the expected value. The pH electrode was calibrated with Radiometer precision buffer solutions S1500 and S1510, with each sample being referenced to the high pH buffer. A 10 μl portion of each well-mixed sample was used for haematocrit determination using a micro-haematocrit centrifuge (Compur). Blood used for measurement of gas tensions (175 μl) was suspended in heparinized saline and re-infused into the animal without any apparent haemolytic effects. Nevertheless, haematocrit declined from 26 to 18 % over the course of the experiments. Plasma bicarbonate concentrations were calculated by application of the Henderson–Hasselbalch equation using values for αCO_2 and $pK'_{1,app}$ from the equations derived by Heisler (1984; note: the sign of the last line term of the α-formula was misprinted, and should read +).

In vitro oxygen dissociation curves were constructed using the mixing method (Haab, Piiper & Rahn, 1960) in which various blood O_2 saturations are prepared for P_{O_2} measurement by anaerobically mixing known proportions of oxygenated and deoxygenated whole blood (Scheid & Meyer, 1978). Samples of blood, pooled from four animals, were equilibrated in intermittently rotating glass cuvettes ($20 \pm 0{\cdot}5$ °C) with gas mixtures containing 1 % or 5 % CO_2 in either air or nitrogen. Two complete curves were constructed from measurements of P_{O_2} at prepared saturations of 10, 30, 50, 70 and 90 %. Measurements of P_{CO_2} and pH were made on P_{50} samples.

RESULTS

Upon exposure to 5 % CO_2 in both the water and the air space of the recirculation system, arterial P_{CO_2} (Pa_{CO_2}) levels rose from the normocapnic control of 9·3 mmHg to values of 35 and 37·5 mmHg after 1 and 2 h, respectively, and gradually approached Pa_{CO_2} levels of about 40 mmHg after 24 h (Fig. 2). During the first 4 h of hypercapnia, the average arterial pH (pHa) decreased from 7·907 to 7·397, rising slightly to 7·433 by 24 h. Arterial plasma bicarbonate concentrations increased from 25·8 mmol l^{-1} at time 0 to approximately 34 mmol l^{-1} within the first 2 h of hypercapnic exposure, reaching levels of 36·5 mmol l^{-1} after 24 h.

Exposure to hypercapnia was also accompanied by a marked elevation in arterial P_{O_2} from control levels of 42–44 mmHg to values in excess of 80 mmHg after 15 and 30 min (Fig. 3). Thereafter Pa_{O_2} levels declined in an exponential fashion, reaching

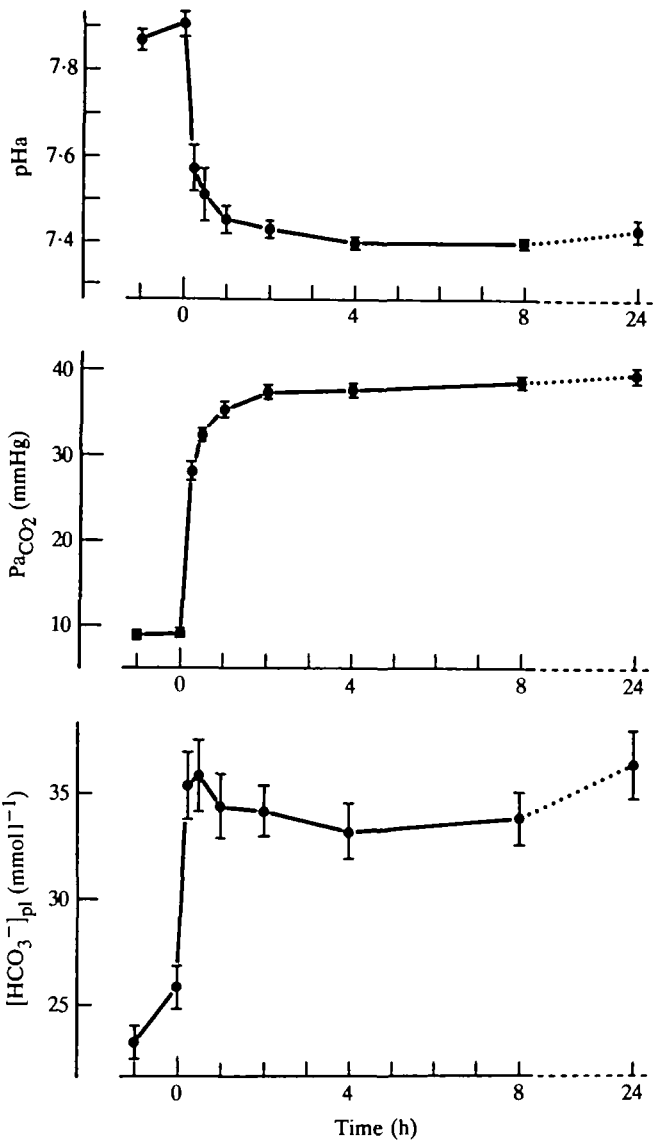

Fig. 2. Changes of arterial pH, P_{CO_2} and plasma bicarbonate concentration during exposure to environmental hypercapnia. Time zero = the onset of hypercapnia ($\bar{x} \pm$ S.E.; number of animals, $N = 9$).

72 mmHg after 8 h and 59 mmHg after 24 h. During the initial respiratory acidosis, an important response of the animal is a considerable CO_2-stimulated hyperventilation (Macintyre & Toews, 1976; Boutilier *et al.* 1979*a*), which leads to an increase in Pa_{O_2} and corresponding decrease in the P_{CO_2} difference between arterial blood and inspired gas (Fig. 4). The elevation of P_{CO_2} following acute exposure to hypercapnia appears to be a slow process, requiring at least 1–2 h before complete equilibrium is

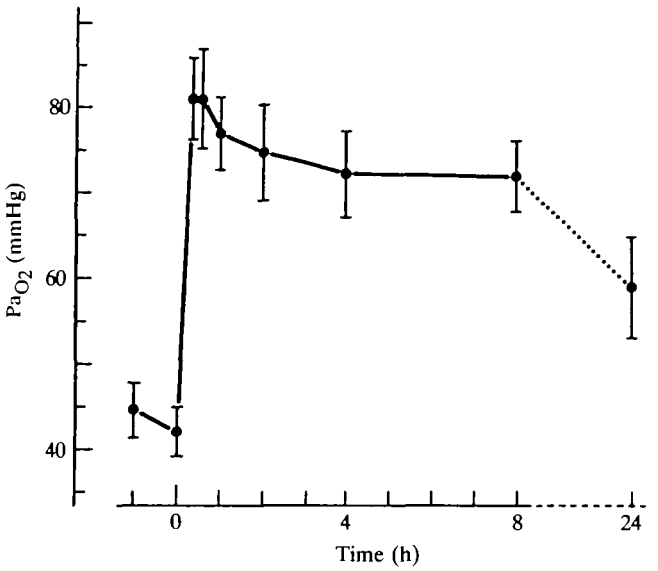

Fig. 3. Changes in arterial blood P_{O_2} during exposure to environmental hypercapnia. Time zero = the onset of hypercapnia ($\bar{x} \pm$ S.E.; number of animals, $N = 9$).

attained. However, following the initial period of CO_2 disequilibrium, it is clear that the animal must continue to hyperventilate, since even after 24 h of exposure, the P_{CO_2} gradient between inspired and arterial blood is only 60 % of that seen when animals are breathing air (Fig. 4).

An analysis of arterial blood O_2 saturation by plotting *in vivo* Pa_{O_2} values on O_2 dissociation curves determined *in vitro* at pH and P_{CO_2} levels characteristic of control and hypercapnic conditions is shown in Fig. 5. O_2 saturation levels at 4 and 8 h (after CO_2 equilibrium had occurred, see Figs 2 and 4) were slightly higher than those seen prior to CO_2 treatment, whereas after 24 h of CO_2 exposure, the O_2 saturation of arterial blood was not significantly different from control conditions (Fig. 5). Certainly, the analysis assumes two steady curve positions even though there are small deviations in pHa and Pa_{CO_2} from each of the curves. However, given the Bohr factor ($\Delta \log P_{50}/\Delta pH$) of $-0{\cdot}23$, it is equally certain that such deviations would hardly affect the general conclusions of this analysis.

During the normocapnic control period, bicarbonate was released from the animals to the water at a rate of 84 μmol h^{-1} kg^{-1} body water, which is only about 12 % of the average rate of NH_4^+ efflux of 662 μmol h^{-1} kg^{-1} body water, resulting in an H^+-equivalent excretion (the difference between NH_4^+ and HCO_3^- release) of 578 $\pm$ 66 μmol h^{-1} kg^{-1} body water ($\bar{x} \pm$ S.E., Fig. 6). Upon exposure to hypercapnia, the release of bicarbonate stopped and reversed to an initial, statistically significant, but small bicarbonate gain which thereafter subsided at a rate of 2·7 μmol h^{-1} kg^{-1} body water between hours 1 and 8 of hypercapnia. Over the last 16 h of hypercapnia, the bicarbonate concentration of the water began to rise again at

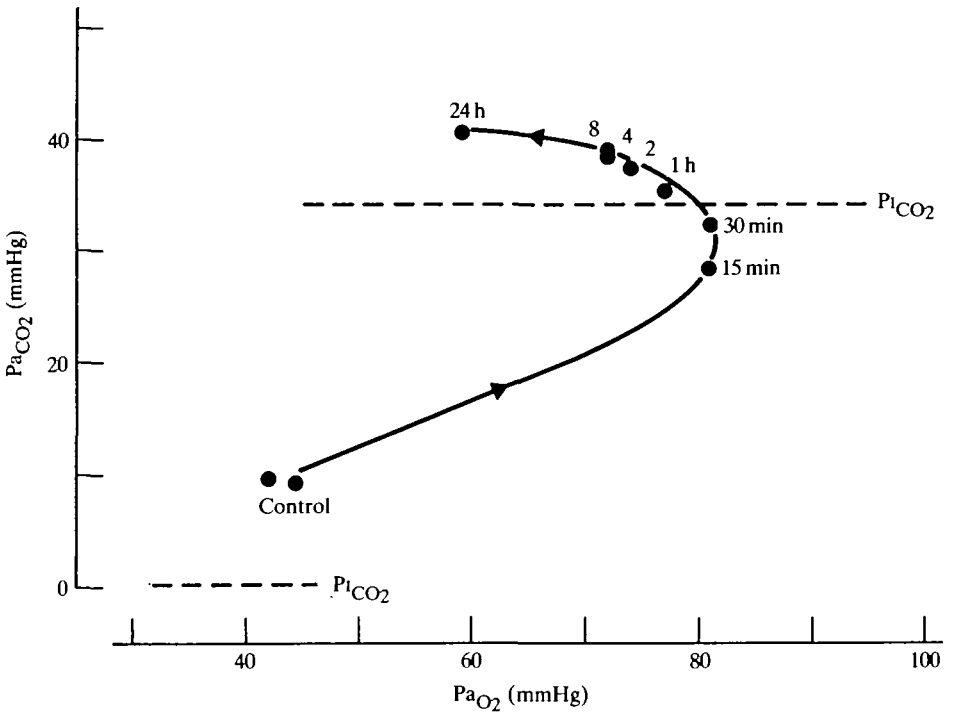

Fig. 4. Changes in arterial blood P_{O_2} and P_{CO_2} during exposure to environmental hypercapnia. Levels of inspired P_{CO_2} during the control period and hypercapnia are indicated by dashed lines. Values are means of data presented in Figs 2 and 3 on nine specimens of *Bufo marinus*.

a rate of 137 μmol h^{-1} kg^{-1} body water. NH_4^+ excretion, however, was increased above the control rate over the entire 24-h period of hypercapnia (771 μmol h^{-1} kg^{-1} body water. This pattern of bicarbonate and NH_4^+ release resulted in a net (hypercapnic minus control) H^+-equivalent excretion of 3·24 mmol kg^{-1} body water over the 24-h hypercapnic period, equivalent to a net gain of bicarbonate from the environment.

When the acid–base status had reached a *quasi* steady-state after 24 h of hypercapnia (Fig. 2; Boutilier *et al.* 1979*a*; Toews & Heisler, 1982), the animals were subjected to an intra-arterial infusion of 4·5 mmol kg^{-1} body water of $NaHCO_3$, resulting in transient rises of arterial pH, P_{CO_2} and bicarbonate concentration (Fig. 7). Analyses of the changes in bicarbonate and NH_4^+ content in the environmental water showed that about 90% of the infused bicarbonate (4·0 mmol kg^{-1} body water) was excreted by the animal within the first 2 h post-infusion (Fig. 7). Although arterial pH and the plasma bicarbonate concentrations decreased following $NaHCO_3$ infusion, both were slightly but significantly elevated above the pre-infusion values 24 h post-infusion (Fig. 7). Bicarbonate infusion had no effect on arterial P_{O_2} levels which ranged from 57·8 ± 4·9 to 63·4 ± 4·6 mmHg ($\bar{x}$ ± S.E.) at the sample periods shown in Fig. 7.

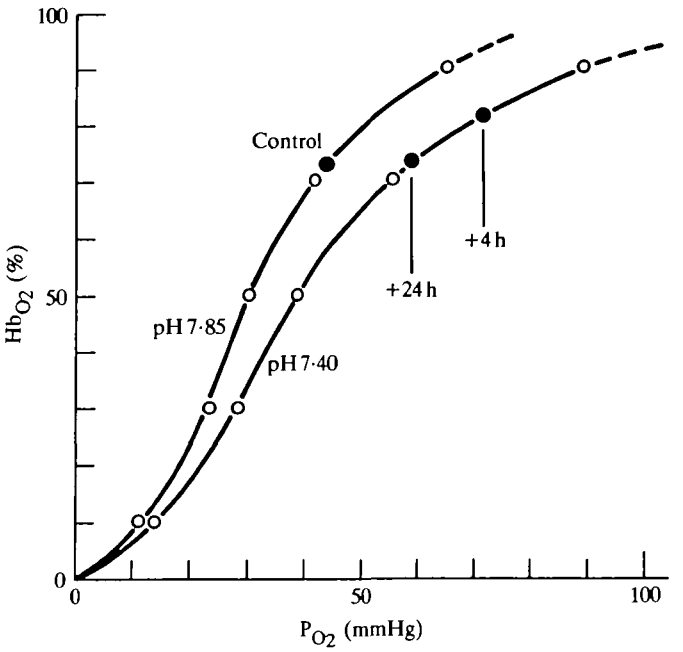

Fig. 5. *In vitro* oxygen dissociation curves for blood of *Bufo marinus* at 20°C. Open circles indicate the means of triplicate measurements made on a single blood pool from four animals. Closed circles are mean *in vivo* arterial blood P_{O_2} values during control conditions and hypercapnia (see Figs 3, 4) on nine *Bufo marinus*. The curves were prepared at P_{CO_2} levels characteristic of normocapnic and hypercapnic animals in the present study. The values for pH were determined on P_{50} blood samples.

DISCUSSION

Exposure of *Bufo marinus* to elevated levels of environmental CO_2 leads to a progressive, albeit relatively slow, elevation in arterial P_{CO_2} values, which attain higher than environmental levels only after 1–2 h of hypercapnia (Figs 2, 4). This initial 1–2 h of net CO_2 uptake by the animal represents the time required for CO_2 equilibration of the various tissue compartments (Toews & Heisler, 1982). The marked increase in ventilation which occurs upon exposure of *Bufo marinus* to elevated ambient CO_2 levels (Macintyre & Toews, 1976) is almost certainly responsible for the considerable reduction in the difference between arterial and inspired P_{CO_2} during continued CO_2 exposure (Fig. 4; Boutilier *et al.* 1979*a*), which probably also applies to the large rise in Pa_{O_2} during hypercapnia. An alternative, though not mutually exclusive, explanation of the rise in Pa_{O_2} during hypercapnia can be based on existing models of O_2 transport in animals with cardiovascular shunts (Wood, 1982; Wood & Hicks, 1985; Wood, Glass, Andersen & Heisler, 1987), or a redistribution of R–L shunt fractions to the systemic cardiac outflow vessels (see Ishimatsu, Hicks & Heisler, 1987).

In comparison with fish and mammals, the comparatively low arterial saturation in amphibians (see normocapnic curve, Fig. 5) can be attributed to systemic venous

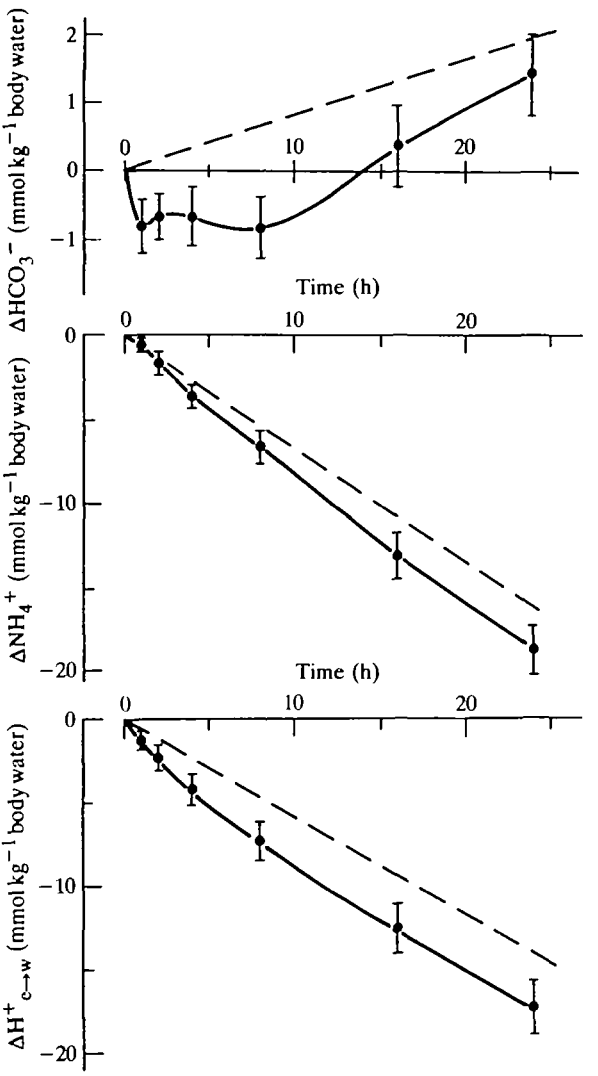

Fig. 6. Changes in the amounts of HCO_3^- and NH_4^+ in the environmental water during hypercapnia. The control line was extrapolated from a 24 h control period immediately before hypercapnia. Cumulative H^+ release from the extracellular space to the environment ($\Delta H^+_{c\rightarrow w}$) is calculated as the difference between the release of NH_4^+ and HCO_3^- ($\bar{x} \pm$ S.E.; number of animals, $N = 9$).

admixture with pulmonary venous blood (right to left cardiac shunt) within the undivided ventricle. Under these physical conditions, the P_{O_2} of a mixture of pulmonary and mixed venous blood is dependent upon the resulting O_2-saturation (Wood, 1982), the principle being the same as for the *in vitro* mixing method for O_2 curve construction (Haab *et al.* 1960; Scheid & Meyer, 1978). Pa_{O_2} is thus a dependent variable of O_2 saturation (Wood, 1982) and increases in arterial oxygen

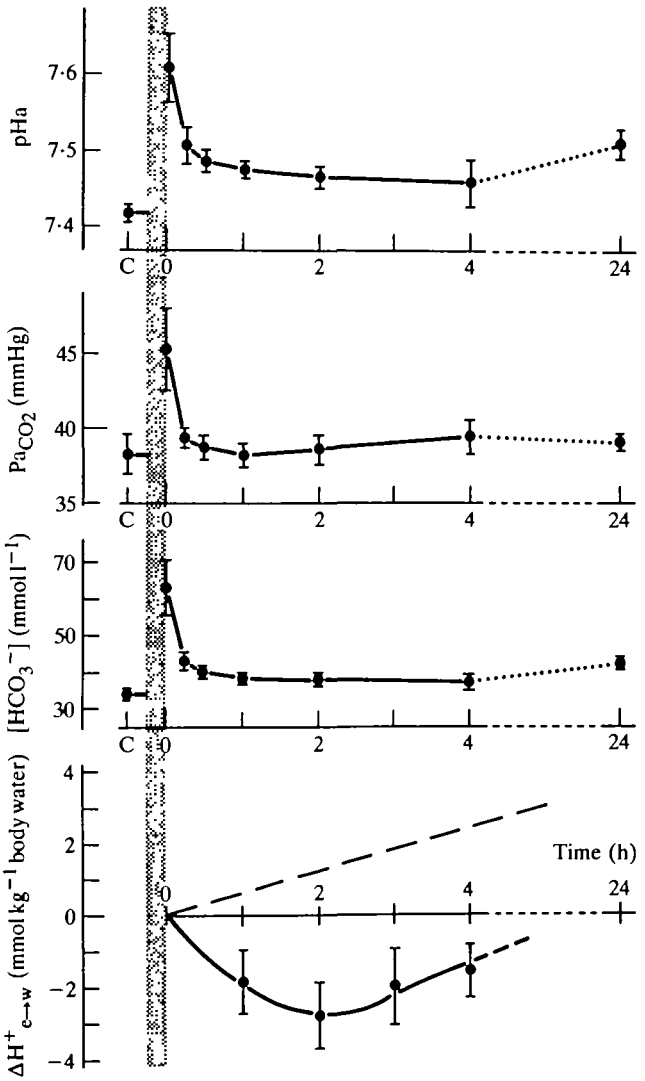

Fig. 7. Arterial pH, P_{CO_2}, plasma bicarbonate concentration and cumulative H^+ excretion to the environmental water following intra-arterial $NaHCO_3$ infusion of 4·5 mmol kg^{-1} body water. The shaded area indicates the period of infusion. The animals had been exposed to environmental hypercapnia (5 % CO_2) for 24 h prior to infusion. Negative values for $\Delta H^+_{e\rightarrow w}$ indicate net base excretion to the environmental water ($\bar{x} \pm$ S.E.; number of animals, $N = 5$). The dashed line represents the prehypercapnic control $\Delta H^+_{e\rightarrow w}$.

tensions during hypercapnia could be interpreted as the result of the pH-induced decrease in haemoglobin O_2-affinity. Hyperventilation, however, could play an important role in elevating the P_{O_2} of the pulmonary venous return, and therefore the Pa_{O_2}, by providing a more saturated precursor for intraventricular mixing. The relationships are complex, however, as the carbon dioxide contents, and therefore

pH values, of the various possible bloodstreams (see Ishimatsu *et al.* 1987; G. M. Malvin & N. Heisler, in preparation) may also change in a dynamic fashion. Regardless of the underlying mechanism, the end result is that the levels of arterial O_2 saturation during a condition of respiratory acidosis were maintained at, or slightly above, those seen in control animals breathing air. Furthermore, it is evident that there is a reciprocal interaction between the two respiratory gases; the gradual increase in Pa_{CO_2} and corresponding decrease in Pa_{O_2} as hypercapnia continues (Fig. 4) are probably the result of diminished ventilation. The latter is well-established for chronic hypercapnic states in mammals where adjustments in cerebrospinal fluid pH, through increases in cerebrospinal fluid bicarbonate concentrations, cause some decrease in ventilatory drive with time.

The reduction in the inspired–arterial P_{CO_2} difference during hypercapnia (Fig. 4) can be viewed as an initial compensatory response to offset further decreases in arterial pH. Though evidence is lacking as to a direct response of the animals to elevated CO_2 levels *per se*, or to a CO_2-mediated change in arterial oxygenation (Fig. 5), the common effect of both mechanisms is a reduction of the hypercapnia-induced change in plasma pH. The initial increase of plasma bicarbonate within the first hour of CO_2 exposure can, at least in part, be attributed to physicochemical action of the extracellular non-bicarbonate buffers. The slope of the apparent extracellular buffer line ($\beta = \Delta[HCO_3^-]/-\Delta pH$) during this period has previously been found to vary between 6·2 (Boutilier *et al.* 1979*a*) and 14 mequiv pH unit^{-1} l^{-1} (Toews & Heisler, 1982). The apparent buffer slope determined in the course of the present study was not significantly different from that of true plasma (20·4 mequiv pH unit^{-1} l^{-1}) when blood samples were equilibrated *in vitro* (Boutilier *et al.* 1979*a*). It is difficult to judge to what extent the elevated levels of counterions and larger volume of the ambient water in the present study were responsible for the comparatively greater degree of initial compensatory accumulation of plasma bicarbonate (Fig. 8).

Some indirect evidence suggests that improved reabsorption of bicarbonate is partially responsible for the improved early pH compensation. Tufts & Toews (1985) described a marked reduction of the initial apparent buffer slope (1·9 mmol pH unit^{-1} l^{-1}) in bladder-bypassed toads, indicating the key role of net bicarbonate reabsorption through the bladder wall. Indeed it has been shown that the apparent *in vivo* buffer slope for intact animals without access to water (6·4 mmol pH unit^{-1} l^{-1}) was not significantly different from that observed for their hydrated counterparts (Boutilier *et al.* 1979*b*). Further evidence for the acid–base-relevant potential of the toad bladder comes from *in vitro* studies on hemibladder preparations, showing increased rates of H^+ and NH_4^+ excretion during respiratory and metabolic acidoses (Frazier & Vanatta, 1971, 1973). The skin of amphibians is also a well-known exchanger of acid–base-relevant ions (see Evans, 1986, for a review). Taken together, these data suggest that mechanisms additional to passive blood buffering, such as transfer of bicarbonate-equivalent ions between the environment and/or various body fluid compartments, may influence the initial *in vivo* buffer slope to a considerable extent. Although an activity-related metabolic acidosis could,

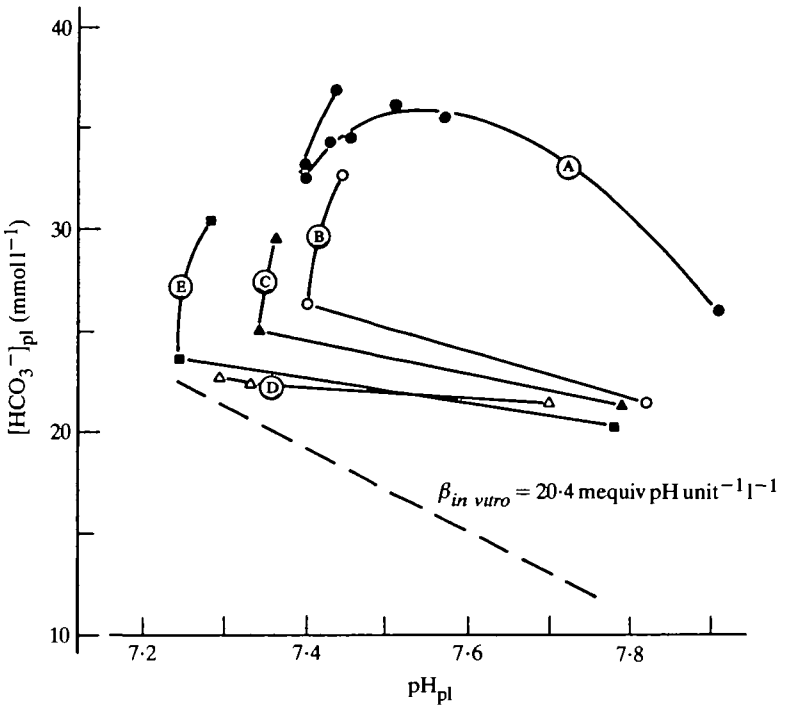

Fig. 8. Relationship between arterial plasma pH and bicarbonate concentration as a function of time after exposure of *Bufo marinus* to environmental hypercapnia (5 % CO_2). (A) Data for all stages of hypercapnia of the present experimental series (i.e. Fig. 2), starting from the normocapnic control values on the far right. (B) Hydrated toads (Toews & Heisler, 1982). (C) Hydrated toads (Boutilier, Randall, Shelton & Toews, 1979*a*). (D) Bladder-bypassed toads (Tufts & Toews, 1985). (E) Dehydrated toads (Boutilier *et al.* 1979*b*). Data in lines B–E include normocapnic control values (farthest right), 1–2 h of hypercapnia (next value left) and 24 h of hypercapnia (next value, usually with higher bicarbonate concentration). Dashed *in vitro* true plasma buffer slope adopted from Boutilier *et al.* 1979*a*. All data for 25 °C except for the present study (20 °C).

in principle, explain the lower *in vivo* buffer slopes described in earlier studies, the increased potential for cutaneous net bicarbonate uptake due to higher sodium and bicarbonate levels in the ambient water of the present study certainly facilitated an earlier rise in plasma bicarbonate (Fig. 8).

The differences between the present study and others utilizing the same degree of hypercapnia (5 % CO_2) are largely due to an earlier contribution by active compensatory mechanisms in the present study (Fig. 8). However, the overall degree of extracellular pH compensation at the end of 24 h of hypercapnia (about 30 %) was not appreciably different from that observed by Toews & Heisler (1982). A similar degree of compensation was achieved in dehydrated toads (about 28 %), which was slightly more than in their hydrated counterparts (Fig. 8). This phenomenon is not easily explained, particularly when one considers that the dehydrated animals experienced a more severe acidosis (Boutilier *et al.* 1979*b*).

There can be little doubt that the bladder plays a dominant role in the overall correction of such acid–base disturbances, since elimination of the bladder as an exchange surface prevents any compensation from occurring in otherwise intact and freely moving animals (Tufts & Toews, 1985). The latter data also imply a relatively low potential for acid–base compensation by the skin and kidneys.

In contrast to fish, a general trend of limited pH compensation during hypercapnia in air-breathing lower vertebrates is apparent (Heisler, 1986*c*). Since air-breathing in lower vertebrates is usually coupled with higher levels of extracellular P_{CO_2} and HCO_3^- for a given pH (Robin, Bromberg & Cross, 1969), the limited compensation in these animals may reflect an inability to accumulate bicarbonate to the extremely high levels that would be required for complete pH restoration during hypercapnia. This limitation does not hold for water-breathing fish with their considerably lower levels of plasma bicarbonate. Their potential for further bicarbonate elevation during hypercapnia, without causing intolerable disturbances in electrolyte balance (i.e. too low $[Cl^-]$), must accordingly be much greater than that of bimodally breathing vertebrates (Heisler, 1986*c*). The plasma bicarbonate concentration in *Bufo marinus* does not become elevated beyond about $30\,mmol\,l^{-1}$, irrespective of the levels of hypercapnic exposure (D. P. Toews, personal communication). This maximal level of bicarbonate or 'bicarbonate threshold' appears to be class-specific, ranging from $22–27\,mmol\,l^{-1}$ in fish to $22–36\,mmol\,l^{-1}$ in amphibians and $40–50\,mmol\,l^{-1}$ in higher vertebrates (Heisler, 1986*c*).

The fact that amphibians are less capable of increasing plasma bicarbonate during hypercapnia may, however, not be related to the constraints of the electrolyte homeostasis or of electrophysiological mechanisms, but may simply be an expression of the inability to gain sufficient amounts of bicarbonate-equivalents from the environment. This study has shown that the overall pH compensation during chronic hypercapnia could not be improved by external intervention, at least in the short term. The higher environmental bicarbonate concentration in the present experiments had little effect on the time taken to compensate as compared to other studies (Fig. 8). Bicarbonate directly infused into the bloodstream increased the plasma concentration only transiently, before being almost completely eliminated to the environment (Fig. 7). Both of these findings are quite consistent with the idea of a 'bicarbonate threshold'. These data, together with comparable results from similar experiments on the aquatic salamander *Siren lacertina* (Heisler *et al.* 1982), the lacertilian reptile *Tupinambis nigropunctatus* (Glass & Heisler, 1986) and the teleost fish *Cyprinus carpio* (Claiborne & Heisler, 1986), supply increasing evidence for a 'bicarbonate threshold' as a major limiting factor for pH compensation during hypercapnia in lower vertebrates.

The main objectives of this study were to quantify the net bicarbonate transfer between animal and environment as a regulatory measure of the acid–base homeostatic system (Fig. 6), to differentiate between ammonia and non-ammonia transfer mechanisms, and to relate the amount gained from the environment to the total rise in the body bicarbonate pool. As suggested earlier, the increases in extracellular and intracellular bicarbonate concentrations during hypercapnia in *Bufo*

are probably larger than can be expected on the basis of CO_2-buffering by intracorporeal non-bicarbonate buffers (Toews & Heisler, 1982). This idea has been pursued by estimating the net changes in bicarbonate pools, the bicarbonate produced by non-bicarbonate buffering, and the bicarbonate transferred by ionic mechanisms between animal and environment (for details of the approach see Heisler, 1982, 1986*a*, 1987*a*,*b*).

This estimate is based on the assumptions that the intracellular compartment of white muscle represents the total intracellular space, that the apparent buffer slope for skeletal muscle of *Bufo marinus*, determined during the initial stages of CO_2 exposure (Toews & Heisler, 1982), is representative of the intracellular non-bicarbonate buffer slope, and that about 50 % of the rise in intracellular bicarbonate can be attributed to intracellular non-bicarbonate buffering of CO_2 (Table 1). On the basis of this estimate, the change in the intracellular bicarbonate pool is about 4·2 mmol kg^{-1} body water. The low apparent buffer value for skeletal muscle (Toews & Heisler, 1982) as compared with other animals (see Heisler, 1982, 1986*a*) leads us to view this estimate with caution. The non-bicarbonate buffer values (β_{NB}) determined for several fish species, for instance, are of the order of 40–60 mmol pH $unit^{-1}$ l^{-1} (Heisler, 1980, 1982, 1986*a*,*c*), substantially higher than the apparent *in vivo* buffer slope of toad skeletal muscle after 24 h of hypercapnic exposure (33 mmol pH $unit^{-1}$ l^{-1}; Toews & Heisler, 1982). Thus, if the non-bicarbonate buffer value of toad skeletal muscle was of the same order as those found in fish, a considerable quantity of the bicarbonate produced through non-bicarbonate buffering of CO_2 in toad skeletal muscle would have had to have been transferred to other body fluid compartments. In fact, when the animal is modelled as a two-compartment system (ICS and ECS) surrounded by a closed third compartment (the environmental water), the model predicts that the initial and longer term increases in the extracellular bicarbonate pool should occur at the expense of that of the intracellular compartment.

From the balance sheet in Table 1, the changes in the total bicarbonate pool (about 7·8 mmol kg^{-1} body water) are estimated to be greater than that which can be attributed to non-bicarbonate buffering plus uptake from the environment (6·4 mmol kg^{-1} body water). Tufts & Toews (1985) suggested that the increases in calcium concentrations in urine and plasma of hypercapnic *Bufo marinus* represented dissolution of calcium carbonate deposits and estimated that approximately 1 mmol kg^{-1} animal of bicarbonate would be generated by this process over the 24 h period of hypercapnia. This factor, together with the assumption of more realistic intracellular buffer values (see above), would make up for all of the difference in the balance sheet in Table 1.

The main limitation of the balance sheet approach displayed in Table 1 is the limited information about the intracellular tissue compartments. A more reliable approach comes from the directly measured ionic transfer, the bicarbonate concentration changes, and the amount of bicarbonate produced in the extracellular space by non-bicarbonate buffering (see Heisler, 1984, 1986*a*, 1987*a*,*b*) and indicates (Fig. 9) that the main part of the rise in extracellular bicarbonate level is due to

Table 1. *Comparison of the changes in body fluid bicarbonate pools (ΔHCO_3^-) with the amount of bicarbonate produced by non-bicarbonate buffering ($\Delta HCO_3^-{}_{NB}$), and introduced to the body fluids by net transfer of bicarbonate equivalents ($\Delta HCO_3^-{}_{x\rightarrow y}$) in response to the transition from normocapnia (index '1') to 24 h of hypercapnia (index '2')*

	ΔHCO_3^- (mmol kg^{-1} body water)		Basis of estimate
Changes in body fluid pools			
Extracellular space (ECS)	+3·63	+3·63	$\Delta HCO_3^-{}_e = [HCO_3^-]_{P_2} \times V_{e_2} - [HCO_3^-]_{P_1} \times V_{e_1}$
Intracellular compartments (ICS)			
White muscle	+0·99		$\Delta HCO_3^-{}_i = [HCO_3^-]_{i_2} \times V_{i_2} - [HCO_3^-]_{i_1} \times V_{i_1}$
Total ICS = white muscle	+4·20	+4·20	
ECS + ICS			
(ICS = white muscle)		+7·83	
Production by non-bicarbonate buffering			
Blood (V_{Bl} = 0·074 of body water)	+0·687	+0·687	$\Delta HCO_3^-{}_{NB_{Bl}} = \beta_{P_1} \times \Delta pH_{P_1} \times F_{CO_2} \times V_{Bl}$
Intracellular compartments:			
White muscle	+0·583		$\Delta HCO_3^-{}_{NB_i} = \beta_{NB_i} \times \Delta pH_i \times \left(\frac{V_{i_1} + V_{i_2}}{2}\right)$
Total ICS = white muscle	+2·471	+2·471	
Net acid–base-relevant ion transfer			
Water → ECS	+3·240	+3·240	$\Delta HCO_3^-{}_{w\rightarrow e} = -\Delta H^+{}_{e\rightarrow w} + \Delta H^+{}_{e\rightarrow w,\ control}$
ECS → ICS	+0·300		$\Delta HCO_3^-{}_{e\rightarrow i} = \Delta HCO_3^-{}_{w\rightarrow e} + HCO_3^-{}_{NB_{Bl}} - \Delta HCO_3^-{}_e$
		+6·40	

V = extracellular or intracellular water volume (index e or i, respectively); F_{CO_2} = blood T_{CO_2}/plasma T_{CO_2} (McDonald, Boutilier & Toews, 1980). Body water compartments calculated using data of Thorson (1964) for *Bufo marinus*; skeletal muscle mass as a function of total body mass was taken from Putnam (1979) for *Bufo boreas*.

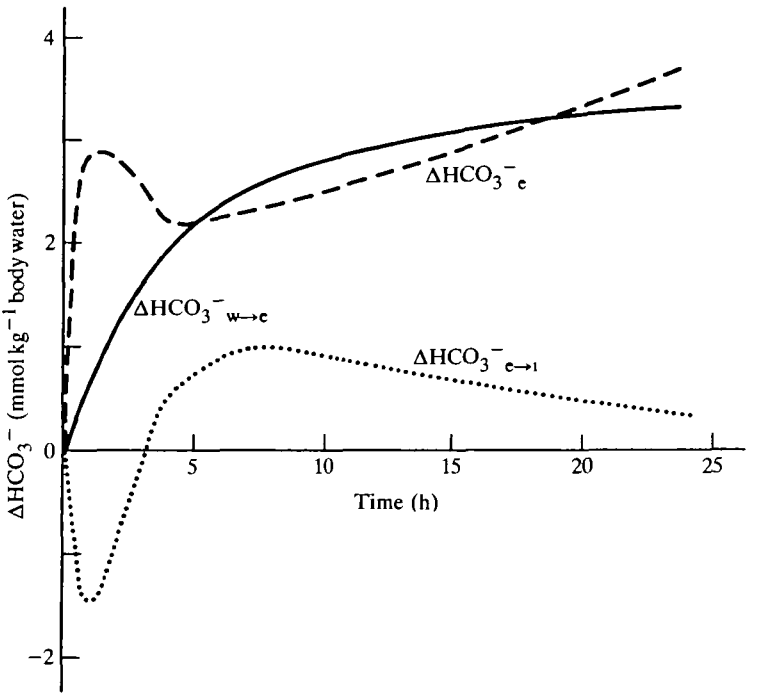

Fig. 9. Changes in the amount of extracellular bicarbonate ($\Delta HCO_3^-{}_e$) and cumulative net transfer of bicarbonate between environmental water and extracellular space ($\Delta HCO_3^-{}_{w\rightarrow e}$) and between extracellular and intracellular space ($\Delta HCO_3^-{}_{e\rightarrow i}$) of *Bufo marinus* after onset of environmental hypercapnia. See text for details.

transepithelial ion transfer. The overall transmembrane transfer is limited to transient shifts of smaller magnitude, and any contribution of bicarbonate originating from the environment is progressively reduced from about 8 h of hypercapnia towards the end of the experiment. The maximal amount transferred into the intracellular fluid space is similar to that observed in fishes (Heisler, Weitz & Weitz, 1976; Toews, Holeton & Heisler, 1983), but the compensatory effect has to be considered to be much lower as a result of the several-fold higher intracellular control bicarbonate concentration in *Bufo*.

The net uptake of bicarbonate-equivalent ions plays an accordingly important role in the limited extracellular pH compensation brought about by exposure to elevated levels of ambient CO_2 (Fig. 9; Table 1). The present experiments have indicated, however, that the incomplete compensation of arterial pH in *Bufo* is not appreciably limited by the availability of bicarbonate or ion exchange counterions in the surrounding water, but is rather attributable to a constitutional inability to accumulate bicarbonate above a certain threshold value.

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