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INTRACELLULAR AND EXTRACELLULAR ACID-BASE REGULATION IN THE TROPICAL FRESH-WATER TELEOST FISH *SYNBRANCHUS MARMORATUS* IN RESPONSE TO THE TRANSITION FROM WATER BREATHING TO AIR BREATHING

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SUMMARY

In the tropical fresh water fish, *Synbranchus marmoratus*, transition from water breathing to air breathing, induced by reduction of oxygen partial pressure (P_{O_2}) in the environmental water below 16 mmHg, causes a considerable rise in the arterial partial pressure of carbon dioxide (P_{CO_2}), from 5·6 to 26 mmHg on the average (half time of the rise between 2 and 6·5 h). The associated fall in arterial plasma pH by about 0·6 units is not compensated by an increase in plasma bicarbonate concentration, whereas the intracellular pH of white skeletal muscle and heart muscle is kept almost constant by elevation of the intracellular bicarbonate concentration. The additional bicarbonate is generated by intracellular non-bicarbonate buffering, and by net transfer into the intracellular space of bicarbonate formed by buffering in blood. Only a relatively small quantity of bicarbonate is taken up from environmental water.

This type of acid-base regulation, with almost complete intracellular pH compensation and only minor bicarbonate uptake (equivalent H^+ release or OH^- uptake) from water, is attributed to several factors. Probably the most important of these is the lack of continuous contact of the gills, which are the main site of ion transfer processes, with the environmental water during air breathing. Regardless of the mechanisms involved, this particular strategy of acid-base regulation provides a constant milieu for the intracellular structures and demonstrates the prevalence of intracellular over extracellular acid-base regulation.

INTRODUCTION

Evolutionary transition from the utilization of water to air as the medium for respiratory gas exchange is coupled with considerable increases in arterial P_{CO_2}. While water breathing fishes exhibit P_{CO_2} values in the range from about 1 to 6 mmHg (for ref.: Heisler, 1980), in amphibians P_{CO_2} is elevated to about 10–20 mmHg (e.g. Reeves, 1972; Boutilier *et al.* 1979*a–c*; Heisler, Ultsch & Anderson, 1980; Heisler

et al. 1982) and in reptiles to a range from about 15 to more than 50 mmHg (e.g. Malan, Wilson & Reeves, 1976; Jackson, Palmer & Meadow, 1974; Jackson & Kagen, 1976; Howell *et al.* 1970; Dill & Edwards, 1935).

The arterial pH values found in species of the above mentioned groups are not as different as could be expected from the broad range of P_{CO_2} values. The increase in P_{CO_2} is in most cases compensated by a proportional rise in bicarbonate concentration, so that the arterial pH values are in a range of $\pm 0{\cdot}2$ around a constant relative alkalinity of 0·7 pH units (pH of neutral water plus 0·7 units; Rahn, 1966, 1967) showing little correlation with P_{CO_2} or with water *vs.* air breathing.

Large changes in the proportion of water to air used as gas exchange media can be found during development from larval to adult amphibians. During metamorphosis of the frog *Rana catesbeiana*, for example, the fractional extent of aerial O_2 exchange is expected to increase from 0 to about 65–70% (values for related anurans, see Rahn & Howell, 1976), which is associated with a 3·3-fold increase in P_{CO_2} and a proportional rise in $[HCO_3^-]$ (Erasmus, Howell & Rahn, 1970/71; Just, Gatz & Crawford, 1973). Similar changes can only rarely be found in adult amphibians under normal environmental conditions. Also air breathing fish have in most cases specialized their gas exchange organs to such a high degree that the animals cannot change the proportion of water and air breathing to a large extent (e.g. *Hoplerythrinus* and *Erythrinus*, Stevens & Holeton, 1978*a*; *Arapaima*, Stevens & Holeton, 1978*b*; *Trichogaster*, Burggren, 1979; *Pantodon*, Schwartz, 1969; *Protopterus*, Johansen, Lomholt & Maloiy, 1976, see also Rahn & Howell, 1976, and Hughes & Singh, 1971) and thus are not subject to large P_{CO_2} changes.

One of the rare species which is capable of switching from exclusive water breathing to exclusive air breathing is the South-American fresh-water teleost *Synbranchus marmoratus*. These animals are found in tropical river basins and associated swamps, where, particularly in the Amazon basin, large areas are subject to water level changes of up to 12 (15) m, between the rainy season and the dry period. When, during the dry season, the water disappears from the swamps, or that which is left becomes stagnant and poorly oxygenated, *Synbranchus* will usually retire from the free water and excavate a labyrinth type burrow which contains a relatively small amount of water (Lüling, 1975). The animal then lives amphibiously as an exclusive air breather in the neighbourhood of, or completely within, its burrow (Lüling, 1975). In contrast, when deep and well oxygenated water becomes available during the rainy season, *Synbranchus* switches immediately to exclusive water breathing as long as the oxygen saturation of the water is sufficient to cover its O_2 demands (personal observation). Unlike other air breathing fish, *Synbranchus* is not equipped with any specialized gas exchange structures such as lungs, modified swim bladders or extensively enlarged surface areas in the buccopharyngeal chambers, and performs its respiratory gas exchange in both modes with the same structures, mainly the gills (Johansen, 1966) and, probably to a smaller extent, the skin.

The aim of the present study is to evaluate the consequences of the complete switch between the two modes of gas exchange, together with the associated large changes in blood P_{CO_2}, on extracellular and intracellular acid-base regulation, plasma electrolyte concentrations, and ion transfer processes between fish and environment.

MATERIALS AND METHODS

Specimens of *Synbranchus marmoratus* were obtained from local fishermen in the Amazon basin in the area of 'Lago do Janauaćа' near the Solimões river, about 35 km upstream from the confluence with the Rio Negro. The fish were caught either in weirs, with hook and line, or dug out of their burrows in the mud of dried-up swampy areas. They were kept in a large water tank for at least 6 days before being used for experimentation. The experiments were performed mainly aboard the RV *Alpha Helix* (Scripps Institution of Oceanography, La Jolla, California USA), anchored on the Solimões river near the entrance channel to 'Lago do Janauaćа'.

Catheterization of the dorsal aorta

The fish were anaesthetized by immersion in 20 g/l urethane solution until all motion had ceased. This took up to half an hour due to the fact that the animals stopped gill ventilation immediately after immersion and switched to air breathing, even when they had been previously adapted to water breathing. When the animals were unable to keep their heads above the water surface they were removed and placed ventral side up on an operating rack.

During surgery the gills were irrigated with a well aerated 3 g/l urethane solution. The body cavity in the area of the spleen was incised mid-ventrally and a large number of small blood vessels (up to about 20) running across the incision area on the inner side of the body wall were tied off before access to one of the splenic arteries was gained. A polyethylene catheter (PE 50) was inserted through the splenic artery into the dorsal aorta and then pushed forward to about 1 or 2 cm behind the junction of the two main dorsal gill arteries. The PE tubing was tied in place with a suture around the splenic artery and, still inside the body cavity, connected to a more flexible and unkinkable thick walled PVC tubing (0·9 mm I.D., 1·8 mm O.D.), which was securely tied to the hind end of the body wall incision. The incision was then tightly closed with three layers of atraumatic sutures (peritoneum, muscular layers and skin). The catheter was filled, and flushed once a day thereafter, with heparinized Ringer solution to prevent blood clotting. In order to remove the anaesthetic quickly from the animal, the gills were flushed with fresh water until the fish became active.

Procedure

Series I: steady state

Two groups, each of six specimens (810–1120 g weight; mean 940 g), were adapted to either exclusive water breathing or air breathing for 4–5¼ days at 30 ± 1 °C. Water breathing was achieved by flushing the experimental aquarium with well aerated water (P_{O_2} > 100 mmHg, P_{CO_2} < 1·5 mmHg). Under these conditions none of the animals made any attempt to breathe air. Moreover, air breathing was also made impossible by placing a screen slightly below the water surface after the first day. Air breathing was provoked by bubbling nitrogen through the experimental tank water.

16–24 h prior to the final phase of the experiment 10 μCi of ^{14}C-DMO (5,5-dimethyl-2,4-oxazolidinedione) for determination of the intracellular pH (Waddell & Butler, 1959) and 20 μCi of ^{3}H-inulin for determination of the extracellular space were injected into each specimen.

The total extracellular space was determined by application of the common inulin distribution method. Plasma samples for the determination of inulin concentration were taken 1, 4, 8, and 12 h after injection and extrapolated to time zero in the usual manner. Excretion of inulin was less than 1·5% of the remaining activity per h, excretion of DMO less than 2%/day, thus being insignificant for the applied methods.

After 4–5 days of adaptation to the respective modes of gas exchange, blood samples were withdrawn anaerobically from the dorsal aorta repeatedly and analysed for pH, P_{CO_2}, and P_{O_2}. After withdrawal of a final, larger blood sample for extracellular references, the fish was sacrificed by injection of anaesthetic and samples of white skeletal muscle and the heart were removed, weighed, dried for 24 h at 105–110 °C, weighed again and then preserved for later analysis for the activity of DMO and inulin. The last blood sample was centrifuged and the plasma also stored for later analysis of DMO and inulin by liquid scintillation counting (method described in detail by Heisler, Weitz & Weitz, 1976*a*), and for determination of $[Na^+]$, $[K^+]$ and $[Cl^-]$.

Series II: transition from water breathing to air breathing

The experiments were conducted in a closed water recirculation system (Fig. 1). The apparatus consisted of a Perspex chamber with a volume of about 14 l, in which a small part was separated and equipped with a set of sintered glass discs at the bottom to serve as an oxygenator or deoxygenator, and a recirculation pump. Temperature was kept constant at 30 ± 0·2 °C by means of a cooling unit attached to a heat exchanger in the bottom of the chamber. In order to mimic the low bicarbonate and sodium concentrations in the water of the burrows of the animals (Table 3), Solimões river water was diluted with distilled water and titrated with H_2SO_4 to achieve the values listed (Table 3).

For determination of bicarbonate concentration, water pH and P_{CO_2} were recorded continuously throughout the course of the experiment by means of electrodes particularly prepared and selected for long-term stability (drift of less than 0·001 units/36 h for pH, less than 0·2 mV/24 h for P_{CO_2}, see Heisler, 1978). P_{O_2} in water was read from the meter at the times of blood sampling and when the animals switched from water breathing to air breathing.

Nine specimens (1030–1535 g weight, mean 1240 g) adapted to exclusive water breathing for at least 4 days were transferred into the experimental chamber 12–24 h before the animals were induced to switch to air breathing. During this control period, water samples were taken every 6–8 h for determination of the control ammonia excretion by the fish. At the end of the control period, 3–4 blood samples were withdrawn anaerobically from the dorsal aorta at intervals of 20–30 min before the oxygenator was switched from CO_2-free air to nitrogen in order to induce air breathing. Further blood samples were withdrawn and water samples were taken 0·5, 1, 2, 3, 5, 7, 10 and 18 h after time 'zero', defined as the time when the animal made its first attempt to breathe air. Urine was collected separately during the control period and during the time intervals 0–5 h, 5–10 h, and 10–18 h via PE catheters introduced into the urinary papilla and fastened by atraumatic sutures.

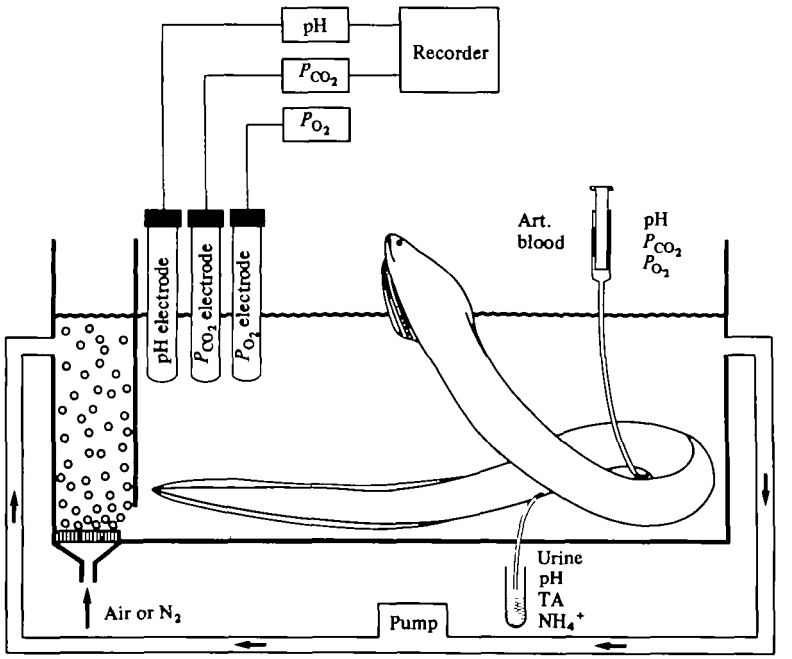

Fig. 1. Experimental set-up. The animals were kept in a closed water recirculation system consisting of an animal box with ventilated air space, a gas exchange unit and a recirculation pump. Changes in water pH and P_{CO_2} were continuously monitored. Air breathing was induced by switching the gas exchange unit from air to nitrogen.

Measurements and calculations

Blood samples withdrawn from the dorsal aorta were analysed for pH, P_{CO_2} and P_{O_2} using a thermostatted (30 ± 0·1 °C) microelectrode set-up (Heisler *et al.* 1976*a*; Heisler, 1978). Plasma was obtained by centrifugation and analysed for [Na^+] and [K^+] by flame photometry (IL 343), and for [Cl^-] by coulometric titration (Radiometer CMT 10).

Intracellular pH, extracellular space and fractional water content were determined as described before (Heisler *et al.* 1976*a*).

Bicarbonate concentrations in plasma and intracellular compartments were calculated from measured plasma pH, or intracellular pH, and P_{CO_2} by application of the Henderson–Hasselbalch equation using CO_2 solubility (α_{CO_2}) and pK'_1 values reported by Severinghaus (1965) for human plasma at 30 °C.

Water bicarbonate was calculated using values for α_{CO_2} [0·0393 mmole/(mmHg L)] and pK'_1 (6·347) reported for pure water (Harvey, 1974). The ammonia concentration in water and urine was determined in alkalinized (pH > 12) samples using gas sensitive ammonia electrodes (Orion). Water [Na^+] and [K^+] were determined by flame photometry (IL 343), water [Cl^-] with Cl^--sensitive electrodes (Orion).

The buffer curve of *Synbranchus* blood was determined by application of the standard CO_2 equilibration method. Samples of blood were shaken at a frequency of 60 Hz and equilibrated at 30 ± 0·5 °C with a gas mixture of CO_2 (1–5%) in air.

Plasma pH was measured, and plasma bicarbonate concentration calculated as described above. The buffer value of true plasma was then calculated as the ratio of change in plasma bicarbonate over the change in plasma pH ($\beta = -\Delta[HCO_3^-]/\Delta pH$).

The blood oxygen dissociation curve was determined by a mixing technique. Samples of blood, pooled from 6 animals, were equilibrated at $30 \pm 0{\cdot}5$ °C with gas mixtures of 1% CO_2, and either air or nitrogen. Various proportions of oxygenated and deoxygenated blood were volumetrically measured in Hamilton syringes, mixed thoroughly, and P_{O_2} was determined in the mixture. This procedure was repeated with gas mixtures containing 4% CO_2. The applied method is a modification of the technique described by Haab, Piiper & Rahn (1960).

For the determination of intracellular nonbicarbonate buffer values, samples of white muscle and heart muscle of 5 specimens were deep-frozen at −80 °C, transported to Germany frozen in dry ice (~40 h) and again stored at −80 °C until further analysis. The muscle samples were pulverized under liquid nitrogen with mortar and pestle, diluted with appropriate Ringer solution by a factor of 2–3 and equilibrated at $30 \pm 0{\cdot}1$ °C with gas mixtures of 1–5% CO_2 in oxygen, after pH in the homogenate had been adjusted to values in the range of those determined under physiological conditions in the intracellular compartments (see Results). pH in the homogenate was recorded continuously by means of a double electrolyte bridge electrode. The homogenate bicarbonate concentration was calculated using the same constants as for plasma because of the similarity in ionic strength and protein concentrations. Details of the procedure and calculation of intracellular buffer values have been described previously (Heisler & Piiper, 1971; Heisler & Neumann, 1980).

RESULTS

Steady-state conditions (Series I)

P_{CO_2} in water-breathing *Synbranchus*, averaging about 5·6 mmHg (Fig. 2), was in the range expected for purely water breathing fish species (see Heisler, 1980, 1982). Plasma pH (Fig. 2), however, was higher than that measured in any other vertebrate, especially for the relatively high temperature of 30 °C, where, on the basis of measured values and of the rule of constant relative alkalinity (Rahn, 1966, 1967), a value around 7·6 would be expected. According to the high pH, the bicarbonate concentration, at about 24 mM, was higher than in water breathing fishes and within the range found in amphibians and reptiles (e.g. Boutilier *et al.* 1979*a–c*; Jackson *et al.* 1974). In air breathing as compared to water breathing specimens, P_{CO_2} was elevated, by almost a factor of 5, to an average of 26 mmHg. This value is in good agreement with the value of 29 mmHg reported for the same species by Johansen (1970). The elevated P_{CO_2} results in a severe drop in plasma pH, by about 0·6 units, whereas bicarbonate concentration stays constant (Fig. 2).

In contrast to the conditions in the extracellular compartment, pH in the two studied intracellular compartments (white skeletal muscle and heart muscle; Fig. 3) was only little affected by the 5-fold increase in P_{CO_2} due to transition from water breathing to air breathing. This resulted from about a 4-fold increase in intracellular bicarbonate concentration. Because of the relatively large scatter in the data from

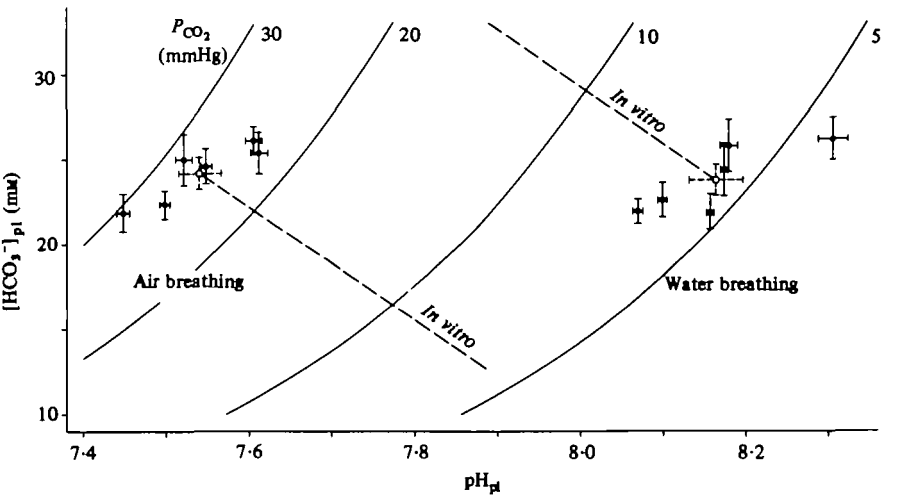

Fig. 2. Arterial plasma pH, P_{CO_2} and bicarbonate concentration in each of six specimens of *Synbranchus marmoratus* acclimated for at least 4 days to exclusive water breathing or air breathing, respectively ($\bar{x} \pm$ S.E.).

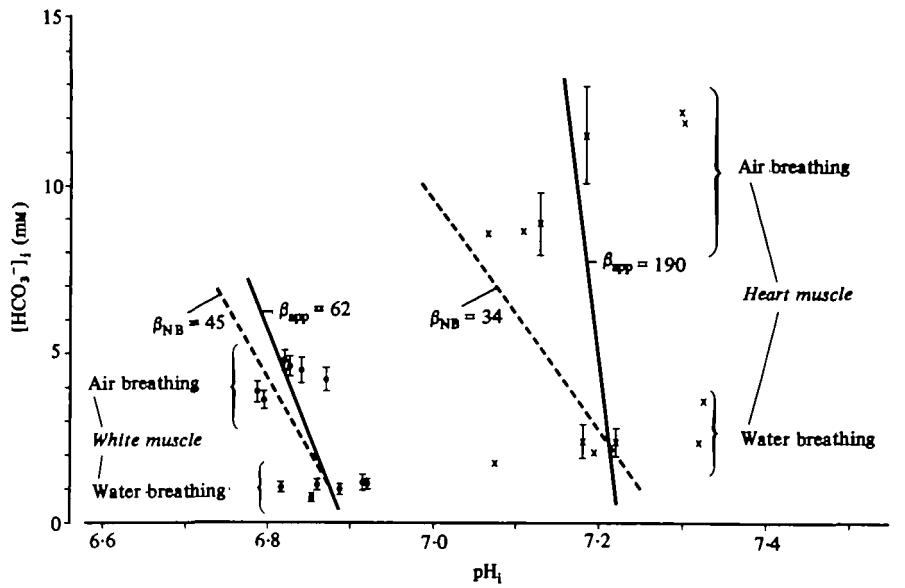

Fig. 3. Intracellular pH and bicarbonate concentrations in white muscle and heart muscle of *Synbranchus* during water breathing and air breathing. The difference in bicarbonate concentration between the chemical non-bicarbonate buffer line (labelled β_{NB}) and actual buffer line (labelled β_{app}) is attributable to trans-membrane bicarbonate (or equivalent OH^- or H^+) transfer. See also text. ($\bar{x} \pm$ S.E.; in several specimens, only one intracellular pH determination was possible for heart muscle.)

Table 1. *Plasma electrolyte concentrations* (mM) *and total extracellular space* (*ECS*, % *of body water*) *in water-breathing and air-breathing* Synbranchus ($\bar{x} \pm$ S.D.)

	$[Na^+]$	$[K^+]$	$[Cl^-]$	$[HCO_3^-]$	ECS
Water-breathing	$125 \pm 4{\cdot}6$	$2{\cdot}34 \pm 0{\cdot}42$	$80{\cdot}5 \pm 3{\cdot}9$	$24{\cdot}2 \pm 1{\cdot}9$	$21{\cdot}8 \pm 0{\cdot}8$
Air-breathing	$133{\cdot}3 \pm 4{\cdot}3$	$3{\cdot}75^* \pm 0{\cdot}51$	$98{\cdot}5^* \pm 4{\cdot}8$	$24{\cdot}4 \pm 1{\cdot}6$	$19{\cdot}9^* \pm 0{\cdot}6$

* Significantly different from water breathing values (*t*-test, $P < 0{\cdot}05$).

individual fish, no statistically significant least square linear regression could be fitted to the respective intracellular pH and bicarbonate concentration values measured in white muscle and heart muscle. Also the average pH values during water breathing and air breathing were not significantly different. In order to gain a minimum estimate of transmembrane net bicarbonate transfer* the apparent intracellular buffer lines of Fig. 3 have been drawn through the points defined by the average values for pH_i and bicarbonate, of water breathing and air breathing fishes, respectively. This most conservative way of fitting the buffer lines results in apparent buffer values ($\beta_{app} = 62$ and 190 meq/(pH·L cell water) for white or heart muscle, respectively), which are higher than the chemical buffer values which have been measured in muscle homogenates to be $45{\cdot}3 \pm 6{\cdot}1$ and $34{\cdot}1 \pm 6{\cdot}8$ mmol/(pH kg intracellular water) for white muscle and heart muscle, respectively ($x \pm$ S.D., $n = 15$ for white muscle, 5 for heart muscle, of 5 specimens). These are in the range of values which have been determined in the same muscle species in other animals (Heisler & Piiper, 1971, 1972; Heisler & Neumann, 1980; Gonzalez, Wemken & Heisler, 1979).

All plasma electrolytes (Table 1) which were measured (except bicarbonate), were increased in concentration under air breathing conditions in comparison with water breathing. This can partly be attributed to a significant reduction of the extracellular space under air breathing conditions (Table 1).

The electrolyte concentrations of the experimental water and of the animal's habitat, are listed in Table 3. These concentrations are orders of magnitudes lower than those in the plasma of the fish.

Arterial P_{O_2} was on the average $32{\cdot}0 \pm 10{\cdot}4$ mmHg ($\bar{x} \pm$ S.D.) during water breathing and $27{\cdot}4 \pm 3{\cdot}1$ mmHg during air breathing, values which are not significantly different from each other. The blood O_2 dissociation curve of *Synbranchus* blood exhibits P_{50} values of 7·0 and 12·2 at P_{CO_2} of 7·2 or 28·8 mmHg and pH values of about 7·8 or 7·45, respectively (Fig. 4). The buffer value of true plasma in blood of $45 \pm 3\%$ haematocrit was determined to be 33·4 meq/(pH·L plasma) ($\bar{x} \pm$ S.D., $n = 8$).

The haematocrit of *Synbranchus* was found to be $48{\cdot}5 \pm 9{\cdot}4\%$ ($\bar{x} \pm$ S.D., $n = 29$) with a range from 38 to 71 %. Because of the scatter no significant differences could be observed between water breathing and air breathing animals.

* The term 'Bicarbonate transfer' is descriptively used, although the mechanisms may involve transfer of OH^- ions, or of H^+ ions in opposite direction; they are indistinguishable by the methods used.

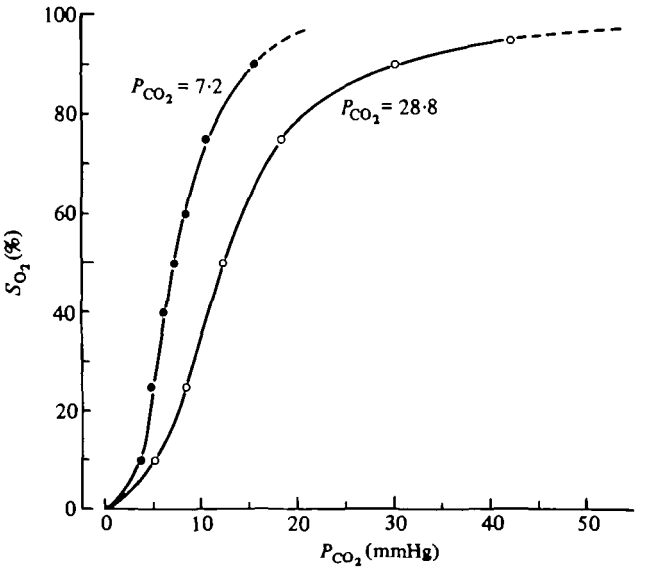

Fig. 4. Oxygen dissociation curves of *Synbranchus* blood, pooled from 6 specimens. Closed circles represent conditions similar to those during water breathing (P_{CO_2} = 7·2 mmHg, pH ~ 7·8), open circles conditions similar to those during air breathing (P_{CO_2} = 28·8 mmHg, pH ~ 7·45).

Transition from water-breathing to air-breathing (Series II)

Upon reduction of the P_{O_2} in the water (P_{W,O_2}) of the recirculation system (Fig. 1) below 24·3 ± 6·5 mmHg ($\bar{x}$ ± S.D.), *Synbranchus* took the first breath of air, but also continued water gill ventilation. During this initial phase of mixed water breathing and air breathing the animal flushed its buccal cavity 2–4 times with air, expired and then dived below the water surface where it ventilated its gills in the normal water breathing fashion. This entire procedure was repeated every 2–5 min. However, when P_{W,O_2} fell below 15·8 ± 5·3 mmHg the animal switched to exclusive air breathing. Then it filled its buccal cavity with air and floated with its head on the water surface with the rostral half of it in air. The breathing cycle started with expiration through both mouth and the single ventral gill opening, then the gills were briefly flushed (1–3 breaths) with water (probably in order to keep the gill surface moist, to allow ion exchanges at the gills, and/or to test the oxygen content of the water) before air was inspired into the buccal cavity again. This procedure was repeated every 5–35 min (in some rare cases even up to 50 min). As time progressed after the switch to exclusive air breathing the length of the interval between air breaths became progressively longer, though these parameters were only loosely correlated.

The control values of the acid-base parameters in arterial plasma (P_{CO_2}, pH and [HCO_3^-]) were similar to those determined in the steady state series (Fig. 5). When air breathing was induced, arterial P_{CO_2} (Fig. 5, upper panel) started to rise towards the values found in air breathing steady state animals. Two clearly distinguishable types of specimens having either 'fast' (n = 4) or 'slow' (n = 5) rises of P_{CO_2} were

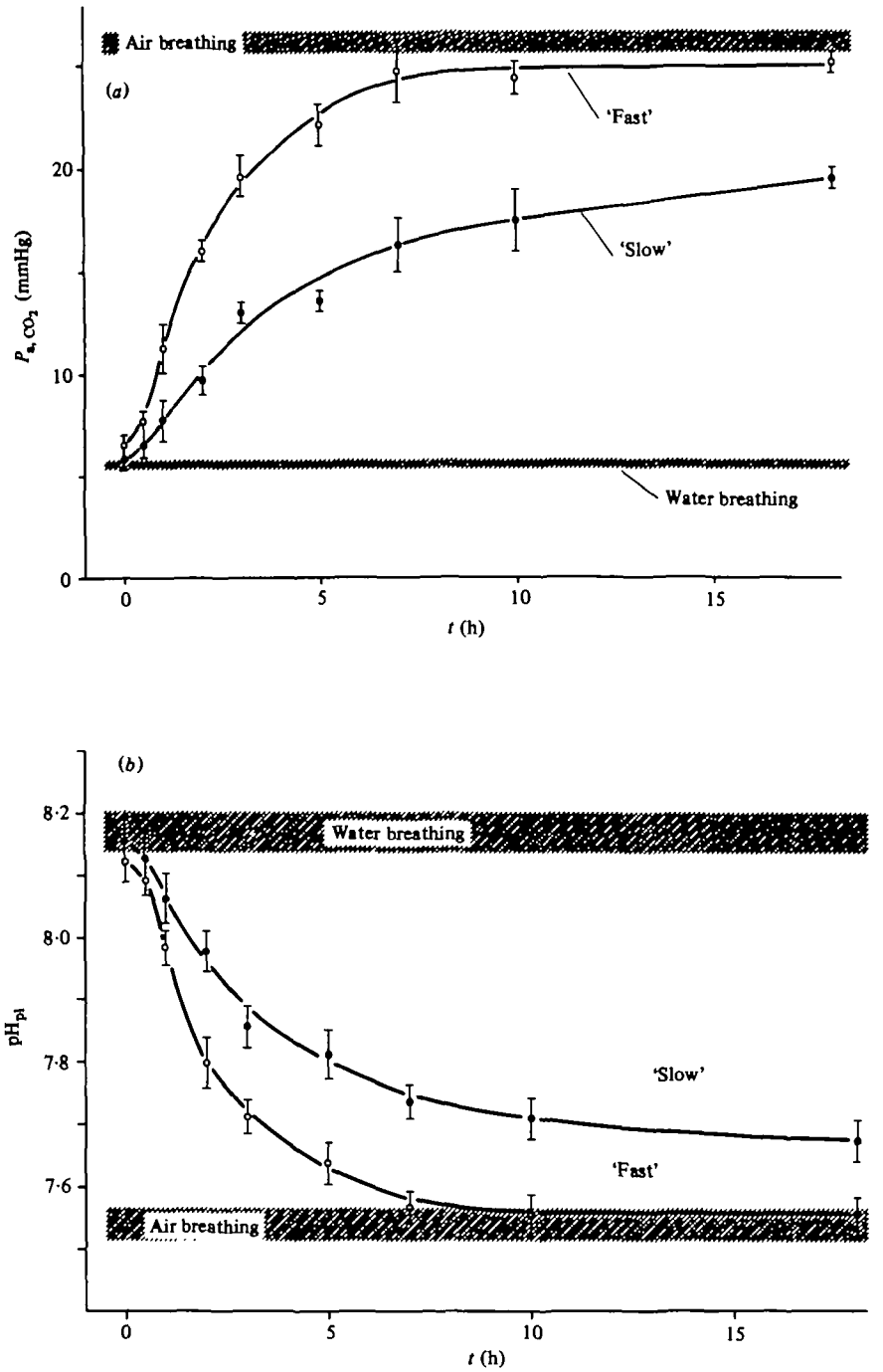

Fig. 5. For legend see opposite.

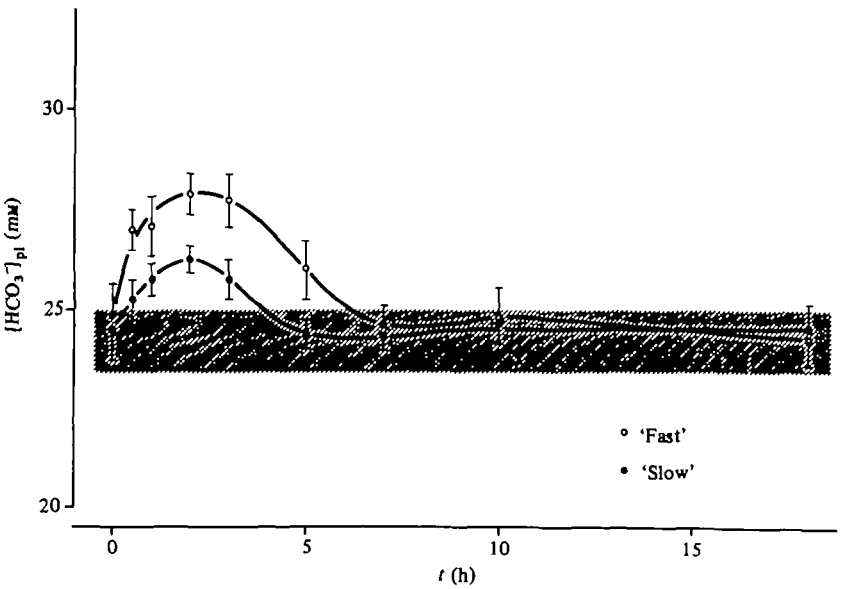

Fig. 5. Kinetics of changes in arterial pH, P_{CO_2} and bicarbonate concentration as a function of time after initiation of air breathing. Shaded areas represent the steady state values ($\bar{x}$+S.E.) determined for the respective type of breathing in series I (Fig. 2).

observed. These time course differences could not be correlated with sex, size or any other apparent parameter. The steady state P_{CO_2} values of air breathing specimens (Series I) were finally also attained in the 'slow' animals, though only after 2·5–3 days. As a result of the increased P_{CO_2}, plasma pH fell accordingly (Fig. 5, middle panel). Plasma bicarbonate concentration was initially increased in both 'slow' and 'fast' specimens, but returned to the original value in about 5–7 h (Fig. 5, lower panel).

Ammonia, as the main nitrogenous metabolic end product, becomes immediately ionized to NH_4^+ in the organism, thereby producing equimolar quantities of bicarbonate. NH_4^+ and bicarbonate are believed to be mainly eliminated via NH_4^+/Na^+ and HCO_3^-/Cl^- ion exchange mechanisms (for references see Heisler, 1980; Maetz, 1974; Evans, 1980), but ammonia is, probably to a lesser extent, also eliminated in its non-ionized form by passive non-ionic diffusion (Heisler, 1982). Regardless of the mechanism of elimination, the difference between NH_4^+ and HCO_3^- appearance in the environmental water (pH < 8) is equivalent to extrusion of H^+ ions.

During the control (water breathing) period, *Synbranchus* released ammonia in considerable amounts at a rate of 4·4 ± 2·1 μmol/(min kg body water) ($\bar{x}$ ± S.D.), which was almost completely balanced by the rate of bicarbonate excretion (or equivalent H^+ uptake) of 4·2 ± 2·0 μmol/(min kg body water) (Fig. 6, upper and middle panel). The net H^+ excretion rate as the difference between NH_4^+ and HCO_3^- release, 0·2 ± 0·2 μmol/(min kg body water) (Fig. 6, lower panel), was not significant. After switching to air-breathing the animals more or less maintained these rates of excretion for a few hours, but then ammonia as well as bicarbonate release started to level off

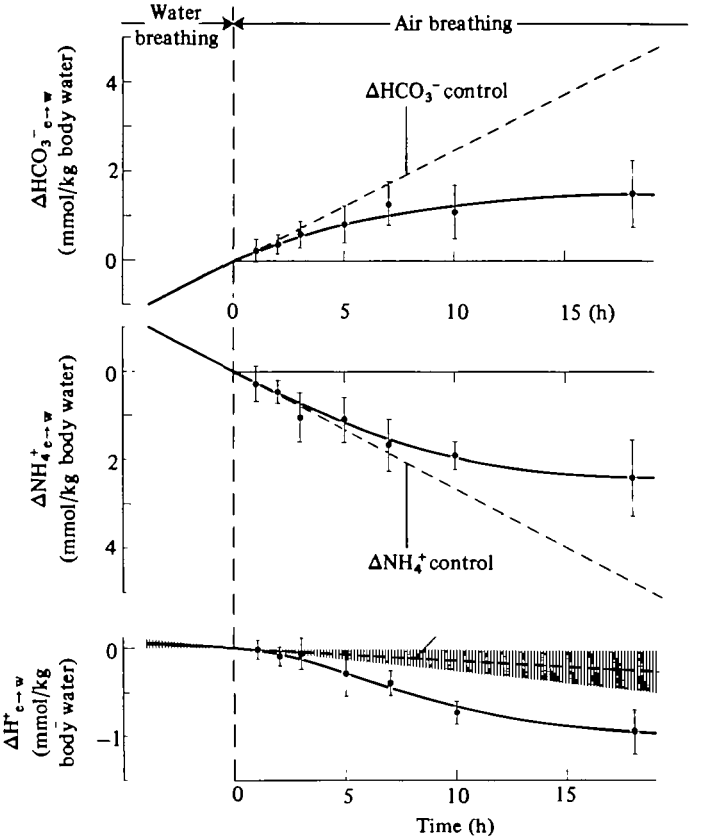

Fig 6. Cumulative excretion of bicarbonate (upper panel) and NH_4^+ (middle panel) from *Synbranchus marmoratus* as a function of time after initiation of air breathing. $\Delta H_{e\to w}$ (lower panel) represents the difference of the above two ions and is the net amount of H^+ ions excreted by the animal ($\bar{x} \pm$ S.E.).

and finally came to about zero after 18 h of air breathing. The net H^+ excretion during air breathing was slightly elevated as compared to the water breathing period, resulting after 18 h in a net gain of about 0·7 mmol bicarbonate/(kg body water) (Fig. 6).

The urine flow rate was extremely variable between individuals and averaged 3·5 ± 2·1 ml/(h kg body weight) ($\bar{x} \pm$ S.D.) during the water breathing control period and changed slightly but insignificantly during air breathing (4·9 ± 2·9 ml/h kg body weight). The urine pH was always close to the respective plasma pH values (pH_u = 8·27 ± 0·18 during water breathing, 7·71 ± 0·31 during air breathing). The rise in net H^+ excretion via the kidneys during air breathing as compared to water breathing during the 18 h of the experiment was insignificant, averaging 0·075 ± 0·29 mmol/(kg body water).

DISCUSSION

Arterial P_{O_2} levels in water breathing and air breathing *Synbranchus* are not significantly different. Based on the present oxygen dissociation curves, they are

sufficient to saturate, respectively, more than 95 or 85% of the haemoglobin, in spite of the pronounced Bohr effect. This relatively tight regulation of arterial P_{O_2}, even after the change of the respiratory medium, suggests that P_{O_2} in these fish is the leading parameter for the regulation of ventilation, whereas during steady state conditions P_{CO_2} and extracellular pH appear to have little influence. After transition from water breathing to air breathing, however, arterial P_{CO_2} rises and pH falls much more slowly than would be expected if aerial ventilation was being immediately adjusted according to the oxygen demand of the animal. Also, the gradual reduction of the ventilatory rate with time after initiation of air breathing suggests that other regulatory mechanisms are involved. Part of the respiratory drive from oxygen-sensitive receptors may then be substituted by input from pH sensitive receptors. Air ventilation would then become progressively reduced when the increased P_{CO_2} is compensated for by bicarbonate accumulation to normalize pH in the intracellular fluid of the receptor.

Also, increased O_2 uptake at the gills, compensating for the diffusive loss of oxygen via the skin to the anoxic water (which would later be reduced by vasoconstriction of the skin vessels), could explain the observed slow rise of P_{CO_2} to the finally observed values. The latter mechanism would reduce the elimination of CO_2 via the skin and thus contribute to the conspicuous rise in arterial P_{CO_2}.

As a result of this primarily oxygen-oriented regulation in *Synbranchus*, the transition from water breathing to air breathing causes a rise in P_{CO_2} similar to the differences found between species of the corresponding evolutionary stages. However, the extracellular bicarbonate concentration in water breathing *Synbranchus* is initially much higher than that of exclusively water breathing fish, but is not further elevated in response to the initiation of air breathing like in amphibians and reptiles, as compared to water breathing fish species or in fish as a result of environmental hypercapnia (e.g. Heisler *et al.* 1976*b*; Cameron & Randall, 1972; Janssen & Randall, 1975; Eddy, 1976; Randall *et al.* 1976; see also Heisler, 1980, 1982). The resultant considerable fall of extracellular pH remains uncompensated, the bicarbonate concentration not even being increased by the amount of bicarbonate produced by the blood non-bicarbonate buffering. The amount must therefore have been transferred to another compartment. In contrast, in the intracellular compartments of white muscle and heart muscle, pH is maintained within relatively narrow limits, in spite of the largely increased P_{CO_2}, by accumulation of bicarbonate. The change in the intracellular bicarbonate pool can be estimated to be 2·4 mmol/kg body water (Table 2, (4)), assuming that the intracellular compartment of white muscle represents the whole intracellular space (normally 50–70% of the intracellular water, see Heisler, 1982). As indicated by the measured slope of the intracellular non-bicarbonate buffer line ($-\Delta[HCO_3^-]_{NB}/\Delta pH$) as compared to the estimated minimal slope of the apparent buffer line ($-\Delta[HCO_3^-]_{actual}/\Delta pH$) (Fig. 3), about two-thirds of this quantity, 1·6 mmol/kg body water, is produced by intracellular non-bicarbonate buffering of CO_2 (Table 2, (9)). The remainder is very likely due to transmembrane bicarbonate (or equivalent H^+ or OH^-) transfer from the extracellular compartment, where about 0·8 mmol/kg body water is produced by non-bicarbonate buffering in the haemoglobin-rich blood (H_t = 48·5%) (Table 2(6)); estimate based on the true plasma

Table 2. *Estimated changes in bicarbonate pools* (ΔHCO_3^-), *bicarbonate production by non-bicarbonate buffering* ($\Delta HCO_3^-{}_{NB}$), *and net bicarbonate transferred* ($\Delta HCO_3^-{}_{x\to y}$) *in response to the transition from water breathing* (*index* 1) *to air breathing* (*index* 2) (*see also text*)

	Basis of estimate	ΔHCO_3^- (mmole/kg body water)	
Pools			
Extracellular space (ECS)	$\Delta HCO_{3e}^- = [HCO_3^-]_{Pl_2}.V_{e_2} - [HCO_3^-]_{Pl_1}.V_{e_1}$	−0·4	−0·4 (1)
Intracellular compartments (ICS):			
White muscle (F_i = 0·5)	$\Delta HCO_3^- = [HCO_3^-]_{i_2}.V_{i_2} - [HCO_3^-]_{i_1}.V_{i_1}$	+1·2	(2)
Heart muscle (F_i = 0·003)		+0·02	(3)
Total ICS = white muscle		+2·4	+2·4 (4)
ECS + ICS (ICS = white muscle)			+2·0 (5)
Non-bicarbonate buffering			
Blood (V_{Bl} = 3 % of b.w.)	$\Delta HCO_3^-{}_{NB_{Bl}} = \beta_{Pl}.\Delta pH_{Pl}.F_{CO_2}.V_{Bl}$	+0·8	+0·8 (6)
Intracellular compartments:			
White muscle (F_i = 0·5)	$\Delta HCO_3^-{}_{NB_i} = \beta_{NB_i}.\Delta pH_i.\left(\frac{V_{i_1}+V_{i_2}}{2}\right)$	+0·8	(7)
Heart muscle (F_i = 0·003)		+0·004	(8)
Total ICS = white muscle		+1·6	+1·6 (9)
Net transfer			
Water → ICS	$\Delta HCO_3^-{}_{w\to e} = -\Delta H^+_{e\to w} + \Delta H^+_{e\to w_{control}}$	+0·7	+0·7 (10)
ECS → ICS	$\Delta HCO_3^-{}_{e\to i} = \Delta HCO_3^-{}_{w\to e} + \Delta HCO_3^-{}_{NB_{Bl}} - \Delta HCO_3^-{}_e$	+1·9	(11)
			+3·1 (12)

V = extracellular or intracellular water volume (index e or i, respectively); F_{CO_2} = blood T_{CO_2}/plasma T_{CO_2}.

buffer value of *Synbranchus* blood, the total CO_2 distribution ratios reported for human blood (Van Slyke & Sendroy, 1928) and, for a conservative estimate, a low assumed blood volume of 3% of the body weight). Moreover, about 10% of the original extracellular bicarbonate pool (~0·4 mmol/kg body water) must have been net transferred to the intracellular space (Table 2, (1)). These calculations are in good accordance with the observed changes in extracellular electrolyte concentrations. The rise in extracellular [Cl^-] in surplus to that expected from the shrinkage of the extracellular space (Table 1) suggests an exchange mechanism of HCO_3^- against Cl^-, whereas the [Na^+] rises slightly less than expected, possibly as an indication of an additional Na^+/H^+ transfer.

The amount of bicarbonate gained from the environmental water, 0·7 mmol/kg body water, must have been transferred to intracellular compartments too, as the extracellular bicarbonate pool was evidently not elevated in response to air breathing (Table 2, (10)).

The rise in the total intracorporal bicarbonate pool (ECS + ICS), as calculated from the changes in bicarbonate concentration (~2·0 mmol/kg body water), is 35% smaller than that which can be calculated from the non-bicarbonate buffer values, the changes in pH and the amount gained from water (3·1 mmol/kg body water; Table 2, (5) *vs.* (12) respectively). This discrepancy (1·1 mmol/kg body water) indicates the presence of limitations for these model calculations like uncertainties about the actual average

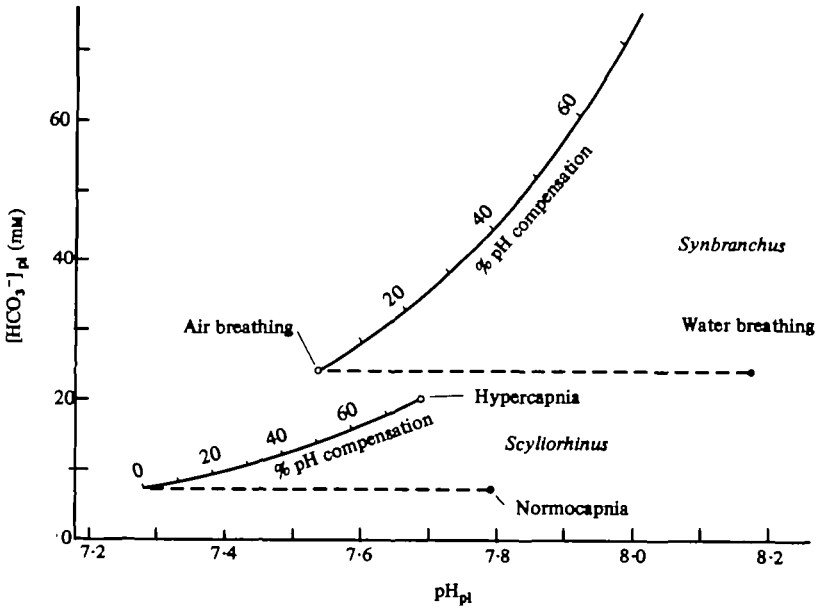

Fig. 7. Comparative presentation of plasma pH and bicarbonate in a water breathing fish (*Scyliorhinus stellaris*, Heisler *et al.* 1976*a*) and *Synbranchus* at normocapnia or water breathing, respectively, and during environmental or air breathing-induced hypercapnia, respectively. In *Scyliorhinus* a small amount of bicarbonate yields 80% compensation, whereas in *Synbranchus* almost 60 mM would be required for the same degree of compensation due to the initially higher bicarbonate concentration.

tissue P_{CO_2}, the apparent average buffer value of the whole organisms and the intracellular pH values, reflected by the relatively large scatter between individual fish specimens. Though the absolute values listed in Table 2 should be taken with reserve, they still provide valuable qualitative information about the relative contributions of single mechanisms and the direction of ion transfer processes.

The relatively small quantity of bicarbonate accumulated additionally in the intracellular compartment during air breathing (Table 2, (4)) evidently suffices to allow tight regulation of intracellular pH by compensation of the increased P_{CO_2}. This is possible only because of the initially low intracellular bicarbonate concentrations (water breathing conditions), which are similar to those in intracellular compartments of exclusively water breathing fish species (Heisler *et al.* 1980; Heisler & Neumann, 1980; Cameron, 1980; Heisler, 1980). In contrast, the extracellular bicarbonate concentration of *Synbranchus* (24 mM) is 3–5 times larger than in exclusively water breathing species (for references see Heisler, 1980, 1982). Consequently, the amount of bicarbonate required for a similar degree of compensation would have to be larger by the same factor (see Fig. 7). If the small quantity accumulated intracellularly (Table 2, (4)) was made fully available to the extracellular compartments, this would have resulted in only about 20% compensation of the pH shift observed between the water breathing and air breathing specimens.

Even if a 4-fold increase in extracellular bicarbonate concentration could be accom-

Table 3. *Environmental water composition of* Synbranchus marmoratus *in Amazonian water sheds and in the experimental set-up (series II)*

(Number of determinations in brackets, when exceeding one.)

Site	$[Na^+]$ (mM)	$[K^+]$ (mM)	$[Cl^-]$ (mM)	$[HCO_3^-]$ (mM)
Solimões river (35 km upstream from the confluence with the Rio Negro)	0·143–0·187 (7)	0·024–0·029 (7)	0·110–0·138 (7)	0·150–1·2 (7)
Rio Negro (10–25 km upstream from Manaus)	0·027	0·007	0·021	0·02–0·09 (4)
Rio Negro (90 km upstream from Manaus)	0·038	0·011	0·025	0·03–0·08 (3)
Lago do Janauaćа				
site 1	0·123	0·032	0·107	0·64
site 2	0·074	0·018	0·068	0·104
site 3	0·034	0·007	0·023	0·06
site 4	0·030	0·005	0·020	0·08
Synbranchus burrows (area of Lago do Janauaćа: all inhabited)				
No. 1	0·025	0·018	0·018	0·030
No. 2	0·084	0·028	0·044	0·094
No. 3	0·014	0·012	0·010	0·021
No. 4	0·022	0·015	0·015	0·043
Experimental water (series II, initial)	0·061–0·097 (9)	0·012–0·019 (9)	0·062–0·078 (9)	0·103–0·157 (9)

plished by uptake of bicarbonate or OH^- from the environment, or release of H^+, this rise would have to be accompanied by an equivalent release of Cl^-, which would deplete the animal of almost all of its chloride. Large reductions of the chloride concentration, however, may cause severe electrophysiologic disturbances, especially at nerve synapses (e.g. Tauc & Gerschenfeld, 1961; Strumwasser, 1962; see also Eccles, 1964 and Eccles & Scheid, 1980). Therefore a large degree of extracellular compensation cannot be expected in animals with high initial bicarbonate concentrations. Two other factors, however, may be more important for the lack of extracellular pH compensation. These are related to the environmental water composition and behaviour of the fish during air breathing.

The larger 'white water' rivers in the Amazon basin like the Solimões and their associated channels and flood lakes carry relatively electrolyte-rich water (see Table 3), whereas in the flooded forest areas and near the influx of 'terra firma' streams and brooks, in the preferred habitats of *Synbranchus* (Lüling, 1975), the electrolyte concentrations may be considerably lower and in the range of typical 'black water' rivers like the Rio Negro. Also in the majority of *Synbranchus* burrows the bicarbonate and sodium concentrations are extremely low (Table 3). Because of these concentrations and the small amount of water in the burrows (a situation which has been simulated during the present experiments) only rather small quantities of bicarbonate and sodium are available to the animals. Resorption of these will soon lead to the limit of ion concentration ratios beyond which higher gradients cannot be established.

Similar limitations are known from dogfish where uptake of bicarbonate from the environment in hypercapnia is terminated when the difference between plasma pH and sea-water exceeds about 1·1 pH units (or a concentration ratio of bicarbonate of

about 20). The fish will even lose bicarbonate to the water when the pH difference is increased (Heisler & Neumann, 1977). Carp also lose bicarbonate to the environment when exposed to a low water pH of 5·1 (Ultsch, Ott & Heisler, 1981). It is doubtful, however, that a low electrolyte concentration is the main factor leading to the observed extracellular non-compensation, as the fish studied in series I (steady state conditions) showed a similar pattern of extracellular acid-base status in spite of the fact that they had been studied in Solimões river water with higher ion concentrations.

The most important factor limiting the bicarbonate resorption from the water appears to be the lack of continuous contact of the gills, as the most potent ion exchange surface of the fish, with the water. During air breathing (see Results), *Synbranchus* flush the gills with water for only the first 2–3 s following an air breath and repeats these ventilatory episodes in a highly intermittent fashion (i.e. ranging from 10–50 min). Even if *Synbranchus* gills could achieve a bicarbonate uptake rate similar to other fish species (15 μmol/(min kg body water); see Heisler, 1982), in spite of the adverse water electrolyte concentrations, the reduced water contact would probably lower the average bicarbonate uptake (or equivalent H^+ extrusion) rate to a small fraction of the above value. This hypothesis is also supported by the fact that after the initial phase of air breathing the release of NH_4^+ and HCO_3^- from the fish to the water is terminated. Whether the nitrogen metabolism under these conditions is reduced to a much lower level, or is switched to another nitrogenous end-product must, however, remain unclear.

If the gills are not capable of gaining sufficient amounts of bicarbonate from the environment because of low external electrolyte concentrations or simply because of the lack of continuous water contact, the kidneys would be a logical alternative. In the present experiments actually rather large urine flows have been measured. The urine, however, contained only small net amounts of H^+ ions, and thus contributed to only 3% of the total amount of bicarbonate utilized for intracellular compensation. This quantity is sufficient to handle a large fraction of the net steady state output of H^+ ions from metabolism, but clearly is too small to contribute significantly to the compensation of the extracellular pH.

CONCLUSION

The absence of extracellular pH compensation after the considerable increase in arterial P_{CO_2} upon transition from water breathing to air breathing, is attributable to a combination of environmental, physiological or behavioural factors. The normally high plasma bicarbonate concentration makes extracellular compensation during air-breathing an unlikely strategy since the obligatory reduction in extracellular chloride would very likely produce severe electrophysiological disturbances. Moreover, the expected net uptake of bicarbonate from the environment is probably limited by low environmental water electrolyte concentrations and/or the extremely reduced contact time of the gill epithelium with water during air breathing. The small quantity of bicarbonate gained from the environment at the beginning of air breathing as well as the bicarbonate produced by extracellular non-bicarbonate buffering are transferred to the intracellular space, and contribute to compensate the intracellular pH. This strategy of utilization of a limited amount of bicarbonate for the protection of the

intracellular pH appears to be the most efficient way to provide a constant milieu for the intracellular structures, and exemplifies the general priority of intracellular over extracellular acid-base regulation in vertebrates.

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