The Journal of Experimental Biology 205, 3587–3600 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4331

Perspective

Temperature and acid–base balance in ectothermic vertebrates: the imidazole alphastat hypotheses and beyond

Richard F. Burton

Institute of Biomedical and Life Sciences, Thomson Building, University of Glasgow, Glasgow G12 8QQ, UK

e-mail: R.F.Burton@bio.gla.ac.uk

Accepted 22 August 2002

Summary

The 'imidazole alphastat hypothesis' states that intracellular and extracellular pH, partly *via* buffering by imidazole groups, change with temperature in a way that keeps imidazole and protein ionization constant, thus maintaining cell function and minimizing shifts of base equivalents and total CO_2, while adjustment of P_{CO_2} involves imidazole-based receptors. 'The hypothesis', which is actually several hypotheses, has been variously perceived and judged, but its underlying conceptual framework remains largely valid, and is reformulated using differential equations requiring less information input than their integral equivalents. Their usefulness is illustrated with published data on temperature responses in fish cells and whole tetrapods. Mathematical modelling allows general principles to be explored with less immediate concern for uncertainties in experimental data and other information. In tetrapods, it suggests that warming is followed by a loss of base equivalents from the body, and that this loss is due to metabolic adjustments that are not part of pH homeostasis. Uncertainties include intracellular buffer values, local variations in P_{CO_2} within the body, the possible role of buffering by bone mineral, and the temperature dependence of pK values for CO_2/HCO_3^- and imidazole groups. The equations utilize a single, notional, temperature-dependent pK value for all non-bicarbonate buffers in a given body compartment. This approximates to the 'passive component' of pH adjustment to temperature change as measured by the homogenate technique. Also discussed are the diversity of cell responses within individual animals, relevant aspects of the control of ventilation, metabolism and transmembrane transport, and the basis of optimum pH–temperature relationships.

Key words: acid–base balance, alphastat, imidazole alphastat, temperature, pH, carbon dioxide tension, vertebrate, fish, amphibian, reptiles, cell pH.

Introduction

The 'imidazole alphastat hypothesis' of Reeves (1972) has inspired many studies on the effects of temperature on acid–base balance. Some writers accept it without question, while others (e.g. Heisler, 1986c) are dismissive. However, it is not one hypothesis, but several, and some of these may apply to varying degrees in different species and tissues. It should be judged accordingly. The purpose of this paper is not to review the extensive literature, but more to analyze and comment on the constituent hypotheses. To help in this, a simple mathematical model is presented. Its use is illustrated, sometimes with new light shed on old data. Discussion of the model's limitations raises other issues that are equally relevant to some published experimental studies. This paper is not concerned with effects of temperature beyond critical limits, where steady-state function is disturbed.

What Reeves (1972) describes as a "*general conceptual framework*" was put forward mainly as a basis for computing relationships between pH, CO_2 tension (P_{CO_2}) and total CO_2 content (C_{CO_2}). It can be regarded as having the following components, of which those marked with asterisks are highlighted by Reeves himself as conclusions. Components (1) and (2) are not so much proposals of 'the hypothesis' as fundamental facts underlying it. (12) is a natural addition (Reeves, 1985).

(1) Equilibrium constants (pK) of chemical reactions are generally temperature-dependent, including those for the protonation of imidazole groups (pK_{Im}), i.e. for the equilibrium: $Im+H^+=ImH^+$. The fractional dissociation, $[Im]/([Im]+[ImH^+])$, is the 'alpha' in 'alphastat'.

(2) Intracellular and extracellular non-bicarbonate buffering is dominated by imidazole groups, notably those of protein histidines.

(3)* In a solution buffered by comparable amounts of imidazole and CO_2/HCO_3^- in closed system, imidazole buffering dominates, so that $\Delta pH/\Delta T$ is close to $\Delta pK_{Im}/\Delta T$, where T is temperature.

(4)* The temperature dependence of pH in extracellular and intracellular fluids *in vivo* is similar to the temperature dependence of pK_{Im} (i.e. $\Delta pH/\Delta T$ is close to $\Delta pK_{Im}/\Delta T$). Equality implies constant imidazole alpha, and thus the preservation of protein net charge.

(5)* Preservation of protein net charge assures "*optimal enzyme activities, protein conformation and structural stability, as well as stable ion and water distribution across cell membranes based on Donnan principles*". (Unlike protein net charge, Donnan equilibria are not now seen as very relevant here. This is because the distribution of most ionic species across cell membranes is influenced by special transport mechanisms.)

(6)* "*If ventilation is regulated to maintain a constant alpha for one compartment, say blood, alpha imidazole will be maintained for other compartments, regardless of the quantitative makeup of the total intracellular buffer value*."

(7)* "*Alphastat control ensures that changes in total carbon dioxide stores are small with body temperature changes, and thus the transients of loading and unloading such stores, and the associated disturbances of intracellular acid–base state are minimized*." The stores consist of HCO_3^-, CO_3^{2-} and dissolved CO_2 (but see Discussion) and the transmembrane movements of HCO_3^- and CO_2 are best treated here as distinct processes (see items 9–10).

(8) Within any body compartment, regulation of P_{CO_2} to produce this constant imidazole ionization as temperature varies should result in nearly constant $[HCO_3^-]$.

(9) Therefore adaptation to varying temperature should not require transfer across cell membranes of acid–base relevant substances other than CO_2 (i.e. of HCO_3^-, H^+, OH^-, or weak acids and bases). With constant imidazole ionization in all body compartments, no such exchanges would be required with the environment either.

(10) The generation and breakdown of HCO_3^- involves one-for-one loss and production of CO_2. Preservation of imidazole ionization therefore minimizes transient changes in the body's content of dissolved CO_2.

(11)* Key protein imidazole groups might function as respiratory receptors governing ventilation.

(12) Key protein imidazole groups might also function as receptors involved in regulating transmembrane transport of HCO_3^- and H^+, and hence both cell pH and the acid–base function of kidneys and gills.

Underlying the whole scheme is an attractive idea that is implied rather than stated: to the extent that protein properties are all similarly affected by pH and temperature through the dominant role of imidazole groups, each imidazole group facilitates pH homeostasis for all others.

Reeves (1972) did not propose that all conditions apply exactly. Thus item (7) refers to changes that are "*small*" or "*minimized*"; in bullfrogs he found no dependence of CO_2 content on temperature in blood and cardiac muscle, but an increase with warming in liver, and possibly ($0.05<P<0.1$) in striated muscle. He acknowledged that inorganic phosphate, and to a small extent N-terminal α-amino groups, contribute to intracellular buffering (item 2). He did not refer to the small imidazole compounds, e.g. carnosine, that abound in some cells (Crush, 1970; Burton, 1983).

Items (4) or (9) are most often singled out as 'the hypothesis'. Reeves (1976a) put the focus more clearly on preservation of protein net charge (Z), as had Stadie et al. (1925) and Austin et al. (1927) in relation to serum and blood. Many authors have since done the same. Indeed, Cameron (1989) suggested that a 'Z-stat' model might be more appropriate than an imidazole alphastat model. But is Z exactly the key variable? Where buffering is the issue, i.e. in relation to changes in $[HCO_3^-]$, it is the ionization and net charge of *all* the non-bicarbonate buffers that matters. The properties of enzymes may depend largely on the states of specific imidazole, and other, groups, but enzyme properties are also much affected by the crowding effect of other proteins present, and that may itself be affected by net charge (Garner and Burg, 1994; Elcock and McCammon, 2001).

The temperature dependence of pK values

Individually, imidazole groups on proteins have diverse values of $\Delta pK_{Im}/\Delta T$, ranging from –0.01 to $-0.02°C^{-1}$ in myoglobin (Bhattacharya and Lecomte, 1997). The value for one histidine in ribonuclease even changes, *in vitro*, from –0.01 to $-0.05°C^{-1}$ above 32°C (Roberts et al., 1969). Therefore values of $\Delta pK_{Im}/\Delta T$ can hardly match those of $\Delta pH/\Delta T$ in every instance (item 4). Despite this variation, one may define a single quantity, $\Delta pK_{prot}/\Delta T$, that characterizes the temperature dependence of protein net charge. This is not a simple mean of individual values, but, for proteins with net charge Z and combined buffer value β_{prot}, is such as to satisfy the equation:

$$\Delta Z/\Delta T = \beta_{prot}(\Delta pK_{prot}/\Delta T - \Delta pH/\Delta T)\,. \qquad (1)$$

For individual buffer groups, and small temperature changes, the value of $\Delta pK/\Delta T$ is calculable from the standard enthalpy change, $\Delta H°$, as $\Delta H°/(2.303\boldsymbol{R}T^2)$, where $\boldsymbol{R}$ is the gas constant ($1.99\,cal\,°C^{-1}\,mol^{-1}$; $1\,cal=4.186\,J$) and T is the absolute temperature.

As discussed by Heisler (1986c), various values of $\Delta pK_{prot}/\Delta T$ have been assumed in the interpretation of experimental results, notably $-0.018°C^{-1}$ as calculated for histidine at 15°C (Edsall and Wyman, 1958), and $-0.021°C^{-1}$, which lies between values for imidazole and 4-methylimidazole (i.e. –0.020 and $-0.022°C^{-1}$, respectively), from the same source and again at 15°C. Measurements on actual proteins are preferable, but few are available, not all of these representative. For plasma proteins near pH 7, Reeves (1976b) found $\Delta H°$ to be $6940\,cal\,mol^{-1}$, corresponding to a value of $\Delta pK_{prot}/\Delta T$ of $-0.018°C^{-1}$ at 20°C. For oxyhaemoglobin near pH 7, he found respective values of $7300\,cal\,mol^{-1}$ and $-0.019°C^{-1}$. For oxyhaemoglobin at pH 6.8–8.0, Wyman had found $\Delta H°$ to be near 6000–7000 $cal\,mol^{-1}$ (Edsall and Wyman, 1958). For proteins extracted from white skeletal muscle of the eelpout *Zoarces viviparus*, van Dijk et al. (1997) obtained a less negative value of $-0.013°C^{-1}$.

Some vertebrate tissues are also buffered by substantial amounts of low molecular mass imidazole compounds, such as L-histidine itself and the dipeptides carnosine, anserine and balenine (=ophidine) (Crush, 1970; Burton, 1983; Abe, 2000). In fish white muscle, containing amounts between 0 and 148 mmol kg^{-1} wet mass, these compounds may be more concentrated than in red muscle, be responsible for much of the buffer capacity, and show a strong correlation with lactic dehydrogenase activity (Abe, 2000). As calculated from figure 2 of Abe and Okuma (1991), the value of $\Delta pK_{Im}/\Delta T$ for carnosine is $-0.018°C^{-1}$, while values for anserine and balenine are both $-0.013°C^{-1}$. From the same figure, the value for histidine is about $-0.020°C^{-1}$ (compared with $-0.018°C^{-1}$ as given above). Hitzig et al. (1994) obtained values of $-0.0166°C^{-1}$ for imidazole (compared with $-0.02°C^{-1}$ as given above) and $-0.0154°C^{-1}$ for carnosine. For several of these compounds there is thus significant disagreement.

Although buffering is dominated by imidazole groups, and concentrations of inorganic phosphate are often very low (e.g. Marjanovic et al., 1998), the latter may sometimes contribute significantly to intracellular buffering (but note that concentrations are sometimes artefactually raised in tissue samples by hydrolysis of organic phosphates; Heisler and Neumann, 1980). Values of $\Delta pK/\Delta T$ for inorganic phosphate are small, i.e. $-0.006°C^{-1}$ for 0–5°C and –0.001 for 35–40°C^{-1} (Seo et al., 1983), or $-0.003°C^{-1}$ overall, as also found by Alberty (1972). Adenosine triphosphate (ATP), because it is present mainly as MgATP, contributes little to buffering. Phosphocreatine, with its pK of 4.6 (Edsall and Wyman, 1958), is not a significant buffer at cell pH. Although taurine has a high pK (8.8 at 37°C), some cells contain enough to make this a significant buffer, and $\Delta pK/\Delta T$ is probably strongly negative (Bevans and Harris, 1999).

Temperature effects are modelled below in terms of a single, notional, non-bicarbonate buffer with a temperature-dependent pK denoted pK*. It stands for all the different non-bicarbonate buffers and buffer groups that might be present. With these denoted by subscripts A, B etc., $\Delta pK^*/\Delta T$ is in principle calculable as follows (Burton, 1973):

$$\Delta pK^*/\Delta T = \frac{\beta_A(\Delta pK_A/\Delta T) + \beta_B(\Delta pK_B/\Delta T) + \dots}{\beta_A + \beta_B + \dots} . \quad (2)$$

In general, data now available give only approximate values in this way, but estimates have been obtained more directly from the effects of temperature on tissue homogenates. Thus, data of Heisler and Neumann (1980) for the dogfish, *Scyliorhinus stellaris*, imply mean values for $\Delta pK^*/\Delta T$ of $-0.017°C^{-1}$ in white skeletal muscle, $-0.012°C^{-1}$ in red skeletal muscle and $-0.016°C^{-1}$ in heart muscle. (I have derived these by recombining separate estimates for phosphate-like and imidazole-like buffers, themselves originally calculated using assumed values for $\Delta pK/\Delta T$ of $-0.002°C^{-1}$ and $-0.021°C^{-1}$, respectively.) The concentrations of inorganic phosphate in the homogenates used could have been raised by hydrolysis of ATP and phosphocreatine, leading to underestimates of $\Delta pK^*/\Delta T$. Heisler and Neumann argued against that and it is also possible that some inorganic phosphate was precipitated by the added calcium (approximately 3.3 mmol l^{-1}).

In a simple solution of non-bicarbonate buffers, free of HCO_3^-, $\Delta pH/\Delta T = \Delta pK^*/\Delta T$. Therefore $\Delta pK^*/\Delta T$ may be estimated by measuring $\Delta pH/\Delta T$. A similar method has been applied to tissue homogenates, with the value of $\Delta pH/\Delta T$ described as the 'passive component' of the pH adjustment to temperature change (Pörtner, 1990; Pörtner et al., 1990; Pörtner and Sartoris, 1999). In fact the original CO_2 and HCO_3^- are retained in the closed system, but this has little effect on $\Delta pH/\Delta T$ (Burton, 1973; van Dijk et al., 1997), so that this should still provide a good estimate of $\Delta pK^*/\Delta T$ (see item 3 of the hypothesis). An important aspect of this method is that continuing metabolism and the hydrolysis of organic phosphates are prevented by rapid dissection and freeze-clamping of the tissue, followed by homogenization in a solution containing KF and nitrilotriacetic acid that precipitate and chelate Mg^{2+} and Ca^{2+}. The method is also used for measuring non-bicarbonate buffer values and *in vivo* pH.

For skeletal muscle of the black racer snake, *Coluber constrictor*, the value for $\Delta pK^*/\Delta T$ thus found averages $-0.013°C^{-1}$ (Stinner et al., 1998). Corresponding values in both the cane toad *Bufo marinus* and the bullfrog *Rana catesbeiana* average $-0.011°C^{-1}$ (Stinner and Hartzler, 2000), and a similar value applies in tissues of the eelpout *Pachycara brachycephalum* (Pörtner and Sartoris, 1999). For white skeletal muscle of *Z. viviparus* the value of $\Delta pK^*/\Delta T$ is about $-0.006°C^{-1}$ (van Dijk et al., 1997). It is not known what component makes this value so much less negative than that of ΔpK_{prot} in the same muscle ($-0.013°C^{-1}$). All these estimates of $\Delta pK^*/\Delta T$ could have been made slightly less negative by the conversion of MgATP, a poor buffer at intracellular pH, to ATP, which is more like inorganic phosphate in its buffer properties (Alberty, 1972), but the concentration in *Z. viviparus* averages only 3.6 mmol kg^{-1} fresh mass (van Dijk et al., 1997).

There is evidently variation amongst tissues and species, but, for modelling of hypothetical, generalized cells, it seems that representative values of $\Delta pK^*/\Delta T$, at least for muscle, may be taken as about -0.011 to $-0.018°C^{-1}$. Muscle containing much carnosine may be expected to yield values in the more negative part of this range. Values need to be more accurately known for calculations on real experimental data.

Other background information

It is a general finding that arterial blood pH falls with increasing temperature. According to the compilation of Heisler (1986c), values of $\Delta pH/\Delta T$ in plasma are mostly -0.005 to $-0.018°C^{-1}$ in reptiles, -0.011 to $-0.018°C^{-1}$ in amphibians and -0.007 to $-0.014°C^{-1}$ in fish, with respective mean values of approximately -0.011, -0.015 and $-0.012°C^{-1}$. Separate studies on a given species have sometimes yielded very disparate results. Intracellular values of $\Delta pH/\Delta T$, again as compiled by Heisler (1986c), have been found to vary between 0 and $-0.029°C^{-1}$, with a mean of approximately $-0.012°C^{-1}$.

Again there are marked differences amongst tissues and species, and values may vary with temperature range (Boutilier et al., 1987; Stinner and Hartzler, 2000). Heisler (1986c) discusses sources of error. The compilation of Ultsch and Jackson (1996) includes more recent data on both plasma and cells.

Within cells, there is thus some similarity between values of $\Delta pH/\Delta T$ and overall $\Delta pK_{Im}/\Delta T$, but exact comparisons are not yet generally possible. Despite this, it is evident that overall imidazole ionization, dependent on the difference, must increase with cooling in some cells and decrease in others. In the case of white skeletal muscle of *Z. viviparus*, values of both $\Delta pK_{prot}/\Delta T$ for dialyzed homogenates and intracellular $\Delta pH/\Delta T$ have been measured, averaging $-0.013°C^{-1}$ (as already noted) and $-0.016°C^{-1}$, respectively, a difference that is not statistically significant. Thus the net charge on these proteins, and the dissociation state of their histidine residues, hardly varies with temperature (van Dijk et al., 1997). The fractional dissociation of carnosine imidazole has been measured directly, by proton NMR, in tail muscle of intact unanaesthetized newts (*Notophthalmus viridescens*); it was independent of temperature between 10 and 30°C, thus showing alphastat regulation for carnosine (Hitzig et al., 1994).

Concentrations of HCO_3^- in arterial plasma are typically 14–40 mmol l^{-1} in reptiles, with the higher values mostly occurring in chelonians, 10–30 mmol l^{-1} in amphibians, and 3-15 mmol l^{-1} in water-breathing fish (Toews and Boutilier, 1986; Heisler, 1986b; Ultsch and Jackson, 1996). The generally lower concentrations in water breathers relate to the fact that P_{CO_2} in these is typically lower than in air breathers (Rahn, 1967). There is a general tendency for plasma $[HCO_3^-]$ to fall with warming, especially in fish, but as discussed more fully below, the trend is usually small or absent in tetrapods (Heisler, 1986b,c; Ultsch and Jackson, 1996). It is reversed in adults, but not juveniles, of *S. stellaris* (Heisler et al., 1980).

It is harder to generalize about intracellular HCO_3^-, especially since absolute concentrations and temperature effects both differ amongst tissues. For modelling, however, reasonable representative values may often suffice. Figures 3–7 of Ultsch and Jackson (1996) suggest that the cytoplasmic pH of muscle and liver is commonly about 0.3–0.6 unit lower than arterial plasma pH. If P_{CO_2} is assumed to be not much higher in the cells than in arterial plasma (but see below), then the Henderson–Hasselbalch equation suggests that $[HCO_3^-]$ in cells should often be about a quarter or half that in plasma.

Intracellular non-bicarbonate buffer values vary with tissue and species, with measurements in skeletal and cardiac muscle of ectothermic vertebrates being typically 25–110 mequiv l^{-1} cell water pH $unit^{-1}$ (Castellini and Somero, 1981; Heisler, 1986a; Milligan and Wood, 1986; Abe, 2000). Many such values must have been significantly raised artefactually by the release of inorganic phosphate from phosphocreatine and ATP (Pörtner, 1989, 1990). With the homogenate technique (Pörtner et al., 1990), this hydrolysis is avoided (see above) and in gastrocnemius muscle of *B. marinus* the non-bicarbonate buffer value has been found in that way to be 29.8 mequiv l^{-1} cell water pH $unit^{-1}$ (Pörtner, 1990). Measured similarly, values in white muscle of *Z. viviparus* average 31 mequiv l^{-1} cell water pH $unit^{-1}$, being slightly higher at 12°C than at 0°C (van Dijk et al., 1997). A small positive error results from the conversion of MgATP to ATP (see above) but, in *Z. viviparus*, the ATP concentration of 3.6 mmol kg^{-1} fresh mass (van Dijk et al., 1997) implies a maximum contribution to the buffer value of 2.1 mequiv l^{-1} fresh mass pH $unit^{-1}$ (i.e. 3.6×0.575; Burton, 1973).

Buffer values in separated plasma, about 6–8 mequiv l^{-1} pH $unit^{-1}$ in mammals, are usually lower in plasma of ectothermic vertebrates and lower still in their interstitial fluid (Cameron and Kormanik, 1982; Heisler, 1986a; Tufts and Perry, 1998).

Other general findings are discussed below. These include the tendency for P_{CO_2} to rise with increasing temperature and, in many tetrapod species, for whole-body CO_2 content to fall.

Modelling the effects of temperature

In thinking generally about the effects of temperature on acid–base balance, as opposed to fully analyzing experimental data, it is helpful to start with a single-compartment model described mainly in terms of differential equations expressing changes per degree rise in temperature. This reduces the number of variables and allows easy exploration of the effects of changing them, but the equations are strictly valid only for small temperature changes. The equations are based on well-established principles (e.g. Edsall and Wyman, 1958; Reeves, 1972; Burton, 1973; Heisler and Neumann, 1980; Heisler, 1986a), but some may be novel. The model is in line with the general conceptual framework of Reeves (1972), but lacks a restrictive focus on imidazole buffering (item 2 of the hypothesis).

This model of what could be cells or extracellular fluid has a constant volume and contains a single, notional, non-bicarbonate buffer having a buffer value β^* and a temperature-dependent pK, denoted pK* as above. Also present is HCO_3^-, for which the buffer value in open system, β_{bic}, is $2.303[HCO_3^-]$. For simplicity, the small amounts of CO_3^{2-} included by Reeves (1972) are ignored here. With the principle of electroneutrality in mind, we may define a quantity N, reflecting the concentrations and net charge of all other (non-buffer) ions present, such that, in terms of equivalents:

$$\Delta[HCO_3^-] = \Delta[H^+]_{bound} - \Delta N, \quad (3)$$

where $[H^+]_{bound}$ corresponds to the protonated form of the buffer. The term ΔN, when positive, can, for example, match movements of HCO_3^- out of the compartment or the production of dissociating lactic acid within it (see below). Effectively, HCO_3^- movements in one direction are equivalent to movements of CO_3^{2-} or OH^- in the same direction or of H^+ in the opposite direction. All are conveniently described as movements of 'base equivalents', so that an increase in N can equate to a loss of base equivalents. Such changes may be part

of the acid–base adjustment or unrelated responses to temperature change. In accordance with the definition of buffer value, buffer ionization depends on the difference between pK* and pH (Heisler, 1986a), such that:

$$\Delta[H^+]_{bound} = \beta^*(\Delta pK^* - \Delta pH)\,. \quad (4)$$

From equations 3 and 4,

$$\Delta[HCO_3^-]/\Delta T = \beta^*(\Delta pK^*/\Delta T - \Delta pH/\Delta T) - \Delta N/\Delta T\,. \quad (5)$$

The total CO_2 content, C_{CO_2}, equals ($[HCO_3^-]+[CO_2]$), where:

$$[CO_2] = SP_{CO_2}\,, \quad (6)$$

where S is the solubility coefficient of CO_2. Therefore,

$$\Delta C_{CO_2}/\Delta T = \beta^*(\Delta pK^*/\Delta T - \Delta pH/\Delta T) - \Delta N/\Delta T + \Delta[CO_2]/\Delta T\,. \quad (7)$$

Given Equation 6, one form of the Henderson–Hasselbalch equation is:

$$pH = pK_1' - \log S + \log([HCO_3^-]/P_{CO_2})\,. \quad (8)$$

From this, since $\Delta\log[HCO_3^-]$ is $\Delta[HCO_3^-]/(2.303[HCO_3^-])$ and $2.303[HCO_3^-]$ is β_{bic},

$$\Delta pH/\Delta T = \Delta pK_1'/\Delta T - \Delta\log S/\Delta T + (1/\beta_{bic})\Delta[HCO_3^-]/\Delta T - \Delta\log P_{CO_2}/\Delta T\,. \quad (9)$$

It is often useful to treat $\Delta pK_1'/\Delta T - \Delta\log S/\Delta T$ as a single term. It happens to be nearly constant at $0.0053°C^{-1}$ between 0 and 40°C (as calculated from data on S and pK_1' given by Reeves, 1976b) and this value is assumed in all calculations below. Two equations follow from Equations 5 and 9:

$$\Delta N/\Delta T = \beta_{bic}(\Delta pK_1'/\Delta T - \Delta\log S/\Delta T) - \beta_{bic}\Delta\log P_{CO_2}/\Delta T + \beta^*\Delta pK^*/\Delta T - (\beta_{bic}+\beta^*)\Delta pH/\Delta T\,, \quad (10)$$

and

$$\Delta\log P_{CO_2}/\Delta T = (1/\beta_{bic} + 1/\beta^*)\Delta[HCO_3^-]/\Delta T + (\Delta pK_1'/\Delta T - \Delta\log S/\Delta T) - \Delta pK^*/\Delta T + (1/\beta^*)\Delta N/\Delta T\,. \quad (11)$$

Another form of the Henderson–Hasselbalch equation is:

$$C_{CO_2}/[HCO_3^-] = 1 + 10^{pK_1'-pH}\,. \quad (12)$$

At typical extracellular and intracellular pH values, the ratio of C_{CO_2} to $[HCO_3^-]$ is little greater than 1, so that the two may be regarded as interchangable for modelling purposes. Moreover, since P_{CO_2} generally rises with increasing temperature (see below) and S falls, their product, $[CO_2]$, tends to be less temperature-sensitive than either, so that $\Delta[CO_2]/\Delta T$ is often a minor term in Equation 7.

The equations may be adapted to a model of several compartments, each of fractional water volume (or mass) V, such that $\Sigma V=1$. Then both $\Delta N/\Delta T$ and $\Delta[HCO_3^-]/\Delta T$ may be averaged for the whole, as $\Sigma\{V\Delta N/\Delta T\}$ and $\Sigma\{V\Delta[HCO_3^-]/\Delta T\}$, respectively. While the terms β^*, β_{bic}, $\Delta pK^*/\Delta T$ and $\Delta pH/\Delta T$ may differ from one compartment to another, the terms $\Delta pK_1'/\Delta T - \Delta\log S/\Delta T$ and $\Delta\log P_{CO_2}/\Delta T$, in the absence of information to the contrary, may be taken as identical in each. Equations 5 and 9 become:

$$\Sigma\{V\Delta[HCO_3^-]/\Delta T\} = \Sigma\{V\beta^*(\Delta pK^*/\Delta T - \Delta pH/\Delta T)\} - \Sigma\{V\Delta N/\Delta T\}\,, \quad (13)$$

and

$$\Sigma\{V\Delta[HCO_3^-]/\Delta T\} = \Sigma\{V\beta_{bic}\}(\Delta\log P_{CO_2}/\Delta T - \Delta pK_1'/\Delta T + \Delta\log S/\Delta T) + \Sigma\{V\beta_{bic}\Delta pH/\Delta T\}\,. \quad (14)$$

Equations 10 and 11 may be adapted likewise.

Initial exploration of the model

Given the set of model equations, what general conclusions may be drawn, using a minimum of specific experimental data and applying other postulates of the alphastat hypotheses? Let us start by modelling either particular cells, or all cells collectively, as a single compartment. The alphastat hypotheses (items 7, 9 and 10) suggest that we consider the special case in which values of both $\Delta N/\Delta T$ and $\Delta C_{CO_2}/\Delta T$ are zero. With the value of $\Delta[HCO_3^-]/\Delta T$ taken as similar to that of $\Delta C_{CO_2}/\Delta T$ (i.e. zero) in accordance with Equation 12, Equation 11 then reduces to the approximation:

$$\Delta\log P_{CO_2}/\Delta T = (\Delta pK_1'/\Delta T - \Delta\log S/\Delta T) - \Delta pK^*/\Delta T\,. \quad (15)$$

Then, from Equation 10, $\Delta pH/\Delta T$ equals $\Delta pK^*/\Delta T$. This accords with the imidazole hypotheses provided that $\Delta pK^*/\Delta T$ reflects only imidazole buffering. From Equation 15, with $\Delta pK^*/\Delta T$ lying, say, between –0.011 and $-0.018°C^{-1}$ (see Introduction), $\Delta\log P_{CO_2}/\Delta T$ is $0.016–0.023°C^{-1}$. Inasmuch as losses and gains of CO_2 or base equivalents are best minimized, the model thus suggests an appropriate relationship between P_{CO_2} and temperature. These values of $\Delta\log P_{CO_2}/\Delta T$ can also be expressed as Q_{10} values of 1.45–1.70. These lie within the much wider range for arterial blood tabulated by Heisler (1986c), namely 1.17–1.63 in reptiles, 1.08–1.89 in amphibians and 1.04–1.95 in fish. (This overall range corresponds to values of $\Delta\log P_{CO_2}/\Delta T$ of $0.0017–0.029°C^{-1}$.) Matches between model and reality cannot establish the correctness of the assumptions for any species, but they do suggest that these could sometimes be about right.

The possibility that water breathers can increase P_{CO_2} as temperature rises has sometimes been dismissed, and muscle P_{CO_2} in *Z. viviparus* may actually fall with rising temperature (van Dijk et al., 1997). In any case, the characteristic fall in plasma pH is partly due, in most fish species studied, to a fall in plasma $[HCO_3^-]$. This is discussed below, as also the temperature dependence of P_{CO_2} in reptiles and amphibians.

Item (6) of the hypothesis, reworded in model terms, states that regulation of P_{CO_2} to maintain constant buffer ionization in one compartment maintains buffer ionization in all others. In the imidazole alphastat scheme all values of $\Delta N\Delta T$ are zero, while the value of $\Delta pH/\Delta T$ in each compartment equals that of $\Delta pK^*/\Delta T$. Equation 10 then reduces to Equation 15 (not now an approximation). If values of $\Delta\log P_{CO_2}/\Delta T$ are to be the same

in each compartment, so must those of $\Delta pK^*/\Delta T$; they are so in the idealized alphastat model since all buffering is due there to similar imidazole compounds. It is noteworthy that buffer values, i.e. β_{bic} and β^*, do not appear in Equation 15, and are thus irrelevant. In reality, the stated conditions do not generally apply. Nevertheless, regulation of blood P_{CO_2} may well help to regulate pH appropriately in different cell types. Indeed, each type is likely to be adapted – whether in evolution or from day to day – to the characteristic temperature dependence of P_{CO_2} to which it is exposed.

The model suggests an advantage, unrelated to pH optima, for the general fall in intracellular pH with rising temperature: were it not to happen, shifts of base equivalents amongst compartments (ΔN) could often be excessive. Modelling cells of a hypothetical fish as a single compartment, suppose that $\Delta pH/\Delta T$ were actually zero. Suppose also that $\Delta \log P_{CO_2}/\Delta T$ were 0–0.03°C^{-1} (encompassing the range of actual averages given above), that intracellular β^* and β_{bic} were, say, 50 and 4 mequiv kg^{-1} water pH unit^{-1}, respectively, and that the value of $\Delta pK^*/\Delta T$ were, say, –0.015°C^{-1}. Then, from Equation 10, $\Delta N/\Delta T$ would lie between –0.73 and –0.85 mequiv kg^{-1} water °C^{-1}, implying a movement of base equivalents into the cells. (The most influential term is $\beta^*\Delta pK^*/\Delta T$.) Suppose now that this shift applied to all cells in the body, that the ratio of extracellular to intracellular water were 0.4 as in the channel catfish *Ictalurus punctatus* (Cameron, 1980) and that the extracellular [HCO_3^-] were initially, say, 7 mmol kg^{-1} water. A temperature rise of 4°C would then suffice to deplete the extracellular fluid of all its HCO_3^-. This is so unrealistic that the postulated constancy of cell pH has to be wrong and the mean intracellular value of $\Delta pH/\Delta T$ must be negative. As discussed below, the lesser reduction in plasma [HCO_3^-] that occurs with increasing temperature in many fish is a separate issue. The argument applies less forcefully to air-breathing vertebrates, in which extracellular [HCO_3^-] is higher, but, as already noted, values of $\Delta pH/\Delta T$ within cells are typically negative even in these animals. As to individual tissues, these may gain or lose base equivalents on warming (e.g. Reeves, 1972; Heisler and Neumann, 1980; Heisler, 1986c; Stinner and Hartzler, 2000).

Equation 10 may be rearranged as:

$$\Delta pH/\Delta T = (\Delta pK_1'/\Delta T - \Delta \log S/\Delta T) - \Delta \log P_{CO_2}/\Delta T - (\Delta N/\Delta T)/\beta_{bic} + (\beta^*/\beta_{bic})(\Delta pK^*/\Delta T - \Delta pH/\Delta T). \quad (16)$$

This includes terms for the active regulation of $\Delta pH/\Delta T$ through adjustments in P_{CO_2} and in N, but it is the passive mechanism of non-bicarbonate buffering that acts more promptly. This is represented by the term $(\beta^*/\beta_{bic})(\Delta pK^*/\Delta T - \Delta pH/\Delta T)$, which may be positive or negative. In the special case that $\Delta pH/\Delta T$ equals $\Delta pK^*/\Delta T$ in the steady state, non-bicarbonate buffering makes no ultimate contribution to homeostasis, regardless of how much buffer is present. This contrasts with the determination of $\Delta pH/\Delta T$ by $\Delta pK^*/\Delta T$ in CO_2-free buffer solutions.

In extracellular fluids, including cerebrospinal fluid, the buffer value, β^*, is often much less than β_{bic} (see Introduction). Then, regardless of the value of $\Delta pK^*/\Delta T - \Delta pH/\Delta T$, the temperature dependence of pH is governed mainly by the values of $\Delta \log P_{CO_2}/\Delta T$ and $(\Delta N/\Delta T)/\beta_{bic}$. Further modelling of the extracellular compartment in isolation is unrewarding, especially when compared with previous treatments of true plasma (e.g. Reeves, 1972; Rodeau and Malan, 1979).

Applying the one-compartment model to fish cells

In its ideal form, the alphastat scheme has proved particularly inappropriate to the water-breathing fish that have been studied, for temperature changes in these produce substantial shifts of base equivalents into and out of both cells and body, as well as changes in plasma [HCO_3^-]. Here we start by modelling all the cells of the body as a single compartment. As in the source papers, it is assumed that $\Delta N/\Delta T$ relates only to shifts of base equivalents across cell membranes, and not to metabolic changes within cells. The first two examples illustrate how old data may be usefully approached in new ways.

The adult dogfish, *S. stellaris*, is notable for its high value of $\Delta \log P_{CO_2}/\Delta T$ in arterial blood, i.e. 0.029°C^{-1} on average as compared with 0.0017°C^{-1} in juveniles (Heisler et al., 1980). Warming leads to net loss of base equivalents from the cells collectively, and also from the whole body. Unusually, extracellular [HCO_3^-] rises (Heisler et al., 1980; Heisler, 1984). In modelling the cells, we may take the following representative values: $\Delta \log P_{CO_2}/\Delta T$, 0.029°C^{-1}; β^*, 45 mequiv kg^{-1} cell water pH unit^{-1}; $\Delta N/\Delta T$, 0.105 mequiv kg^{-1} cell water °C^{-1} (Heisler and Neumann, 1980; Heisler, 1984). Based on an intracellular [HCO_3^-] of *"about 1 mmol l^{-1}"* (Heisler and Neumann, 1980), β_{bic} is 2.3 mequiv kg^{-1} cell water pH unit^{-1}. The value of $\Delta pK^*/\Delta T - \Delta pH/\Delta T$ may now be estimated from Equation 10. If the value of $\Delta pK^*/\Delta T$ is taken to be between –0.011 and –0.018°C^{-1} (see Introduction), that of $\Delta pK^*/\Delta T - \Delta pH/\Delta T$ must be 0.0025–0.0028°C^{-1}. For white muscle, which makes up most of the fish, the corresponding difference is also positive, approximately 0.001°C^{-1}, since $\Delta pK^*/\Delta T$ is approx. –0.017°C^{-1} (see Introduction) and $\Delta pH/\Delta T$ is approx. –0.018°C^{-1} (Heisler et al., 1980). The estimated difference of 0.0025–0.0028°C^{-1} for all cells collectively, multiplied by β^*, implies that non-bicarbonate buffering generates base equivalents at 0.11–0.13 mequiv kg^{-1} cell water °C^{-1}. The value chosen for whole-body β^* is based somewhat arbitrarily on measurements on white, red and cardiac muscle, all possibly raised artefactually by inorganic phosphate (see Introduction). If a lower value is used, say 30 mequiv kg^{-1} cell water pH unit^{-1}, the estimate of $\Delta pK^*/\Delta T - \Delta pH/\Delta T$ becomes 0.0037–0.0042°C^{-1} and the quantities of base equivalents generated on warming are 2.4% lower.

The mean value of $\Delta pK^*/\Delta T - \Delta pH/\Delta T$ may also be estimated for the intracellular compartment of *I. punctatus*. Data of Cameron and Kormanik (1982) suggest the following representative values. For the whole body: $\Delta \log P_{CO_2}/\Delta T$,

0.0164°C^{-1}; $\Sigma\{V\Delta N/\Delta T\}$, 0.056 mequiv kg^{-1} cell water °C^{-1}. For all cells: V, 0.726; ΔpH/ΔT, –0.0148°C^{-1}; $\Delta[HCO_3^-]/\Delta T$, –0.028 mmol kg^{-1} water °C^{-1}; β*, 35 mequiv kg^{-1} water pH unit^{-1}. For the extracellular fluid: V, 0.274; ΔpH/ΔT, –0.0141°C^{-1}; $\Delta[HCO_3^-]/\Delta T$, –0.097 mmol kg^{-1} water °C^{-1}; β*, say 4 mequiv kg^{-1} water pH unit^{-1} (chosen as slightly below the value of approximately 5.8 for blood with zero haematocrit). The extracellular value of ΔpK*/ΔT is unknown, but not very critical. If it is taken as, say, –0.013 to –0.019°C^{-1} (see Introduction), then, from Equation 13, the intracellular value of ΔpK*/ΔT–ΔpH/ΔT is 0.0003–0.0006°C^{-1}, again positive. This seems small enough to suggest item (4) of the alphastat scheme and implies very little generation of HCO_3^- by buffering. From Equation 5, the value of $\Delta N/\Delta T$ for the cells is 0.039–0.048 mequiv kg^{-1} water °C^{-1}.

With increasing temperature, there is a net loss of base equivalents from the cells of both these species, and a rise in P_{CO_2}. These effects can be seen as alternative ways of lowering cell pH. According to Equation 10, for constant intracellular values of β_{bic}, β*, ΔpK*/ΔT and ΔpH/ΔT, reduction in the value of $\Delta N/\Delta T$ from x to zero in a model fish would require that the value of $\Delta\log P_{CO_2}/\Delta T$ be raised by x/β_{bic}. In *S. stellaris* the value of $\Delta\log P_{CO_2}/\Delta T$ would thus need to be approximately 0.075°C^{-1}. Such a high, perhaps unattainable, value does not explain why the shifts in base equivalents occur in the real fish, since, with only minor changes in ΔpK*/ΔT or ΔpH/ΔT, $\Delta N/\Delta T$ could be zero even at constant P_{CO_2}.

The air-breathing swamp eel *Synbranchus marmoratus* contrasts with these two species in that, collectively, the cells take up base equivalents on warming, i.e. about 0.25 mequiv kg^{-1} cell water °C^{-1} (Heisler, 1984). Here, therefore, the value of ΔpK*/ΔT must be more negative than that of ΔpH/ΔT. Indeed, values of ΔpH/ΔT are only –0.009 and –0.003°C^{-1}, respectively, in white skeletal muscle and heart.

White skeletal muscle of *Z. viviparus* is of interest for its high value of $\Delta N/\Delta T$ (van Dijk et al., 1997). From the mean value of $\Delta[HCO_3^-]/\Delta T$, i.e. –0.27 mmol kg^{-1} cell water °C^{-1}, and from estimates, already noted, of ΔpH/ΔT *in vivo* and of ΔpK*/ΔT and β*, the value of $\Delta N/\Delta T$ is calculated from Equation 5 as 0.58 mequiv kg^{-1} cell water °C^{-1}. If all the cells were like this, large temperature changes would have major implications for extracellular homeostasis.

Applying the model to tetrapods: the protein titration hypothesis of Stinner et al. (1998)

In detailed studies of *C. constrictor*, Stinner and Wardle (1988) and Stinner et al. (1998) found an increase in whole-body CO_2 stores with cooling, and with it increases in both C_{CO_2} and pH in arterial plasma and skeletal muscle. Little evidence was found for changes in either lactate or the balance of inorganic anions and cations that would suggest shifts of base equivalents. It was concluded that changes in whole-body CO_2 stores result from changes in protein ionization coupled with ventilatory regulation of P_{CO_2}, such that the overall value of $\Delta pK_{prot}/\Delta T$ is more negative than that of ΔpH/ΔT. Thus there is titration of proteins by carbonic acid (along with other non-bicarbonate buffers), rather than a maintenance of their overall ionization state as in item (4) of the hypothesis.

Stinner et al. (1998) extended this idea to other reptiles and amphibians. Whole-body CO_2 stores increased with cooling in all 13 species studied (Stinner and Wardle, 1988; Stinner et al., 1994, 1998). The changes took many hours. Mean values of $\Delta C_{CO_2}/\Delta T$ ranged from –0.02 mmol kg^{-1} body mass °C^{-1} in *R. catesbeiana*, to –0.21 mmol kg^{-1} body mass °C^{-1} in the tortoise, *Testudo graeca*. Presumably the range would be even greater if expressed in terms of body water. Only in the bullfrogs do the results seem close to the alphastat prediction of constant tissue CO_2 content.

The further analysis by Stinner et al. (1998) may be described in terms of the one-compartment model, in which Equation 7 shows the determinants of $\Delta C_{CO_2}/\Delta T$. The term $\Delta N/\Delta T$ is regarded as negligible on the basis of the findings for *C. constrictor*. As already noted, the term $\Delta[CO_2]/\Delta T$ is also trivial here. Thus Equation 7 reduces to:

$$\Delta C_{CO_2}/\Delta T = \beta^*(\Delta pK^*/\Delta T - \Delta pH/\Delta T)\,. \qquad (17)$$

Stinner et al. (1998) took the whole-body value of ΔpH/ΔT as approximating that for arterial plasma and found a linear relationship between that and whole-body $\Delta C_{CO_2}/\Delta T$ (10 species; r=–0.93). The values of ΔpH/ΔT are mostly taken from other studies over similar ranges of temperature (Howell et al., 1970; Jackson et al., 1974; Malan et al., 1976; Bickler, 1981; Wood et al., 1981; Nicol et al., 1983). The equation of the regression line is:

$$\Delta C_{CO_2}/\Delta T = 8.24(-0.022 - \Delta pH/\Delta T)\,. \qquad (18)$$

In accordance with Equation 17, this suggested for the whole body a mean non-bicarbonate buffer value, β*, of 8.24 mequiv kg^{-1} body mass pH unit^{-1} and a mean value for ΔpK*/ΔT of –0.022°C^{-1}.

As Stinner et al. (1998) pointed out, this value of $\Delta pK^*/\Delta T$ is reasonable for some small imidazole compounds. However, the real value is probably no more negative than –0.018°C^{-1} (see Introduction). As for that whole-body value of β*, it may be re-expressed in terms of body water using a representative body water content of, say, 76% (Deyrup, 1964; Bentley, 1976); it then becomes 10.8 mequiv kg^{-1} water pH unit^{-1}. This is little above the 8.1 mequiv l^{-1} pH unit^{-1} calculated for plasma of *C. constrictor*, despite the greater contribution of the cells, where β* is presumably much higher (see Introduction). It therefore seems improbably low. Next, the assumption that the whole-body value of ΔpH/ΔT approximates that for arterial plasma may be inappropriate, since values of ΔpH/ΔT in *C. constrictor* averaged –0.009°C^{-1} in muscle and –0.0028°C^{-1} in arterial plasma. (Modelling of the sort to be described next, but starting with Equations 13 and 18, also shows the assumption to be implausible.)

The data may be better modelled by treating the body water as two compartments, intracellular and extracellular, and taking account of data on P_{CO_2}. Values of $\Delta\log P_{CO_2}/\Delta T$ are assumed to be the same in both compartments, both for simplicity and because the average differences in P_{CO_2}

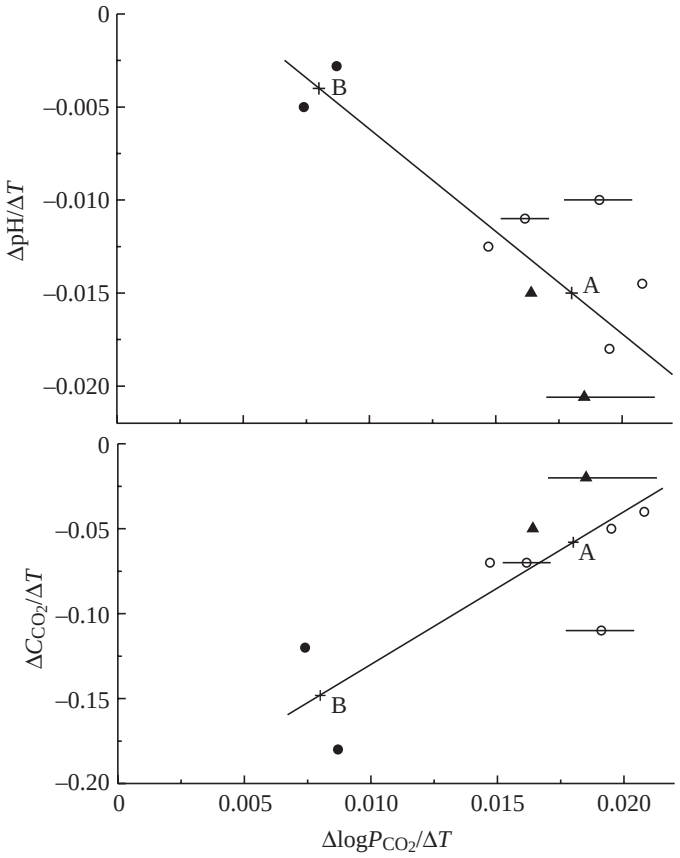

Fig. 1. The temperature dependence of arterial pH and P_{CO_2} and of whole-body CO_2 stores (C_{CO_2}) in tetrapods. Mean values of $\Delta pH/\Delta T$ and of $\Delta C_{CO_2}/\Delta T$ (mmol kg^{-1} body mass $°C^{-1}$) are as tabulated by Stinner et al. (1998). Mean values of $\Delta \log P_{CO_2}/\Delta T$, in $°C^{-1}$, are from the following sources (where there is more than one per species, the means and ranges of individual means are shown): filled circles, *Coluber constrictor* (Stinner et al., 1998), *Varanus exanthematicus* (Wood et al., 1981); open circles, *Trachemys scripta* (Jackson et al., 1974; Jackson and Kagan, 1976; Hitzig, 1982), *Alligator mississippiensis* (Davies, 1978), *Chrysemys picta* (Nicol et al., 1983; Glass et al., 1985), *Chelydra serpentina* (Howell et al., 1970), *Dipsosaurus dorsalis* (Bickler, 1981); filled triangles, *Bufo marinus* (Howell et al., 1970), *Rana catesbeiana* (Howell et al., 1970; Reeves, 1972; Mackenzie and Jackson, 1976). As described in the text, the crosses A and B show representative values for the two distinct groups of species (i.e. filled circles, and open circles together with filled triangles).

between blood and cells in these air breathers are likely to be small (Burton, 2001). Again $\Sigma\{V\Delta[HCO_3^-]/\Delta T\}$ is taken as approximating $\Delta C_{CO_2}/\Delta T$. Equation 18 is assumed to apply exactly. Plausible values, representing all species collectively, are allotted to other parameters. The water content of the body is again taken as 76%.

Fig. 1 shows the correlations between $\Delta pH/\Delta T$ and $\Delta \log P_{CO_2}/\Delta T$ in arterial plasma and between $\Delta C_{CO_2}/\Delta T$ and $\Delta \log P_{CO_2}/\Delta T$ for the whole body. The nine species fall into two groups and the mean values for each group of $\Delta pH/\Delta T$ and $\Delta \log P_{CO_2}/\Delta T$ are shown by crosses marked A and B. For group A they are, respectively, $-0.015°C^{-1}$ and $0.018°C^{-1}$. For group B they are, respectively, $-0.004°C^{-1}$ and $0.008°C^{-1}$. Values of $\Delta C_{CO_2}/\Delta T$ corresponding to groups A and B, calculated from Equation 18, are –0.058 and –0.148 mmol kg^{-1} body mass $°C^{-1}$, or –0.076 and –0.195 mmol kg^{-1} water $°C^{-1}$, respectively.

For the extracellular and intracellular fluids, respectively, the values of V are taken as 0.4 and 0.6 and the bicarbonate buffer values, β_{bic}, are taken as 60 and 24 mequiv kg^{-1} water pH $unit^{-1}$. From these parameters and the data of the previous paragraph, the value of $\Delta pH/\Delta T$ for the cells is calculated using Equation 14. For both sets of data it is $-0.0141°C^{-1}$. The important point here is not its exact value, which depends on the chosen parameters, but the fact that the mean values for the two groups of species are plausibly modelled as similar. This seems a reasonable postulate (despite differences within groups) if optimum cell function depends on the relationship between intracellular pH and temperature.

The parameters β^* and $\Delta pK^*/\Delta T$ for extracellular fluid are now allotted plausible values, say 5 mequiv kg^{-1} water pH $unit^{-1}$ and $-0.018°C^{-1}$, respectively (see Introduction). Then the extracellular values of $\Delta N/\Delta T$, calculated from Equation 10, are 0.123 mequiv kg^{-1} water $°C^{-1}$ for group A and 0.008 mequiv kg^{-1} water $°C^{-1}$ for group B. Warming therefore leads to a loss of base equivalents from the extracellular fluid. These calculations may be repeated for the whole body using Equation 13, with values for β^* and $\Delta pK^*/\Delta T$ in the intracellular fluid taken, say, as 25 mequiv kg^{-1} water pH $unit^{-1}$ and $-0.0130°C^{-1}$, respectively. Then the whole-body value of $\Delta N/\Delta T$, i.e. $\Sigma\{V\Delta N/\Delta T\}$, is 0.087 mequiv kg^{-1} water $°C^{-1}$ for group A and 0.183 mequiv kg^{-1} water $°C^{-1}$ for group B. These are almost equal, but opposite in sign, to the respective values of $\Delta C_{CO_2}/\Delta T$ given above. (That this is about equally true of the two group means was arbitrarily achieved by adjusting the value of $\Delta pK^*/\Delta T$.) For groups A and B, the sums $\Sigma\{V\Delta N/\Delta T\}+\Delta C_{CO_2}/\Delta T$ are, respectively, +0.011 and –0.012 mequiv kg^{-1} water $°C^{-1}$. These small differences correspond to the titration of proteins and other buffers (Equation 13).

The model compares and integrates data from two groups of species, but the diagonal lines in Fig. 1 can also represent the changes in a single hypothetical individual as the value of $\Sigma\{VN\}$ alters after temperature changes. After warming, there is a loss of gaseous CO_2 from its body and, as modelled, this loss is nearly matched by a loss of base equivalents. These come partly from the cells, with the CO_2 generated from HCO_3^- and H^+ ions (and almost entirely so in the case of group B). The reduction in $[HCO_3^-]$ in the cells is matched by a fall in P_{CO_2} that keeps the value of $\Delta pH/\Delta T$ constant. (For the P_{CO_2} to fall even as CO_2 is generated from HCO_3^-, ventilatory adjustments to P_{CO_2} would have to be rapid; the many hours needed to achieve a steady state would thus reflect slow changes in N rather than slow gas exchange.) If the value of $\Sigma\{V\Delta N/\Delta T\}$ were zero, $\Delta C_{CO_2}/\Delta T$ would be positive instead of negative, i.e. 0.036 mmol kg^{-1} water $°C^{-1}$ (calculated from Equations 13 and 18).

No one set of parameters can be right for all species, and each species or individual should ideally be modelled with its own set. Moreover, data for real cells are generally for particular muscle tissue rather than for the whole intracellular compartment. The chosen parameters are broadly in line with data given in the Introduction, but the constant intracellular value of $\Delta pH/\Delta T$ in the model ($-0.0141°C^{-1}$) is more negative than the values of –0.009, –0.012 and $-0.007°C^{-1}$ measured in skeletal muscle of *C. constrictor*, *R. catesbeiana* and *B. marinus*, respectively (Stinner et al., 1998; Stinner and Hartzler, 2000). It is closer to the mean whole-body intracellular value ($-0.0151°C^{-1}$) obtained by Bickler (1982) in the lizard *Dipsosaurus dorsalis*, itself more negative than his values for skeletal and cardiac muscle (–0.0098 and $-0.0104°C^{-1}$, respectively).

According to the model, $\Delta[HCO_3^-]/\Delta T$ for the extracellular fluid is negative, having values of –0.14 and $-0.08\ mmol\ l^{-1}\ °C^{-1}$, respectively, for groups A and B (calculated from Equations 5 or 9). Some values determined for real arterial plasma in these tetrapods are similar in sign and magnitude (Wood et al., 1981; Stinner and Wardle, 1988; Stinner et al., 1998), but others do not differ significantly from zero (Jackson et al., 1974; Bickler, 1981; Nicol et al., 1983; Stinner et al., 1994). Shifts of base equivalents ($\Delta N/\Delta T$) between compartments are hard to quantify experimentally, because accurate analyses are needed for all ions present. The shifts seem insignificant in *D. dorsalis* (Bickler, 1984) and, although they do occur in *B. marinus* and *R. catesbeiana*, a consistent trend is not evident (Stinner and Hartzler, 2000). Neither these discrepancies and uncertainties, nor the arbitrariness of some model parameters, invalidate the semi-quantitative conclusions summarized next.

Two important conclusions have emerged. Firstly, it is shown that mean intracellular values of $\Delta pH/\Delta T$ could be similar in the two groups of species. Secondly, the net loss of gaseous CO_2 following a rise in temperature could be due largely to titration of HCO_3^- as base equivalents are lost from cells and body (or proton equivalents gained). The latter idea is absent from the model of Stinner et al. (1998), but was originally suggested by Stinner (1982) for the snake *Pituophis melanoleucus*. Bickler (1984) did not find evidence for a major role of excretion in the acid–base responses of *D. dorsalis* to temperature. Moreover, in none of the species can the loss of base equivalents be due mainly to excretion of HCO_3^- since the accompanying reduction in whole-body CO_2 stores is measured as gaseous CO_2. It is therefore more likely that the whole-body gains and losses of base equivalents involve metabolic adjustments to intracellular concentrations of organic ions (see below). Because $\Delta pH/\Delta T$ does not alter, these would not be homeostatic for pH. A major temperature-dependent process modifying N, and best developed in species where C_{CO_2} changes most, should now be sought. Although the model is made consistent with the relationship of Equation 18, that remains unexplained.

Discussion

The equations provide a convenient approach to the effects of temperature on pH, P_{CO_2}, $[HCO_3^-]$ and buffer ionization. They can be used both to explore the effects of varying buffer properties etc. in hypothetical animals and to complement previous analyses of experimental data. The tetrapod model unites various facts, ideas and uncertainties in what may be less a true description than a step towards better understanding. Indeed there are yet more uncertainties involved and some of these are discussed under the next heading. Also discussed below are other parts of the imidazole alphastat scheme (items 5, 11 and 12) that do not relate directly to the model, and three phenomena that are excluded from the strict alphastat scheme. These are metabolic adjustments to non-buffer ions within cells, and movements of base equivalents both amongst cells of different types and between body and environment.

Some limitations of the model in interpreting measurements on real animals

In the model 'total CO_2' consists just of the dissolved gas and HCO_3^- ions, and their reactions are treated in terms of a straightforward apparent equilibrium constant, pK_1'. However, this has been found in many studies to decrease with increasing pH (e.g. Dill et al., 1937; Boutilier et al., 1985; Heisler, 1986a). This effect can markedly influence estimates of intracellular $[HCO_3^-]$ when this is calculated from P_{CO_2} and pH (Reeves, 1976a). Unfortunately, the influence of pH on pK_1' varies from study to study and a relationship quantified for arterial plasma may be wrong for cells, especially when some of the cell water is 'bound' (Garner and Burg, 1994). Uncertainties regarding $\Delta pK_1'/\Delta T$ and $\Delta[HCO_3^-]/\Delta T$ are much less. This effect of pH is not fully understood. 'CO_2' as measured gasometrically exists not only as free HCO_3^- and dissolved gas (plus minute amounts of carbonic acid), but as CO_3^{2-} (generally in small amounts), as carbamate (barely studied outside of erythrocytes), possibly as the compound $H_2CO_3.HCO_3^-$ (Covington et al., 1981) and as ion pairs of HCO_3^- with cations such as Na^+, Mg^{2+} and Ca^{2+}. Boutilier et al. (1985) and Burton (1987) discuss these and other uncertainties in calculating '$[HCO_3^-]$' from pH and P_{CO_2}. According to formulae given by Heisler (1986a), values of $\Delta pK_1'/\Delta T - \Delta \log S/\Delta T$ for solutions resembling protein-free plasma are about $0.0053°C^{-1}$ for 0–25°C, as above, and about $0.0069°C^{-1}$ for 25–35°C. As discussed in the next paragraph, the intracellular P_{CO_2} cannot be assumed to be exactly that of accessible extracellular fluids.

The single-compartment model is homogeneous, unlike both real extracellular space and real cells. Regarding P_{CO_2}, this is generally higher in venous than arterial plasma and higher still in interstitial fluid (Pörtner and Sartoris, 1999), and cells vary in their relationships to blood vessels. As modelled by Burton (2001), the discrepancy between arterial and mean whole-body interstitial or cellular P_{CO_2} varies inversely with arterial P_{CO_2} and is therefore greatest in water-breathing fish. How far the discrepancy varies with temperature is unclear, because it depends also on respiratory quotient, oxygen tensions, the relative solubilities of the two gases, and the possible

disequilibrium of CO_2 in blood. As modelled for the whole body, average interstitial and cellular P_{CO_2} in some fish can be more than twice the arterial P_{CO_2}. For real cells C_{CO_2} has sometimes been calculated from cell pH and arterial P_{CO_2} by the method of Cameron (1980); for fish especially, the results could be much too low. Equation 14 is based on the assumption that $\Delta\log P_{CO_2}/\Delta T$, but not necessarily P_{CO_2}, is the same in all compartments.

Cytoplasm is heterogeneous too. Much of a cell may be taken up with acidic organelles or the very alkaline mitochondrial matrix. In addition, local variations in net fixed charge density on proteins and membranes must cause inhomogeneities of pH and $[HCO_3^-]$. Estimates of cell pH made using DMO (5,5-dimethyloxazolidine-2,4-dione) yield values that approximate to averages for the whole cell contents, but, more exactly, what is averaged is 10^{-pH} (Waddell and Bates, 1969). There is little quantitative information on mitochondrial pH *in vivo* and on its temperature sensitivity in ectotherms. However, Moyes et al. (1988) have studied mitochondria isolated from red muscle of the carp, *Cyprinus carpio*: provided extramitochondrial pH varied as *in vivo*, the transmembrane pH gradient remained constant. If this gradient is generally insensitive to temperature in ectothermic vertebrates, then values of $\Delta pH/\Delta T$ in cells obtained with DMO should reflect cytosolic values. Cell $[HCO_3^-]$ may be calculated from pH and P_{CO_2}. With pH values obtained by the DMO method, the resulting $[HCO_3^-]$ averaged over all subcompartments, each with its fractional volume V and concentration $[HCO_3^-]$, equals $1/\{\Sigma(V/[HCO_3^-])\}$, where $\Sigma V=1$. Given, for example, two subcompartments of equal volume differing in pH by 0.3, the true mean $[HCO_3^-]$ is 12% higher than that calculated from the pH measured by DMO. Pörtner and Sartoris (1999) give a detailed analysis of the effects of cytoplasmic heterogeneity on pH measurements and calculations of $[HCO_3^-]$.

The model compartments are of constant volume, but in reality osmotic water movements may occur. They should result from gain or loss of HCO_3^- through buffering, as in erythrocytes, and sometimes from transmembrane shifts of base equivalents with other ions, as when HCO_3^- leaves with Na^+ (but not when it exchanges with Cl^-). The resulting volume changes should generally be small, but there may also be substantial effects of temperature on fluid volumes that are not due to acid–base changes. In *D. dorsalis*, inulin space increases after warming, with no significant change in total body water (Bickler, 1982). In *B. marinus* and *R. catesbeiana*, Stinner and Hartzler (2000) found substantial reductions in ion concentrations on cooling, due to increased body hydration. Measurements of extracellular space may depend on the marker used (Poole-Wilson and Cameron, 1975; Cameron and Kormanik, 1982).

Generally missing from discussions of temperature effects are extracellular buffers that are not in solution. In *I. punctatus*, there is no release of Ca^{2+} or phosphate from bone in hypercapnia (Cameron, 1985), but this does not mean that bone has no role in buffering. In mammals, buffering of acid by bone may result in the dissolution and excretion of bone Ca^{2+}, but another mechanism involves release of HCO_3^- with Na^+ and K^+ rather than with Ca^{2+} (Green and Kleeman, 1991; Burton, 1992; Jackson, 1999). In turtles, bone and shell are important in buffering metabolic acidosis, with CO_2 being released with Ca^{2+}, Mg^{2+} and Na^+ (Jackson, 1999; Jackson et al., 2000). Since there is little information on temperature effects, it is at least possible that bone mineral, and shell in turtles, play a significant buffering role in the adjustment of ectotherms to temperature change. In modelling, such buffering would contribute to ΔN. The two major extracellular proteins, collagen and elastin, can contribute little to buffering at the normal pH of extracellular fluid, through lack of appropriate buffer groups (Hartman and Bakerman, 1966; Winlove et al., 1992).

Metabolic adjustments to non-buffer organic ions

The term $\Delta N/\Delta T$ may include changes in the concentrations of organic ions (e.g. lactate, phosphocreatine) resulting from altered metabolism. (One may also think in terms of gains and losses of protons, but '$\Delta N/\Delta T$' is the relevant term in the equations.) Lactate, especially, has long been known to participate in pH homeostasis in mammals (Giebisch et al., 1955; Siesjö, 1973). However, in relation to pH-temperature relationships in ectothermic vertebrates, such metabolic adjustments in non-buffer ions were long neglected (Pörtner, 1987). They could either disturb pH homeostasis or be regulated as part of it, and complete negative-feedback loops for the homeostasis of pH in relation to temperature have yet to be established. Changes resulting from disturbances other than of temperature within its critical limits, e.g. in hypoxia or exercise, are not our concern here.

Whatever the role of lactate, it is a potential contributor to the term $\Delta N/\Delta T$ and its concentration has often been determined in acid–base studies. A rise with increasing temperature has been observed in the plasma of *B. marinus* (Stinner et al., 1994) and, in *C. constrictor*, there is both a small rise in mean concentration from 15 to 35°C and a big one at 1–7°C (Stinner and Wardle, 1988). However, in other resting, undisturbed reptiles, the low blood or plasma concentrations may be independent of temperature (e.g. Wood et al., 1981; Bickler, 1981; Bickler, 1984; Stinner et al., 1998).

Metabolite concentrations are affected by other factors too, and, in muscle of fish such as the trout *Oncorhynchus mykiss*, concentrations of lactate, phosphocreatine and ATP are very rapidly disturbed by handling (Dobson and Hochachka, 1982; Tang and Boutilier, 1991). In white muscle of resting *O. mykiss*, killed quickly to minimize this effect, Lehoux and Guderley (1997) found an increase in lactate concentration of about 3 mmol kg^{-1} muscle between 8 and 22°C (possibly, as the authors suggest, reflecting increased metabolic rate or excitation of the trout), while mean muscle pH fell by $0.011°C^{-1}$. Concentrations of ATP, ADP, AMP, fructose 6-phosphate and fructose 1,6-biphosphate changed little, but the latter three were too low to affect N much anyway.

Cell diversity: shifts of base equivalents amongst cells

So far the cells of an individual have been modelled as a single compartment. However, these can differ significantly in regard to $\Delta pH/\Delta T$ (e.g. Malan et al., 1976; Bickler, 1982; Cameron and Kormanik, 1982; Heisler, 1986b,c; Toews and Boutilier, 1986; Boutilier et al., 1987; Ultsch and Jackson, 1996). Accordingly, the sign, as well as magnitude, of $\Delta N/\Delta T$ could vary from one kind of cell to another, even for shared values of $\Delta \log P_{CO_2}/\Delta T$ (Equation 10). At the same time, any contributions of metabolic adjustments to $\Delta N/\Delta T$ could vary too. It is therefore possible that base equivalents shift simultaneously into and out of different cell types in response to temperature changes (without necessarily much net effect on extracellular $[HCO_3^-]$). For juvenile and adult *S. stellaris*, Heisler and Neumann (1980) calculated that warming leads to movements of base equivalents out of heart muscle and into white and red skeletal muscle. One may speculate that the situation is sometimes somewhat comparable with that postulated for hypercapnia in mammals (Burton, 1980a, 1992) in which base equivalents shift into cells that actively and effectively regulate their intracellular pH and out from other ('altruistic') cells that do so less effectively. Some shifts of base equivalents following a temperature change could then be governed more by changing transmembrane ionic gradients (notably for H^+, HCO_3^-, Na^+, K^+ and Cl^-) than by set points for intracellular pH homeostasis. This whole topic has been little explored.

Exchanges of base equivalents between body and environment

We have seen that homeostasis is possible in some vertebrates with minimal renal or transepithelial exchanges of base equivalents with the environment (item 9). The exchanges are insignificant in *S. marmoratus* (Heisler, 1984), but base equivalents are lost in response to warming, about 0.04 mequiv kg^{-1} body water $°C^{-1}$, in *S. stellaris* (Heisler, 1978) and *I. punctatus* (Cameron and Kormanik, 1982). This loss accompanies the net shift from the cells discussed above. Shifts of base equivalents must always be linked to shifts of other ions in accordance with the principle of electroneutrality; depending on diet, and the composition of the surrounding water in freshwater species, this may be another reason for animals to limit exchanges with the environment, especially when there are frequent large temperature fluctuations. A complicating issue is the excretion of acid or base linked to dietary intake and metabolism, since their rates are also generally temperature-dependent.

The assessment of urinary acid or base excretion may involve measurement of titratable acidity. Confusingly, this can be defined and measured in several ways (Burton, 1980b). It is usual to titrate the urine to the pH of the arterial plasma, but in the present context the endpoint would more appropriately be the whole-body mean pH, if that were known. In either case, the endpoint pH would then vary with temperature, with the curious result that acid urine of a given composition would have a higher measured titratable acidity if collected from a cooler animal. Buffering of the urine by phosphate must increase this effect. Although the procedures of Cameron and Kormanik (1982) are unclear, their positive values of $\Delta N/\Delta T$ for whole *I. punctatus* could only be partially explained in this way.

What determines the optimum temperature dependence of pH?

At a given temperature, the optimum pH of interstitial fluid, and so indirectly that of arterial plasma, probably relates to key proteins on particular cell surfaces (Burton, 2001, 2002). Nevertheless, an obvious effect of acute alkalaemia due to hyperventilation in ourselves is hypocalcaemic tetany as plasma albumin binds more Ca^{2+}. The binding is to imidazole groups (Pedersen, 1972), but the effect of temperature, whether in mammals or ectotherms, is unknown.

Within cells, many enzyme systems are affected by pH and temperature (Somero, 1981, 1986; Nattie, 1990). Immediate effects, as studied *in vitro*, are best known, but major metabolic reorganization, and even changes in cell structure, may occur *in vivo* with thermal acclimation (Jones and Sidell, 1982; Guderley and Gawlicka, 1992; Pörtner et al., 1998). As already noted, protein ionization could be critical to many cellular processes through the effects of macromolecular crowding. More specific in effect, pH influences membrane channels and carriers that are not themselves involved directly in pH homeostasis (e.g. Chen et al., 1996; Tavi et al., 1999; Wiebe et al., 2001), a field of research expanding too rapidly for review here. Cardiac contractility in mammals is very sensitive to intracellular pH (and presumably temperature) through its effects on various Ca^{2+} mechanisms (Tavi et al., 1999; Balnave and Vaughan-Jones, 2000). Although negligible contributors to buffering, ionizing groups on proteins other than imidazole can confer sensitivity to pH within the physiological range, i.e. sulphydryl and α-amino groups (Edsall and Wyman, 1958) and even carboxyl groups (Chen et al., 1996). Temperature sensitivity has not been studied in all these instances.

Because movements of base equivalents between compartments must be accompanied by movements of other ions, changes in, for example, extracellular $[K^+]$ and intracellular $[Na^+]$, or their avoidance, may turn out to have some role in determining the optimal acid–base adjustments to changing temperature (see also the earlier comment on ionic gradients). Effects of temperature on the ionic composition of plasma may be significant, especially in fish, but are inconsistent and often small (Hitzig, 1982; Bickler, 1984; Burton, 1986b; Stinner and Hartzler, 2000).

There are so many possible interactions of pH and temperature that the main determinants of the overall optimum value of $\Delta pH/\Delta T$ are unknown. They probably vary from one tissue to another, and could sometimes represent compromises linked to no single process. Regarding effects unrelated to protein ionization, an idea still current is that homeostasis is of 'relative alkalinity' or $[OH^-]/[H^+]$ ratio (Rahn, 1967; Howell et al., 1970), but this has no known physicochemical or functional significance (Burton, 2002).

Imidazole groups as pH sensors in pH homeostasis

There is evidence that the temperature-dependent control of ventilation involves imidazole groups in *T. scripta elegans* (Hitzig, 1982; Hitzig and Nattie, 1982) and more clearly so in mammals (Nattie, 1990). It is unnecessary for regulated values of $\Delta pH/\Delta T$ in plasma or cerebrospinal fluid to equal the relevant values of $pK_{Im}/\Delta T$ (Burton, 1986a).

Cytoplasm is generally more alkaline than the pH that would correspond to electrochemical equilibrium for H^+ and HCO_3^- ions, so that the regulation of intracellular pH must involve transmembrane transport. Where this involves a set point for pH, this would be expected to show an appropriate temperature dependence, and Marjanovic et al. (1998) obtained evidence for this with respect to the Na^+-H^+ exchanger (and perhaps the H^+-Na^+/Cl^--HCO_3^- exchanger) in skeletal muscle (isolated, and at low P_{CO_2}) of the frogs *Rana temporaria* and *R. pipiens*. Nattie (1990) had suggested a role for imidazole groups associated with the Na^+-H^+ exchanger and this is becoming increasingly likely (Wiebe et al., 2001).

Conclusions

How the original imidazole alphastat hypotheses (‘the hypothesis’) are perceived and judged depends on which are deemed central, on how strictly generalizations should apply, and on what species and tissues are in mind. Nowadays much more emphasis is given both to transmembrane shifts of base equivalents and to metabolic adjustments, but otherwise Reeves’s ‘general conceptual framework’ and emphasis on imidazole ionization remain broadly valid. The mathematical models serve to clarify those of the alphastat hypotheses that concern buffering, CO_2 and shifts of base equivalents, but they conform to strict imidazole alphastat thinking just to the extent that non-bicarbonate buffering is dominated by imidazole groups. The evident role of imidazole groups as pH sensors in acid–base homeostasis is a separate issue.

There have been so many studies of pH–temperature relationships that it is hard to co-ordinate them all, paying due attention to differences in animal and sample treatments, methods of analysis etc. As illustrated, the models provide a convenient approach to the complexities of real data, and the treatment of tetrapod data has even suggested new hypotheses to be tested experimentally. However, exact quantitation can be hampered by various uncertainties, e.g. regarding the temperature dependence of pK values (for both CO_2/HCO_3^- and non-bicarbonate buffers) and, especially in fish, of variations in P_{CO_2} within the body. Extracellular buffering, as by bone mineral, may prove to have been given too little attention, both in the models and generally. Another area of ignorance is what actually defines optimum pH–temperature relationships; much molecular detail is now becoming available on pH effects, but often these are only studied at constant temperature.

References

Abe, H. (2000). Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. *Biochem. (Moscow)* **65**, 757-765.

Abe, H. and Okuma, E. (1991). Effect of temperature on the buffering capacities of histidine-related compounds and fish skeletal muscle. *Nippon Suisan Gakkaishi* **57**, 2101-2107.

Alberty, R. A. (1972). Calculation of the standard Gibbs free energy enthalpy, and entropy changes for the hydrolysis of ATP at 0°, 25°, 37°, and 75°C. In *Horizons of Bioenergetics* (ed. A. San Pietro and H. Gest), pp. 135-147. New York: Academic Press.

Austin, J. H., Sunderman, F. W. and Camack, J. G. (1927). Studies in serum electrolytes. II. The electrolyte composition and the pH of serum of a poikilothermous animal at different temperatures. *J. Biol. Chem*. **72**, 677-685.

Balnave, C. D. and Vaughan-Jones, R. D. (2000). Effect of intracellular pH on spontaneous Ca^{2+} sparks in rat ventricular myocytes. *J. Physiol. Lond.* **528**, 25-37.

Bentley, P. J. (1976). Osmoregulation. In *Biology of the Reptilia*, vol. 5 *Physiology A* (ed. C. Gans and W. R. Dawson), pp. 365-412. London: Academic Press.

Bevans, C. G. and Harris, A. L. (1999). Regulation of connexin channels by pH – direct action of the protonated form of taurine and other aminosulfonates. *J. Biol. Chem*. **274**, 3711-3719.

Bhattacharya, S. and Lecomte, J. T. J. (1997). Temperature dependence of histidine ionization constants in myoglobin. *Biophys. J.* **73**, 3241-3256.

Bickler, P. E. (1981). Effects of temperature on acid–base balance and ventilation in desert iguanas. *J. Appl. Physiol*. **51**, 452-460.

Bickler, P. E. (1982). Intracellular pH in lizard *Dipsosaurus dorsalis* in relation to changing body temperature. *J. Appl. Physiol*. **53**, 1466-1472.

Bickler, P. E. (1984). Effects of temperature on acid and base excretion in a lizard, *Dipsosaurus dorsalis*. *J. Comp. Physiol. B* **154**, 97-104.

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

Boutilier, R. G., Iwama, G. K., Heming, T. A. and Randall, D. J. (1985). The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. *Respir. Physiol*. **61**, 237-254.

Burton, R. F. (1973). The roles of buffers in body fluids: mathematical analysis. *Respir. Physiol.* **18**, 34-42.

Burton, R. F. (1980a). The role of intracellular buffers in acid–base disturbances: mathematical modelling. *Respir. Physiol.* **39**, 45-61.

Burton, R. F. (1980b). Acid and base excretion: assessment and relationships to diet and urine composition. *Comp. Biochem. Physiol.* **66A**, 371-375.

Burton, R. F. (1983). The composition of animal cells: solutes contributing to osmotic pressure and charge balance. *Comp. Biochem. Physiol*. **76B**, 663-671.

Burton, R. F. (1986a). The role of imidazole ionization in the control of breathing. *Comp. Biochem. Physiol*. **83A**, 333-336.

Burton, R. F. (1986b). Ionic regulation in fish: the influence of acclimation temperature on plasma composition and apparent set points. *Comp. Biochem. Physiol*. **85A**, 23-28.

Burton, R. F. (1987). On calculating concentrations of ‘HCO_3’ from pH and PCO_2. *Comp. Biochem. Physiol.* **87A**, 417-422.

Burton, R. F. (1992). The roles of intracellular buffers and bone mineral in the regulation of acid–base balance in mammals. *Comp. Biochem. Physiol.* **102A**, 425-432.

Burton, R. F. (2001). Differences in pH between interstitial fluid and arterial blood in water-breathing and air-breathing vertebrates. *Physiol. Biochem. Zool*. **74**, 607-615.

Burton, R. F. (2002). Evolutionary determinants of normal arterial plasma pH in ectothermic vertebrates. *J. Exp. Biol.* **205**, 641-650.

Cameron, J. N. (1980). Body fluid pools, kidney function, and acid–base regulation in the freshwater catfish *Ictalurus punctatus*. *J. Exp. Biol.* **86**, 171-185.

Cameron, J. N. (1985). The bone compartment in a teleost fish, *Ictalurus punctatus*: size, composition and acid–base response to hypercapnia. *J. Exp. Biol.* **117**, 307-318.

Cameron, J. N. (1989). Acid–base homeostasis: past and present perspectives. *Physiol. Zool.* **62**, 845-865.

Cameron, J. N. and Kormanik, G. A. (1982). Intracellular and extracellular acid–base status as a function of temperature in the freshwater channel catfish, *Ictalurus punctatus*. *J. Exp. Biol.* **99**, 127–142.

Castellini, M. A. and Somero, G. N. (1981). Buffering capacity of vertebrate muscle: correlations with potentials of anaerobic function. *J. Comp. Physiol*. **143**, 191-198.

Chen, X.-H., Bezprozvanny, I. and Tsien, R. W. (1996). Molecular basis of proton block of L-type Ca^{2+} channels. *J. Gen. Physiol*. **108**, 363-374.
Covington, A. K., Robinson, R. A. and Sarbar, M. (1981). Determination of carbonate in the presence of hydroxide. Part 2: Evidence for existence of a novel species from first derivative potentiometric titration curves. *Anal. Chim. Acta* **130**, 93-102.
Crush, K. G. (1970). Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol*. **34**, 3-30.
Davies, D. G. (1978). Temperature-induced changes in blood acid–base status in the alligator, *Alligator mississipiensis*. *J. Appl. Physiol*. **45**, 922-926.
Deyrup, I. J. (1964). Water balance and the kidney. In *Physiology of the Amphibia* (ed. J. A. Moore), pp. 251-328. New York: Academic Press.
Dill, D. B., Daly, C. and Forbes, W. H. (1937). The pK′ of serum and red cells. *J. Biol. Chem*. **117**, 569-579.
Dobson, G. P. and Hochachka, P. W. (1982). Role of glycolysis in adenylate depletion and repletion during work and recovery in teleost white muscle. *J. Exp. Biol.* **129**, 125-140.
Edsall, J. T. and Wyman, J. (1958). *Biophysical Chemistry*, vol. 1. New York: Academic Press.
Elcock, A. H. and McCammon, J. A. (2001). Calculation of weak protein-protein interactions: the pH dependence of the second virial coefficient. *Biophys. J*. **80**, 613-628.
Garner, M. M. and Burg, M. B. (1994). Macromolecular crowding and confinement in cells exposed to hypertonicity. *Am. J. Physiol*. **266**, C877-C892.
Giebisch, G., Berger, L. and Pitts, R. F. (1955). The extrarenal response response to acute acid–base disturbances of respiratory origin. *J. Clin. Invest*. **34**, 231-245.
Glass, M. L., Boutilier, R. G. and Heisler, N. (1985). Effects of body temperature on respiration, blood gases and acid–base status in the turtle *Chrysemys picta bellii*. *J. Exp. Biol.* **114**, 37–51
Green, J. and Kleeman, C. R. (1991). Role of bone in regulation of systemic acid–base balance. *Kidney Internat*. **39**, 9-26.
Guderley, H. and Gawlicka, A. (1992). Qualitative modification of muscle metabolic organization with thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem*. **10**, 123-132.
Hartman, B. K. and Bakerman, S. (1966). Titration of salt-extracted human skin collagen. *Biochem*. **5**, 2221-2228.
Heisler, N. (1978). Bicarbonate exchange between body compartments after changes of temperature in the larger spotted dogfish (*Scyliorhinus stellaris*). *Respir. Physiol.* **33**, 145-160.
Heisler, N. (1984). Role of ion transfer processes in acid–base regulation with changes of temperature in fish. *Am. J. Physiol.* **246**, R441-R451.
Heisler, N. (1986a). Buffering and transmembrane ion transfer processes. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 3-47. Amsterdam: Elsevier.
Heisler, N. (1986b). Acid–base regulation in fishes. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 309-356. Amsterdam: Elsevier.
Heisler, N. (1986c). Comparative aspects of acid–base regulation. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 397-450. Amsterdam: Elsevier.
Heisler, N. and Neumann, P. (1980). The role of physico-chemical buffering and of bicarbonate transfer processes in intracellular pH regulation in response to changes of temperature in the larger spotted dogfish (*Scyliorhinus stellaris*). *J. Exp. Biol.* **85**, 99-110.
Heisler, N., Neumann P. and Holeton G. F. (1980). Mechanisms of acid–base adjustment in dogfish (*Scyliorhinus stellaris*) subjected to long-term temperature acclimation. *J. Exp. Biol.* **85**, 89-98.
Hitzig, B. M. (1982). Temperature-induced changes in turtle CSF pH and central control of ventilation. *Respir. Physiol*. **49**, 205-222.
Hitzig, B. M. and Nattie, E. E. (1982). Acid–base stress and central chemical control of ventilation in turtles. *J. Appl. Physiol.* **53**, 1365-1370.
Hitzig, B. M., Perng, W.-C., Burt, T., Okunieff, P. and Johnson, D. C. (1994). ^{1}H-NMR measurement of fractional dissociation of imidazole in intact animals. *Am. J. Physiol.* **266**, R1008–R1015.
Howell, B. J., Baumgardner, F. W., Bondi, K. and Rahn, H. (1970). Acid–base balance in cold-blooded vertebrates as a function of body temperature. *Am. J. Physiol.* **218**, 600-606.
Jackson, D. C. (1999). The role of turtle shell in acid–base buffering. In *Regulation of Tissue pH in Plants and Animals. A Reappraisal of Current Techniques. Society for Experimental Biology Seminar Series*, **68** (ed. S. Egginton, E. W. Taylor and J. A. Raven), pp. 215-231. Cambridge: Cambridge University Press.
Jackson, D. C., Crocker, C. E. and Ultsch, G. R. (2000). Bone and shell contribution to lactic acid buffering of submerged turtles *Chrysemys picta bellii* at 3°C. *Am. J. Physiol*. **278**, R1564-R1571.
Jackson, D. C. and Kagen, R. D. (1976). Effects of temperature transients on gas exchange and acid–base status of turtles. *Am. J. Physiol*. **230**, 1389-1393.
Jackson, D. C., Palmer, S. E. and Meadow, W. L. (1974). The effects of temperature and carbon dioxide breathing on ventilation and acid–base status of turtles. *Respir. Physiol*. **20**, 131-146.
Jones, P. L. and Sidell, B. D. (1982). Metabolic responses of striped bass (*Morone saxatilis*) to temperature acclimation. II. Alterations in metabolic carbon sources and distributions of fiber types in locomotory muscles. *J. Exp. Zool.* **219**, 163-171.
Lehoux, E. A. and Guderley, H. E. (1997). Thermally induced changes in intracellular pH and modulators of phosphofructokinase in trout white muscle. *J. Exp. Biol.* **200**, 931-939.
Mackenzie, J. A. and Jackson, D. C. (1978). The effect of temperature on cutaneous CO_2 loss and conductance in the bullfrog. *Respir. Physiol.* **32**, 313-323.
Malan, A., Wilson, T. L. and Reeves, R. B. (1976). Intracellular pH in cold-blooded vertebrates as a function of body temperature. *Respir. Physiol*. **28**, 29-47.
Marjanovic, M., Elliott, A. C. and Dawson, M. J. (1998). The temperature dependence of intracellular pH in isolated frog skeletal muscle: Lessons concerning the Na^+-H^+ exchanger. *J. Membr. Biol.* **161**, 215-225.
Milligan, C. L. and Wood, C. M. (1986). Tissue intracellular acid–base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* **123**, 123-144.
Moyes, C. D., Buck, L. T. and Hochachka, P. W. (1988). Temperature effects on pH of mitochondria isolated from carp red muscle. *Am. J. Physiol.* **254**, R611-R615.
Nattie, E. E. (1990). The alphastat hypothesis in respiratory control and acid–base balance. *J. Appl. Physiol*. **69**, 1201–1207.
Nicol, S. C., Glass, M. L. and Heisler, N. (1983). Comparison of directly determined and calculated plasma bicarbonate concentration in the turtle *Chrysemys picta bellii* at different temperatures. *J. Exp. Biol.* **107**, 521-525.
Pedersen, K. O. (1972). Binding of calcium to serum albumin II. Effect of pH via competitive hydrogen and calcium ion binding to the imidazole groups of albumin. *Scand. J. clin. Lab. Invest*. **29**, 75-83.
Poole-Wilson, P. A. and Cameron, I. R. (1975). ECS, intracellular pH, and electrolytes of cardiac and skeletal muscle. *Am. J. Physiol*. **229**, 1299-1304.
Pörtner, H. O. (1987). Contributions of anaerobic metabolism to pH regulation in animal tissues: theory. *J. Exp. Biol.* **131**, 69-87.
Pörtner, H. O. (1989). The importance of metabolism in acid–base regulation and acid–base methodology. *Can. J. Zool*. **67**, 3005-3017.
Pörtner, H. O. (1990). Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol*. **81**, 275-288.
Pörtner, H. O., Boutilier, R. G., Tang, Y. and Toews, D. P. (1990). Determination of intracellular pH and P_{CO_2} after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol*. **81**, 255-274.
Pörtner, H. O., Hardewig, I., Sartoris, F. J. and van Dijk, P. L. M. (1998). Energetic aspects of cold adaptation: critical temperatures in metabolic, ionic and acid–base regulation? In *Cold Ocean Physiology. Society for Experimental Biology Seminar Series*, **66** (ed. H. O. Pörtner and R. C. Playle), pp. 88-120. Cambridge: Cambridge University Press.
Pörtner, H. O. and Sartoris, F. J. (1999). Invasive studies of intracellular acid–base parameters: quantitative analyses during environmental and functional stress. In *Regulation of Tissue pH in Plants and Animals. A Reappraisal of Current Techniques. Society for Experimental Biology Seminar Series*, **68** (ed. S. Egginton, E. W. Taylor and J. A. Raven), pp. 69-98. Cambridge: Cambridge University Press.
Rahn, H. (1967). Gas transport from the external environment to the cell. In *Development of the Lung. Ciba Foundation Symposium* (ed. A. V. S. de Reuck and R. Porter), pp. 3-23. London: Churchill.
Reeves, R. B. (1972). An imidazole alphastat hypothesis for vertebrate acid–base regulation: tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* **14**, 219-236.
Reeves, R. B. (1976a). The interaction of body temperature and acid–base balance in ectothermic vertebrates. *Annu. Rev. Physiol*. **39**, 559-586.
Reeves, R. B. (1976b). Temperature-induced changes in blood acid–base status: pH and PCO_2 in a binary buffer. *J. appl. Physiol*. **40**, 752-761.
Reeves, R. B. (1985). Alphastat regulation of intracellular acid–base state? In

Circulation, Respiration, and Metabolism (ed. R. Gilles), pp. 414-423. Berlin: Springer-Verlag.

Roberts, G. C. K., Meadows, D. H. and Jardetzky, O. (1969). Nuclear magnetic resonance studies of the structure and binding sites of enzymes. VII. Solvent and temperature effects on the ionization of histidine residues of ribonuclease. *Biochem.* **8**, 2053-2056.

Rodeau, J. L. and Malan, A. (1979). A two-compartment model of blood acid–base state at constant or variable temperature. *Respir. Physiol.* **36**, 5-30.

Seo, Y., Murakami, M., Watari, H., Imai, Y., Yoshizaki, K., Nishikawa, H. and Morimoto, T. (1983). Intracellular pH determination by a ^{31}P-NMR technique. The second dissociation constant of phosphoric acid in a biological system. *J. Biochem.* **94**, 729-734.

Siesjö, B. K. (1973). Metabolic control of intracellular pH. *Scand. J. clin. Lab. Invest.* **32**, 97-104.

Somero, G. N. (1981). pH-temperature interactions on proteins: principles of optimal pH and buffer system design. *Mar. Biol. Lett.* **2**, 163-178.

Somero, G. N. (1986). Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* **251**, R197-R213.

Stadie, W. C., Austin, J. H. and Robinson, H. W. (1925). The effect of temperature on the acid–base-protein equilibrium and its influence on the CO_2 absorption curve of whole blood, true and separated plasma. *J. Biol. Chem.* **66**, 901-920.

Stinner, J. N. (1982). Ventilation, gas exchange and blood gases in the snake, *Pituophis melanoleucus*. *Respir. Physiol.* **47**, 279-298.

Stinner, J. N. and Hartzler, L. K. (2000). Effect of temperature on pH and electrolyte concentration in air-breathing ectotherms. *J. Exp. Biol.* **203**, 2065-2074.

Stinner, J. N., Hartzler, L. K., Grguric, M. R. and Newlon, D. L. (1998). A protein titration hypothesis for the temperature-dependence of tissue CO_2 content in reptiles and amphibians. *J. Exp. Biol.* **201**, 415-424.

Stinner, J. N., Newlon, D. L. and Heisler, N. (1994). Extracellular and intracellular carbon dioxide concentration as a function of temperature in the toad *Bufo marinus*. *J. Exp. Biol.* **195**, 345–360.

Stinner, J. N. and Wardle, R. L. (1988). Effect of temperature upon carbon dioxide stores in the snake *Coluber constrictor* and the turtle *Chrysemys scripta*. *J. Exp. Biol.* **137**, 529-548.

Tang, Y. and Boutilier, R. G. (1991). White muscle intracellular acid–base and lactate status following exhaustive exercise: a comparison between freshwater- and seawater-adapted rainbow trout. *J. Exp. Biol.* **156**, 153-171.

Tavi, P., Han, C. and Weckström, M. (1999). Intracellular acidosis modulates the stretch-induced changes in E–C coupling of the rat atrium. *Acta Physiol. Scand.* **167**, 203-213.

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

Tufts, B. and Perry, S. F. (1998). Carbon dioxide transport and excretion. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 229-281. San Diego: Academic Press.

Ultsch, G. R. and Jackson, D. C. (1996). pH and temperature in ectothermic vertebrates. *Bull. Alabama Mus. Nat. Hist.* **18**, 1-41.

van Dijk, P. L. M., Hardewig, I. and Pörtner, H. O. (1997). Temperature-dependent shift of pH_i in fish white muscle: contributions of passive and active processes. *Am. J. Physiol.* **272**, R84-R89.

Waddell, W. J. and Bates, R. G. (1969). Intracellular pH. *Physiol. Rev.* **49**, 285-329.

Wiebe, C. A., DiBattista, E. R. and Fliegel, L. (2001). Functional role of polar amino acid residues in Na^+/H^+ exchangers. *Biochem. J.* **357**, 1-10.

Winlove, C. P., Parker, K. H., Ewins, A. R. and Birchler, N. E. (1992). The polyelectrolyte properties of elastin. *J. Biomech. Engin.* **114**, 293-300.

Wood, S. C., Johansen, K., Glass, M. L. and Hoyt, R. W. (1981). Acid–base regulation during heating and cooling in the lizard, *Varanus exanthematicus*. *J. Appl. Physiol.* **50**, 779-783.