

THE BONE COMPARTMENT IN A TELEOST FISH, *ICTALURUS PUNCTATUS*: SIZE, COMPOSITION AND ACID-BASE RESPONSE TO HYPERCAPNIA

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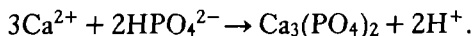
Accepted 29 November 1984

SUMMARY

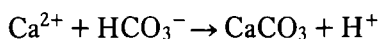
An estimate of the total mass of bone in the Channel catfish *Ictalurus punctatus* Rafinesque, was obtained by dissection. The wet weight of bone constituted $16.3 \pm 1.9\%$ (\pm s.d.) of the total (live) wet weight, and $25.0 \pm 2.1\%$ of the dry weight. Of the dry skeletal material, $66.3 \pm 11.1\%$ was soluble in strong acid. The acid-soluble material was about half mineral salts, consisting of $19.5 \pm 2.21\%$ Ca^{2+} and $27.6 \pm 3.22\%$ PO_4^{3-} , with minor fractions of Mg^{2+} (0.33%) and CO_3^{2-} (1.67%). The pH values of fluid compartments associated with skull and vertebral bone tissues were 7.420 ± 0.026 and 7.444 ± 0.017 (\pm s.e.), respectively, at a normocapnic plasma pH of 7.868 ± 0.020 . In response to external hypercapnia (7.5 Torr), the blood response consisted of an immediate decrease in pH, and a subsequent compensatory rise in both pH and $[\text{HCO}_3^-]$. This compensatory phase was accompanied by a net apparent H^+ excretion to the water. The participation of the mineral salts of the bone compartment in compensation appeared to be negligible, since there was no significant change in either blood $[\text{Ca}^{2+}]$ or $[\text{PO}_4^{3-}]$, nor any significant increase in calcium efflux to the water. The intracellular pH values of the bone compartments were only slightly higher than other tissues, and the changes in pH during hypercapnia were similar in bone and white muscle. Thus, the bone compartment in the fish appears to be well regulated, relatively refractory to acute acid-base disturbance, and does not serve as an ion source during acid-base compensation.

INTRODUCTION

The mineral salts that strengthen the skeletons of animals must be derived from the appropriate ions in body fluids. In vertebrate bone, the predominant salts are apatites. These have varying composition and formulae, which might be characterized as: $x\text{Ca}_3(\text{PO}_4)_2 \cdot y\text{CaCO}_3 \cdot z\text{H}_2\text{O}$. The $\text{Ca}_3(\text{PO}_4)_2$ usually is most abundant and is formed at physiological pH primarily by the reaction:



The lesser amounts of CaCO_3 present are formed by the reaction:



and there are varying amounts of bicarbonate and water of hydration also incorporated into the matrix (Poyart, Bursaux & Fréminet, 1975a; Neuman & Mulryan, 1967; Weiss & Watabe, 1978). The formation reactions in either case are attended by the generation of H^+ ion, and any reaction leading to dissolution of these crystalline materials will consume an equal quantity of H^+ ions. The present investigation was prompted by recent parallel investigations of invertebrate skeletal compartments (Wood & Cameron, 1985; Cameron & Wood, 1985; Cameron, 1985; Henry, Kormanik, Smatresk & Cameron, 1981; DeFur, Wilkes & McMahon, 1980) which indicated that intracellular (or intracompartamental) pH is high, and that these alkaline compartments can contribute significantly to the acute compensation of acid-base disturbances. Studies on mammalian bone have also indicated a significant bone buffer pool (Poyart *et al.* 1975a; Poyart, Fréminet & Bursaux, 1975b), which is known under some circumstances to participate in acute buffering of acid-base disturbances (Bettice & Gamble, 1975; Levitt, Turner, Sweet & Pandiri, 1956). Acellular bone in fishes may be mobilized during scale regeneration (Weiss & Watabe, 1978) and in response to various hormone treatments (Mugiya & Watabe, 1977). The immediate objective in these studies was to characterize the bone compartment of a teleost fish—i.e. to measure its size, composition and the pH of associated fluid compartments—and to assess its participation in the acute buffering of acidosis brought about by experimental hypercapnia.

METHODS AND MATERIALS

The experiments were conducted on Channel catfish, *Ictalurus punctatus*, obtained from a commercial catfish farm, fed a pelleted chow, and maintained at $21 \pm 1^\circ\text{C}$ in running, dechlorinated tap water. The water contained $5\text{--}6\text{ mequiv l}^{-1}$ Na^+ and Cl^- , and approximately 2 mequiv l^{-1} Ca^{2+} and HCO_3^- . The fish weighed between 750 and 2000 g, and were free of external infections or parasites. For determinations of the total skeleton weight, the fish were weighed live, killed by a sharp blow to the skull, and all bone material was cleaned of as much adhering tissue as possible. The remaining tissue included with the bone measurements was judged to be small, and was further corrected for by comparing the wet weight of samples of the skeleton, which had been cleaned to the same extent as the whole carcasses, with the same samples after very painstaking complete cleaning. The maximum error from adhering tissue was estimated as no more than 3% of the wet weight.

Dry weights of whole carcasses, as well as of tissue and bone samples, were determined by drying in an oven at 65°C until constant weight was reached in successive weighings. For tissues, overnight was generally sufficient, whereas whole carcasses or skeletons took several days. The dry material was then ground or broken up, added to pre-measured volumes of 2.00 mol l^{-1} HCl and allowed to extract for several days with occasional agitation. The liquid and remaining solids were separated by filtration, and stored for later analysis. Subsequent titrations of the acid extracts indicated a large excess of acid, quite sufficient to dissolve all acid-soluble mineral material.

Protocol for intracellular pH and flux studies

The catfish were prepared for these studies by catheterization of the dorsal aorta according to the method of Soivio, Nyholm & Westman (1975) under general anaesthesia with MS-222. They were then placed in darkened acrylic plastic chambers equipped with temperature control, circulation and aeration, as described by Cameron & Kormanik (1982). At least 24 h was allowed for recovery from surgery and acclimation to the chambers.

For both the control and experimental hypercapnia series, the following time intervals were designated: -2 to 0, 0 to 2, 2 to 4, 4 to 8, and 8 to 24 h. At time 0, 10 μCi of ^3H -labelled inulin and 4 μCi of ^{14}C -labelled DMO (5, 5-dimethyl-2, 4-oxazolidinedione) were infused slowly into the dorsal aortic catheter in a total volume of 0.4–0.7 ml physiological saline. For controls, there was no other treatment, and for the hypercapnic series, the air supply to the aeration column was replaced with a mixture of 1 % CO_2 in air ($P_{\text{CO}_2} = 7.5$ Torr). Blood samples (1.0–1.5 ml) were taken at -2, 0, 2, 4, 8, 24, and in some cases 25 h. Haematocrit determined at the beginning and end showed that less than 15 % of the total blood volume was withdrawn during the course of the experiments. Measurements of the various fluxes were carried out over each interval by appropriate water sampling, and for the larger fish, a complete water exchange was carried out immediately after the 8 h sample to avoid accumulation of ammonia and other metabolites in the closed volume (approximately 13 l).

At the end of the experimental period, a larger final blood sample was drawn, and the fish quickly killed by a sharp blow to the skull. After determining the wet weight, samples of white muscle, red muscle, vertebral bone, skull bone, brain and heart ventricle were removed, blotted and quickly weighed to the nearest 0.1 mg. The tissue samples were then dried as described above.

Analysis and calculations

Blood samples were collected from the catheters in 2 ml syringes. A 50- μl subsample was transferred directly to a capillary type pH microelectrode (Radiometer G297), and a second portion injected into a temperature-jacketed P_{CO_2} electrode cuvette (Radiometer E5036/D616). The remainder was centrifuged for 10 s to remove the red cells. Two 20- μl subsamples were used for analysis of total CO_2 with a conductometric analyser (Capni-Con 3). The remaining plasma was immediately frozen for later analyses.

Intracellular pH was assessed as described by Cameron (1980) and Cameron & Kormanik (1982). The dried tissue and bone samples were combusted in a Packard sample oxidizer, along with injection standards and aliquots of dried plasma. Completely combusted organic material is collected as CO_2 , which contains the ^{14}C label, and H_2O , which contains the ^3H label. All samples were counted with quench correction, and the counts converted to d.p.m. for analysis. The tissue water was corrected for trapped extracellular water using the labelled inulin data. The water contained in the bone compartment after extracellular correction was treated as an intracellular compartment, even though some of the fluid was probably acellular. The intracellular pH was then calculated from the plasma acid-base parameters and the distribution

of DMO between intra- and extracellular water using the formula given by Waddell & Bates (1969).

Intracellular $[\text{HCO}_3^-]$ was estimated by assuming that the pK' for bicarbonate was the same in intra- and extracellular fluids. The formula used was:

$$[\text{HCO}_3^-]_i = \{10^{(pH_i - pK')} + 1\} \{[\text{HCO}_3^-]_e\} / \{10^{(pH_e - pK')} + 1\},$$

where i and e denote intra- and extracellular, respectively.

Ammonia was measured in the plasma at each sampling interval simply to make sure that the accumulation of ammonia in the external water did not stress the fish or lead to acid-base changes. In no case was the concentration above $200 \mu\text{mol l}^{-1}$. The plasma ammonia was assayed employing the enzymatic reaction of 2-oxyglutarate to glutamate (Sigma 170-UV). The ammonia concentration in the water samples was measured at each sample interval with the phenolhypochlorite method (Solorzano, 1969), and ammonia flux calculated as the change in total ammonia, taking time and volume into account.

Calcium and magnesium were measured in both water and plasma samples by atomic absorption, with samples in 1 % HCl and using lanthanum 'swamping'. Some plasma samples were also assayed for calcium with a cresolphthalein complexone colourimetric assay (Sigma 586). Phosphate was assayed in plasma samples with the Fisk/Subbarow method (Sigma 670). A number of assays were also carried out on the acid extracts of bone and tissue samples, using the same procedures. An indirect method was first used to measure the carbonate content of bone and tissue samples: pre-measured aliquots of the extraction acid were titrated to pH 7.000 with 0.2 mol l^{-1} NaOH, and these titration values compared to aliquots of acid from each tissue after extraction. Since phosphate is two-thirds titrated at that pH, the total titration alkalinity of the sample (from the difference) minus two-thirds of the total phosphate was taken as the carbonate content. In most cases the calculated value was within the error limits of the titration procedure, indicating that only traces of carbonate were present.

The total CO_2 pool of fresh bone samples was then measured directly in the following manner. The bone was finely minced in a humid atmosphere, then weighed portions between 200 and 400 mg were placed in divided-bottom flask, with the minced bone on one side, and 1 ml of 0.5 mol l^{-1} H_2SO_4 added to the other. The flask was then tightly stoppered, and connected *via* a length of PE160 tubing to a pressure transducer (Stratham P23dB). The flask was immersed in a temperature bath, and when the pressure trace showed that thermal equilibrium had been reached, the flask was tipped to mix the minced bone with the acid. The flask was then agitated until the pressure trace showed that the acidification, and subsequent evolution of CO_2 , was complete. The system was calibrated by injection of a known volume of air, and results were corrected to STPD. The method was checked by repeating the procedure with weighed portions of NaHCO_3 .

The apparent H^+ excretion of the fish was measured by titration of duplicate 10-ml water samples to pH 4.000 with 0.02 mol l^{-1} HCl, and by calculating the apparent flux as the difference in titration alkalinity between successive samples. The detection limit of this method, as judged by repetitive titrations and standard acid or base additions to the aquarium systems, was $20 \mu\text{equiv}$ change. The apparent H^+ excretion was then calculated as the excretion of total ammonia minus the change in titration

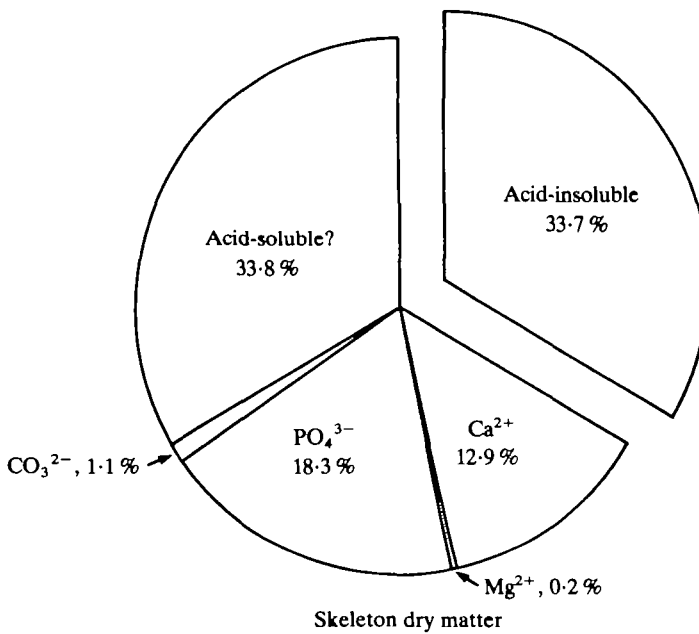


Fig. 1. The composition of the dry matter of the skeleton. The data were taken from 19 skull and vertebral bone samples from six fish. The portion marked 'Acid soluble?' represents material lost upon acidification, but not chemically identified.

Table 1. *Summary of the chemical composition of individual samples of skull and vertebral bone*

	Mean	± s.e.
% Water	43.1	0.69
% Acid weight loss (AWL)	66.3	2.55
As % of AWL:		
Calcium	19.50	0.51
Magnesium	0.33	0.01
Phosphate	27.60	0.74
Carbonate	1.67	0.09

Means are from 19 bone samples.

alkalinity, sign observed. All results given here are positive to denote a net apparent H⁺ excretion (or base uptake).

RESULTS

The skeletal compartment

The distribution of wet and dry material in both the whole carcasses and the bone compartment is shown in Fig. 1 and Table 1. Data for the ratio of wet to dry whole carcass weight were taken from Cameron (1980), and the rest of the values were measured in this study. The whole skeletons were $50.8 \pm 0.9\%$ water by weight, and

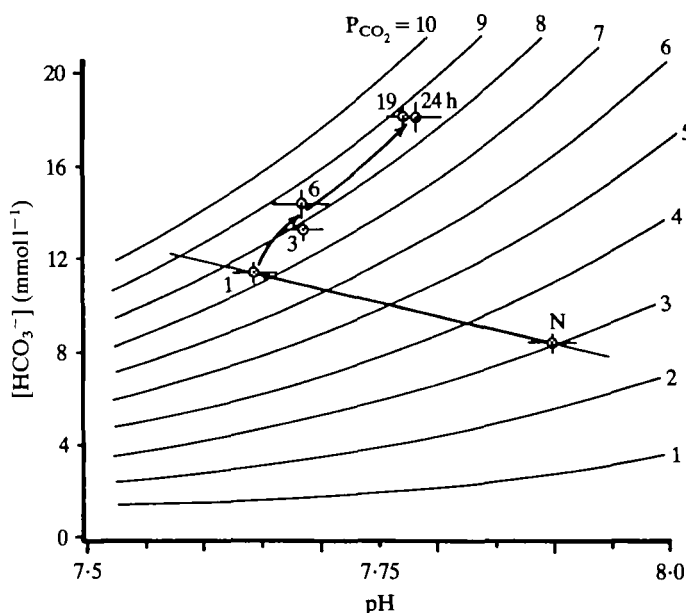


Fig. 2. A pH-HCO₃⁻ diagram showing the time course of blood compensation of hypercapnia in the six treated fish. Horizontal and vertical bars represent \pm s.e., and the numbers beside each point give the time elapsed since the onset of hypercapnia. The control data are labelled N. The diagonal line through the control value shows the passive non-bicarbonate buffer line for true plasma ($\beta = 16$).

$12.2 \pm 0.9\%$ of the total body water was contained in the skeleton. Approximately two-thirds of the skeletal dry material was soluble (or extractable) in acid, and of this roughly half was accounted for by the various inorganic ion analyses shown in Table 1.

Acid-base status of blood, bone and tissues

The blood acid-base status data for control and hypercapnic fish are given in a conventional pH-HCO₃⁻ diagram in Fig. 2. The initial values are shown for the -2 to 0 h period, and the values for the rest of either the control (normal air) or experimental (hypercapnic) period are given up to 24 h. By 24 h the pH compensation of the blood was about 50%.

The intracellular pH data for the six tissue compartments sampled are given in Table 2. The white muscle and vertebrae values showed a significant decline compared with controls after 24 h of hypercapnia, whereas red muscle, skull bone, brain and ventricle did not. Even the significant changes were small, however, as emphasized by the pH-HCO₃⁻ diagram for the tissues (Fig. 3). The values shown were calculated from the measured pH_i values (Table 2), a pK' value for bicarbonate assumed to be the same as plasma (6.174, calculated from data shown in Fig. 2), and an assumed tissue P_{CO₂} 0.7 Torr above that of plasma in both control and hypercapnic treatments. The plasma control point is shown at the right, with a line drawn through it at the achieved value after 24 h. That is, the line drawn has the slope of a line drawn through the time 0 and 25 h points of Fig. 2. The achieved, or effective buffer value (β) for the tissues after 24 h hypercapnia may be calculated as $d[\text{HCO}_3^-]/dpH$. The

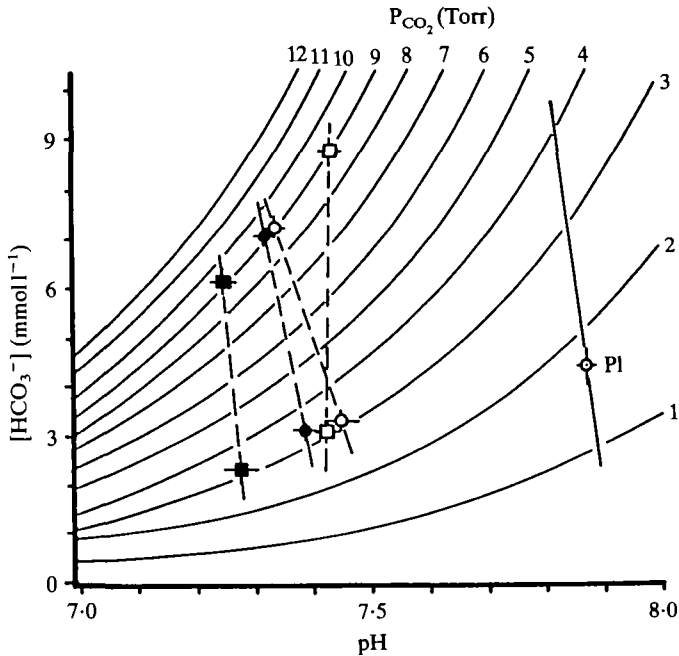


Fig. 3. A pH-HCO₃⁻ diagram showing effective buffering achieved in the plasma (P1; from Fig. 2) and in various tissues after 24 h hypercapnia. See text for complete discussion. (●) White muscle; (■) red muscle; (□) skull bone; (○) vertebrae.

Table 2. *Summary of plasma pH and intracellular pH values from 12 catfish*

Tissue	Control	Hypercapnic	<i>P</i>	% Trapped ECF
Plasma	7.868 ± 0.007	7.780 ± 0.018	<0.01	
White muscle	7.382 ± 0.012	7.328 ± 0.016	<0.05	7.71 ± 0.42
Red muscle	7.278 ± 0.048	7.277 ± 0.024	NS	21.91 ± 0.99
Ventricle	7.558 ± 0.052	7.574 ± 0.029	NS	33.28 ± 1.47
Brain	7.557 ± 0.035	7.491 ± 0.020	NS	9.53 ± 0.27
Skull bone	7.420 ± 0.026	7.439 ± 0.052	NS	46.99 ± 1.85
Vertebrae	7.444 ± 0.017	7.353 ± 0.039	<0.05	28.66 ± 0.75

The values are taken after either 24 h of control conditions, or 24 h of hypercapnia.

All data are shown as mean ± s.e., with *N* = 6 for heart and brain, 12 for plasma and 30 for the other tissues. All data for 20°C.

The *P* values were calculated from unpaired *t*-tests. NS, not significant.

Control and hypercapnic data for percentage trapped ECF were combined, since there was no significant difference.

values were: vertebrae 42, white muscle 71, red muscle > 3600 and brain (not shown) 83. The achieved buffer values for skull bone and ventricle were essentially infinite.

There were no significant differences in either the percentage tissue water or the percentage of trapped extracellular fluid between control and hypercapnic groups. The values, given in Table 2, were similar to those reported earlier for muscle, heart and brain (Cameron, 1980).

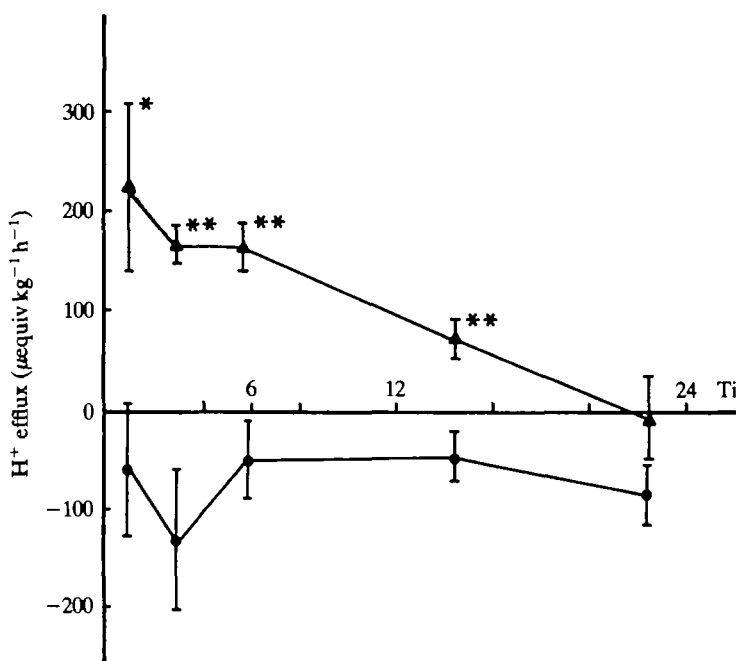


Fig. 4. The apparent net H^+ flux in six control fish (circles) and six hypercapnic fish (triangles). The vertical bars represent ± 1 s.e., and the asterisks denote values significantly different from controls ($P < 0.05$, unpaired t -test).

Table 3. *Summary of whole animal fluxes and blood concentrations of calcium and ammonia for control and hypercapnic catfish*

Parameter	Control	Hypercapnic	P
Ammonia efflux	2.96 ± 0.67	1.69 ± 0.32	< 0.05
Apparent net H^+ flux	-1.30 ± 0.36	2.47 ± 0.36	< 0.001
Calcium net flux	0.030 ± 0.441	-0.027 ± 0.352	NS
Plasma [ammonia]	200 ± 40	108 ± 13	< 0.05
Plasma $[Ca^{2+}]$	2.44 ± 0.20	2.49 ± 0.25	NS
Plasma $[PO_4^{3-}]$	2.57 ± 0.36	2.69 ± 0.52	NS

The mean total fluxes for a 24-h period are given for the six fish in each treatment group, expressed as $\text{mequiv kg}^{-1} \text{h}^{-1} \pm \text{s.e.}$

Concentrations are given as mequiv l^{-1} .

NS, not significant.

The responses of [Ca] and H^+ flux to hypercapnia

The apparent net H^+ flux for control fish was somewhat erratic, but generally negative (Fig. 4; Table 3). Immediately after the onset of hypercapnia, however, the apparent net H^+ flux became strongly positive, and remained so for many hours. If part of the compensation was occurring by acidification and dissolution of the mineral salts of bone, the rate of net calcium efflux would have been expected to increase, and the plasma $[Ca^{2+}]$ might also have risen. As shown in Table 3, there was no significant rise in the net calcium efflux rate, nor was there any significant change in the

mean plasma $[Ca^{2+}]$. Ammonia did not appear to play a significant role in the compensation either, since the rate of ammonia efflux actually fell during the hypercapnic period.

The net transfer of acidic equivalents, or apparent HCO_3^- transfer, may be estimated from the data in Fig. 4. By taking the difference between control and hypercapnic rates for each period and multiplying that difference by the length of the period, an integrated difference is obtained of $3.77 \text{ mequiv kg}^{-1} \text{ h}^{-1}$, or $5.54 \text{ mequiv kg}^{-1}$ fish water. For comparison, this rise in $[HCO_3^-]$ is about 8.9 mmol kg^{-1} water for plasma (about 3 % of body weight), and 3.8 mmol kg^{-1} water for white muscle, the largest compartment.

DISCUSSION

Composition of the skeleton

The proportion of the total body weight contained in the skeletal compartment does not seem to have been measured previously for a teleost fish. Cameron (1975) gave a breakdown of various other tissues for the arctic grayling, showing that 68.5 % of the total body weight was in tissues other than skin and skeleton. Heisler (1978) gave similar figures for various tissues of an elasmobranch, which totalled 70.1 % excluding skeleton and gills. The 16.3 % reported here for the skeleton of the Channel catfish (Table 1) is in line with these values, particularly when the probability of species-to-species difference is admitted. For comparison, the exoskeleton of an invertebrate recently studied was 27 % of the wet weight (the blue crab, *Callinectes sapidus*; Cameron & Wood, 1985). Adhering tissue after dissection may have biased the value for catfish slightly upwards, but some skeletal elements such as fin rays and gill arch supports were not included.

The skeletal compartment has a much lower water content than the other body tissues (50.8 % *vs* about 80 %), presumably because it is mineralized. Although 66.3 % of the dry material of the skeleton was acid-soluble, only about half of the acid-soluble material was accounted for by the inorganic analyses performed (Table 1). Calcium phosphate tends to have a highly variable composition in hydrated crystals, but might be represented by $Ca_{10}(PO_4)_6(OH)_2$. By this formula, the molar ratio of calcium to phosphate should be 1.67, compared to the 1.70 ratio which can be calculated from the data in Table 1. When the small amounts of magnesium and carbonate are taken into account, the agreement with this formula is still good. The direct manometric measurements of bone CO_2 agree reasonably well with those of Weiss & Watabe (1978), but are quite different from those performed on the invertebrate (crab) exoskeleton, which contained roughly 16 times as much carbonate as phosphate. Some fraction of the acid-soluble material not accounted for as inorganic ions would consist of water of hydration in the crystal matrix, and a further portion of acid-soluble proteins.

The bone fluid compartment

The water compartment in whole skeletons was 50.8 % of the wet weight, and in bone samples (Table 1) it was 43.1 %. As shown by the inulin distribution space,

47 % of the water contained in skull bone and 29 % of that in vertebrae comprised part of the extracellular space (Table 1). Although it is not clear whether the remainder is truly intracellular fluid, the fact that it does not equilibrate with inulin, but does allow entry of DMO, indicates that it is separated from the true extracellular fluid space by one or more cell membranes. It could be argued that the apparently intracellular fluid of bones was actually extracellular, but was simply slow to equilibrate. In earlier studies, however, the inulin space and DMO estimates of intracellular pH have been shown to be stable in Channel catfish between 4 and 24 h, which would argue strongly against any slowly-equilibrating pool (Cameron, 1980; Cameron & Kormanik, 1982).

In any case a primary objective of the present study was to determine whether there was a substantial alkaline fluid pool associated with the mineralized portion of the bone, and there clearly was not. Since DMO concentrates strongly in alkaline regions, due to its pK of about 6.1, any significant alkaline pool would have shown up as a high 'intracellular' pH value for the ECF-corrected bone fluid pool. The data in Table 2 show intracellular pH values similar to other tissues.

There may be a link between the differences in pH of the skeletal fluid compartments and in the chemical composition of the mineralized portions in vertebrates and invertebrates. In the crab, where carbonate predominates in nearly a 16:1 ratio (Cameron & Wood, 1985), the pH of the carapace fluid pool is 0.3–0.5 units above the blood, and 0.8–1.0 units higher than the tissues. The solubility product of carbonates requires this alkaline protection, whereas the solubility product of calcium phosphate allows the maintenance of a solid phase at more normal tissue pH values. Alkaline environments have been found in other calcium carbonate-forming tissues, such as the mollusc shell (Campbell & Boyan, 1974) and the avian shell gland (Simkiss, 1970).

The response to hypercapnia

Hypercapnia has a very rapid and pervasive effect of lowering the pH of all internal fluids, due to rapid diffusive equilibration of CO₂ and the subsequent readjustment of the carbonic acid system. It has been shown that there is a slower compensatory reaction to hypercapnia in fish that typically consists of the rise in bicarbonate and pH shown in Fig. 2, and is accomplished mainly by ionic transfers across the gills (Cameron & Randall, 1972; Heisler, 1982). The intracellular pH of most tissues follows a similar pattern, which is thought also to involve ionic exchanges between intra- and extracellular fluids (Heisler, 1978, 1982). The hypercapnic acidification of the bone fluid compartment offers at least the potential for a buffering response which would involve dissolution of the mineral salts by reversal of the formation reactions given above. If this were occurring, at least two consequences should be observable: an efflux of Ca²⁺ from the bone compartment; and a decrease in the intracellular pH of the bone fluid compartment beyond that initially caused by the hypercapnia.

There were no increases in circulating [Ca²⁺] or [PO₄³⁻], as shown by the data in Table 3, nor was there any increase in the net Ca²⁺ efflux from the fish. The ionic data, then, do not suggest any significant participation of the bone pool in the compensation of hypercapnic acidosis.

The DMO data also do not show any participation of the bone pool. In order for the bone to act as a compensating pool, the pH of the bone fluid pool would have to decrease, but the 24 h hypercapnic data for skull bone actually show a higher value than the control (Table 2). The pH_i value for vertebral bone is lower than the control, but the achieved buffer value of 42 is still reasonably high. If this bone were acting as a sacrificial proton sink, its achieved buffer value would be much lower.

In conclusion, the bone compartment of the Channel catfish contains a significant reservoir of mineral salts, and a significant associated fluid pool which is separate from the extracellular fluid. There does not, however, appear to be any significant compensatory response to hypercapnic acidosis which can be attributed to the bone compartment.

Ms Anna Garcia provided able technical assistance throughout the course of the study. This work was supported by NSF Grants PCM80-20982 and PCM83-15833.

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