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Invasive studies of intracellular acid–base parameters: quantitative analyses during environmental and functional stress

Introduction

In recent years, studies of the effects of environmental variables on the physiology and biochemistry of different animal species have increasingly included an analysis of acid–base status and regulation. pH values in different body compartments are widely accepted to play a key role in the maintenance of physiological function or its limitation under functional or environmental stress. pH affects protein function in metabolism and O_2 transport. Also, acid–base and metabolic regulation are interdependent processes such that changes in pH may affect metabolic rate, the mode of catabolism and energetic parameters. Ideally, these analyses should not only describe correlated changes in the different processes under investigation, but should also provide a quantitative picture of the changes involved and the processes responsible for them (Heisler, 1989b).

However, acid–base regulation not only means adjustment or defence of pH, which is traditionally seen as being the key acid–base parameter determining regulatory processes, but it may, under certain conditions and with the help of the respective membrane carriers (see below), also give priority to the regulation of the levels of base (carbonate, bicarbonate) or acid (carbonic acid, proportional to $P\text{co}_2$) in the respective body fluids. In that sense, pH would become a dependent variable. Also, for some treatments it is not pH which is of interest but rather the activity of protons (pH $= -\log a_{H^+}$), when protons contribute to some biochemical reactions in a concentration-dependent manner (see equation 1, p. 73, as an example). In general, biochemical treatments of acid–base regulation usually focus on intracellular pH (pH_i) as a key parameter related to protein function, whereas physiological, cellular and especially whole-animal studies have always considered the close interrelationships between pH and the CO_2/bicarbonate system in intracellular and extracellular fluids (Siggaard-Andersen, 1974). In support of the latter concept, the involvement of the species CO_2 and bicarbon-

ate as substrates or products in enzymatic reactions has become apparent (Pörtner, 1989; Walsh & Milligan, 1989; Hardewig, Pörtner & Grieshaber, 1994), justifying the adoption of physiological concepts of acid–base regulation in metabolic biochemistry.

Acid–base regulation is an energy-dependent process because some of the acid–base equivalents are transported by H^+-ATPases or by secondary active processes, for example *via* the Na^+/H^+ exchanger, which depends upon the Na gradient established by Na^+/K^+-ATPase. It has recently been suggested that certain species are capable of modulating the cost of acid–base regulation as a means of adjusting the rate of energy turnover to environmental requirements (Reipschläger & Pörtner, 1996). In addition, the significance of metabolism for acid–base regulation has been increasingly discussed. Interest has focused not only on disturbances of the acid–base status by metabolism, but also on the contribution to acid–base homeostasis by metabolism. These investigations have been applied to both aerobic (Atkinson & Camien, 1982; Häussinger *et al.*, 1988; Atkinson & Bourke, 1995; Pörtner, 1989; 1995) and anaerobic metabolism (Hochachka & Mommsen, 1983; Pörtner, Heisler & Grieshaber, 1984; Pörtner, 1987a; 1989).

A full set of acid–base parameters in whole-animal research

Bearing these general ideas in mind, studying acid–base status involves the choice of an appropriate methodology for the analysis of acid–base parameters. Acid–base regulation occurs at systemic, cellular and subcellular levels. This chapter focuses on intracellular acid–base parameters. For research concentrating on environmental and functional issues, these parameters need to be largely investigated in whole animals, unconfined and as close as possible to their natural situation (for example dwelling in burrows), or in animals during and after exercise. Sometimes, acid–base balance needs to be studied after long-term exposures (between hours and months) to fluctuations in environmental variables such as O_2, CO_2, salinity, temperature, or, most recently, hydrogen peroxide (Abele-Oeschger, Sartoris & Pörtner, 1997), whereas short-term modifications (between seconds and minutes) occur with the use of muscular activity during attack and escape (e.g. Milligan, 1996).

Microelectrode and fluorescent probes and, to some extent, ^{31}P-NMR are most suitable for cellular and subcellular investigations of acid–base parameters (see Schwiening & Thomas and Kinsey & Moerland, this volume). At higher levels of complexity, in whole animals, microelectrode and fluorescent probes are no longer applicable. Historically, the

first reliable method to be used in whole animals was the measurement of the pH-dependent distribution of weak acids and bases, in particular the weak acid dimethyloxazolidine-dione (DMO), between intracellular and extracellular spaces (Waddell & Butler, 1959; for review see Roos & Boron, 1981). In brief, DMO is infused into the animal *via* an indwelling catheter and pH_i is calculated from DMO distribution and the measured values of extracellular pH (pH_e; Table 1). By using radio-labelled DMO, it is possible to determine pH_i invasively in not just one but various tissues collected from the same individual animal. However, the measurement of rapidly occurring pH_i changes, for example during muscle activity, is limited by the velocity of DMO distribution. Further disadvantages arise from the fact that pH_i can only be mathematically estimated. To do this, the following parameters must be measured: pH_e, water content of the tissue, and concentrations of radiolabelled inulin and DMO in the tissue and plasma to allow evaluation of intracellular and extracellular DMO. Each of the necessary measurements has its own inherent errors. Since these errors may be additive, this leads to a relatively high variability in calculated pH_i values. More recently, pH_i in isolated tissues and whole animals has been investigated by the use of ^{31}P-nuclear magnetic resonance (^{31}P-NMR; Kinsey & Moerland, this volume; van den Thillart & van Waarde, 1996; Wasser, Lawler & Jackson, 1996) and by an improved and reliable version of the homogenisation technique (Pörtner *et al.*, 1990). ^{31}P-NMR requires an *in vivo* analysis of the immobilised animal. Therefore, the homogenate technique is most suitable to investigate acid–base parameters in tissue samples collected from the exercised animal or even from animals in the field.

For whole-animal approaches, a quantitative picture should include co-ordinated analyses of intracellular and extracellular acid–base parameters. Quantification of acid–base parameters in all compartments will reveal the net movement of acid–base equivalents across membranes or epithelial layers (cf. Heisler, 1989a). As a corollary, treatment of the acid–base status is complete with quantitative knowledge of changes in pH, P_{CO_2} and apparent bicarbonate levels in the compartments of interest as well as knowledge of non-bicarbonate buffers resisting such changes by proton binding or release.

Homogenate analyses of pH_i

The homogenate technique allows for a clear allocation of pH values to the experimental condition and for the elimination of time delays in pH_i assessment. This method follows the freeze-stop technique, which
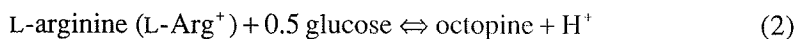
was established for determining the metabolite status of shock frozen tissues (Wollenberger, Ristau & Schoffa, 1960). Previous versions of the homogenate technique were applied to biopsy samples of human muscle (e.g. Sahlin, Harris & Hultman, 1975; Sahlin *et al.*, 1976). Samples need to be taken in such a way that the resting and experimental states of the tissues are maintained under control and experimental conditions. Control and experimental animals are therefore anaesthetised prior to tissue sampling and decapitation, if required, to eliminate the influence of a potential stress response (Pörtner *et al.*, 1990; 1991b; Pörtner, MacLatchy & Toews, 1991a; Tang & Boutilier, 1991). The frozen tissue is ground under liquid nitrogen using mortar and pestle, and the tissue powder is then thawed in a medium (in a volume about five times the wet weight of the tissue) containing potassium fluoride (KF) and nitrilotriacetic acid (NTA), thereby removing Mg^{2+} and Ca^{2+} and preventing ATP-dependent metabolism, which occurs through the action of Mg^{2+}-dependent and Ca^{2+}-dependent ATPases and kinases. ATP-dependent anaerobic metabolism is responsible for the pH changes that occur in tissue homogenates after subcellular structures have been destroyed. According to model calculations (Pörtner *et al.*, 1990), any distortion in the measured pH values due to the pH of the medium, dilution by the medium or mixing with intracellular or extracellular fluids can be disregarded. The special merits of the homogenate technique are the simple methodological procedure, low costs, low variability and the small sample volume required (in reaction tubes with a small enough volume, analysis is possible with sample sizes down to 20 mg fresh weight).

On the practical side, pitfalls in the use of the homogenate technique can be avoided when it is considered that CO_2 condensation needs to be minimised during the process of homogenate preparation. The use of clean liquid nitrogen and dewars, mortars and pestle free of rime during a short but efficient grinding procedure under a nitrogen atmosphere excludes mixing with condensating CO_2. (It is usually sufficient to grind on the bottom of a box, e.g. Styrofoam, thereby allowing the evaporating nitrogen to fill up the volume above mortar and pestle.) pH is best measured in a thermostatted capillary pH electrode, but pH microelectrodes have also been used (Krause & Wegener, 1996) after preparation and centrifugation of the homogenate in a closed (usually 0.5 ml) Eppendorf cap. If required, complete tissue extraction can be ensured by treatment of the closed Eppendorf cap with ultrasound (Sommer, Klein & Pörtner, 1997). The pH electrode and supernatant (inside the capillary electrode) are thermostatted to the experimental temperature of the animal in order to avoid temperature artefacts. NTA

supports rapid binding of Ca^{2+} and Mg^{2+}. However, the concentration of NTA needs to be minimised because at too high levels this substance releases protons, a process minimised or excluded by the formation and precipitation of magnesium or calcium fluorides.

The method has been used so far in studies of invertebrate (annelid, mollusc, sipunculid) and vertebrate (amphibian, fish, reptilian, mammalian) tissues (e.g. Pörtner, *et al.*, 1990; 1991a; 1991b; 1998; Hardewig *et al.*, 1991a; 1991b; Tang & Boutilier, 1991; Schulte, Moyes & Hochachka, 1992; Boutilier *et al.*, 1993; Branco, Pörtner & Wood, 1993; Ferguson, Kiefer & Tufts, 1993; Claiborne, Walton & Compton-McCullough, 1994; Kiefer, Currie & Tufts, 1994; Wang, Heigenhauser & Wood, 1994; 1996a; 1996b; Day & Butler, 1996; Pörtner, Finke & Lee, 1996; Schmidt *et al.*, 1996; Zielinski & Pörtner, 1996; Sommer *et al.*, 1997; Larsen, Pörtner & Jensen, 1997; see also Wood & Wang, this volume). Recently, the homogenate technique has been used to quantify passive and active contributions to temperature-induced changes in pH_i and to study the relevance of Mg^{2+} in acid–base regulation (van Dijk, Hardewig & Pörtner, 1997; Sartoris & Pörtner, 1997a; 1997b; see below).

The accuracy of the method for each individual pH measurement is confirmed by the demonstration of a strong correlation of measured pH_i changes with changes in metabolic parameters (Pörtner *et al.*, 1991b; 1996). Phosphagen breakdown and anaerobic glycolysis are predominantly responsible for metabolic changes in the acid–base status of working muscle starting beyond the anaerobic threshold (Pörtner *et al.*, 1996). These relationships have been studied in invertebrate (molluscan, arthropod, sipunculid) muscles in which the phosphagen is phospho-L-arginine (PLA) instead of phosphocreatine and where octopine, other opines or lactate are formed (Grieshaber *et al.*, 1994). A speciality compared to glycolysis and phosphocreatine depletion in vertebrate muscle is that L-arginine released during PLA depletion may be absorbed in octopine formation. Because ATP content is initially maintained at the expense of phosphagen breakdown, intracellular alkalosis is characteristic for the initial stages of anaerobic muscle activity (e.g. Chih & Ellington, 1985) based on equation (1):

$$\text{Phospho-L-arginine (PLA}^-) + \text{MgADP}^- + \text{H}^+ \Leftrightarrow \tag{1}$$
$$\text{L-arginine (L-Arg}^+) + \text{MgATP}^{2-}$$

$$\text{L-arginine (L-Arg}^+) + 0.5 \text{ glucose} \Leftrightarrow \text{octopine} + \text{H}^+ \tag{2}$$

After initial alkalisation, glycolytic ATP and H^+ production become predominant during maintained *anaerobic* muscle activity, causing pH_i

to fall below the control level (see equation 2; for details, Pörtner, 1987a).

Analyses in the mantle musculature of various squid species have revealed a highly significant linear correlation between changes in pH_i and glycolytic end-product (octopine) accumulation (Fig. 1), the formation of octopine being equivalent in its acidifying effect to lactate generation. The linearity of the relationship between octopine concentration and pH_i suggests a well-balanced response of intracellular physicochemical and metabolic buffering processes as well as proton equivalent ion exchange between tissue and extracellular space to glycolytic proton generation *in vivo*. An *in vivo* buffer value largely independent of pH results, which is higher than the non-bicarbonate buffer value (β_{NB}) determined for control conditions (resting muscle). The latter only reflects physicochemical buffering as measured *in vitro* (see Non-bicarbonate buffer values, p. 82). The increase in slope of the apparent buffer line compared to β_{NB} indicates how metabolic proton consumption can reduce the degree of glycolytic acidification (see Fig. 1). Phosphagen degradation is the main process associated with proton consumption, whereas ATP hydrolysis to ADP and Pi contributes a small amount of surplus protons. Metabolic processes that further reduce acidosis during activity include the deamination of AMP or adenosine further along the ATP degradation pathway, or the metabolism of dicarboxylic acids (malate, aspartate) in the early stages of anaerobic mitochondrial metabolism. The latter occurs when excessive oxygen requirements cannot be met by increased oxygen supply (Pörtner, 1987a).

These compensatory or additive processes in metabolism may change the slope of the octopine/pH relationship with no disruption of linearity in such a way that they either take place at a constant rate or are triggered by falling pH_i. The net channelling of proton equivalents into the extracellular space is minimal in squid (Pörtner, 1994; 1997). A fall in phospho-L-arginine levels with pH_i due to pH dependence of arginine kinase (equation 1) and a linear rise of H^+ binding by inorganic phosphate with falling pH were seen in squid mantle (see Pörtner et al., 1996). These observations support the conclusion that release of inorganic phosphate during phosphagen hydrolysis is the main process affecting the degree of glycolytic acidification. The extensive use of phosphagen in *Illex illecebroses* (Pörtner, 1993) may also explain at least part of the difference seen between the squid species in Figure 1. The formation of octopine (removal of L-arginine), development of acidosis, and accumulation of free ADP co-operate in such a way that transphosphorylation of the phosphagen supports higher performance levels. Moreover, the available data strongly suggest that the develop-
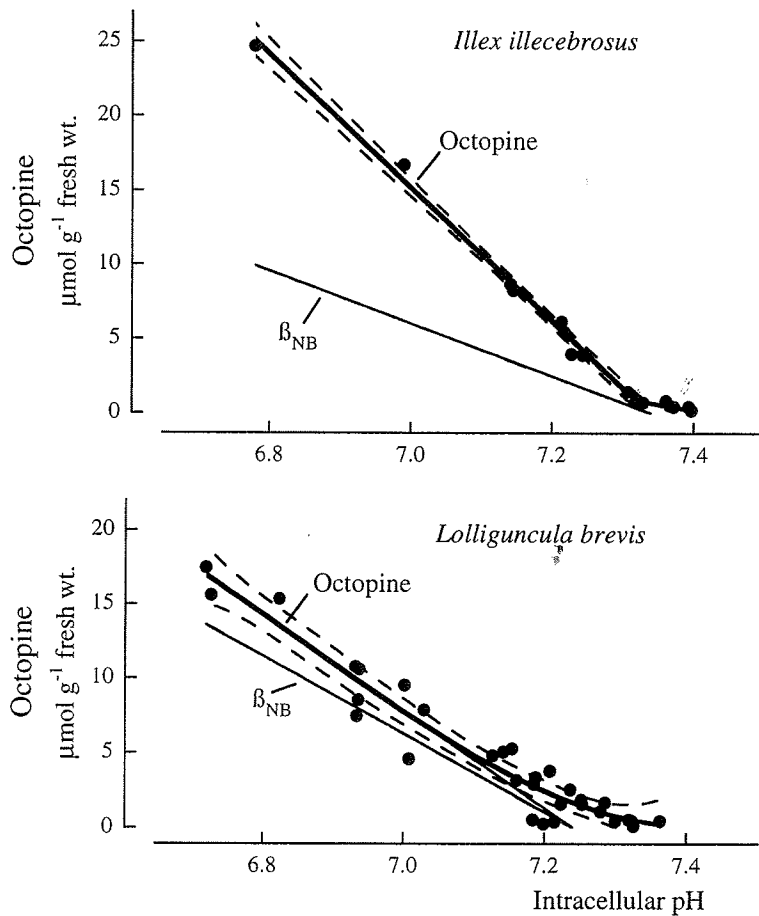
Fig. 1. Comparison of the relationship between octopine levels and pH_i in mantle tissues of squids *Lolliguncula brevis* and *Illex illecebrosus*. This relationship reflects the extent to which glycolytic protons are buffered *in vivo*. Non-bicarbonate buffer lines (β_{NB}) determined *in vitro* reflect how physicochemical buffering alone would resist glycolytic acidification. The additional buffering on top of the non-bicarbonate buffer value is due to the transphosphorylation of the phosphagen triggered by the decrease in pH_i and the rise in free ADP levels. Modified after Pörtner *et al.* (1996). Base release from the tissue into the blood may occur at the onset of muscular activity and explains the initial drop in pH_i at rather constant octopine levels (Pörtner, 1994). Solid lines were determined by regression analysis and delineate significant relationships ($p<0.05$); dashed lines represent 95 per cent confidence intervals.

ment of acidosis may protect the adenylates from being largely degraded during fatigue (Pörtner et al., 1993; 1996).

Intracellular P_{CO_2} and bicarbonate levels

The analysis of pH_i and total CO_2 levels (C_{CO_2}) in the homogenate allows the quantification of cellular bicarbonate and P_{CO_2} levels by calculation. In a first step, total CO_2 levels measured in the homogenate have to be corrected for extracellular water and C_{CO_2} content by use of an adequate marker for extracellular space (for example radiolabelled inulin) to obtain intracellular C_{CO_2} levels (Pörtner et al., 1990). The calculation requires the use of pH_i and the apparent dissociation constant of the CO_2/apparent bicarbonate system, pK''', as well as the physical solubility of CO_2, α, which can be derived according to Heisler (1986a):

$$P_iCO_2 = C_{CO_2} / (10^{pH_i - pK'''} \cdot \alpha + \alpha) \tag{3}$$

Note that apparent bicarbonate includes all bicarbonate and carbonate species according to their effect in cellular buffering. For a full set of equations, see Pörtner et al. (1990).

Overall, the calculation of intracellular C_{CO_2} requires analysis or considerations of parameters analogous to those needed for the determination of pH_i from DMO distribution. In consequence, sources of error similar to those involved in DMO measurements arise, and values of intracellular P_{CO_2} calculated from the intracellular C_{CO_2} concentration (Pörtner et al., 1990; 1991a; 1991b; 1996; 1998; Boutilier et al., 1993; Reipschläger & Pörtner, 1996) exhibit a relatively large degree of variation. Variability is reduced by highly accurate estimates of pH_i, as with the homogenate technique which determines pH with much less signal-to-noise ratio than the DMO or ^{31}P-NMR methods. This means that each individual pH value has the same significance as that given to just the average value when using the DMO method. Estimations of intracellular P_{CO_2} would not be possible if based on individual DMO-derived pH_i values, due to their inaccuracy.

With adequate knowledge of the relationships between pH_i and pH_e, P_{CO_2} and bicarbonate levels, intracellular acid–base parameters in isolated muscle tissues could be varied and clamped by setting the adequate values in the extracellular medium (Reipschläger & Pörtner, 1996). That way it could unequivocally be demonstrated that among all acid–base parameters, only a decrease in pH_e (rather than pH_i) was suitable to cause metabolic depression during environmental stress in Sipunculus nudus (Fig. 2). The mechanisms responding to the decrease in pH_e are currently under investigation. A model suggests that a switch

Table 1. *The influence of pH heterogeneity in cellular compartments on compartmental levels of total CO_2 and calculated values of intracellular P_{CO_2}*

Calculations of DMO and CO_2 distribution follow a modified Henderson-Hasselbalch equation:

$$pH_i = pK' + \log[c_i/c_e \ (10^{pH_e - pK'} + 1) - 1] \tag{a}$$
$$c_i = (F_c \cdot c_c + F_m \cdot c_m)/(F_c + F_m) \tag{b}$$
$$[HDMO]_m = [HDMO]_c \ \text{or} \ (\alpha \cdot P_{CO_2})_m = (\alpha \cdot P_{CO_2})_c \tag{c}$$
$$[DMO^-]_m/[DMO^-]_c = 10^{\Delta pH} \ \text{or} \ '[HCO_3^-]'_m /'[HCO_3^-]'_c = 10^{\Delta pH} \tag{d}$$

where pK′ is the apparent dissociation constant of HDMO or pK′′′ of the CO_2/ HCO_3^- system (see text), c is the concentration of ([HDMO] + [DMO⁻]) or the C_{CO_2} in mean intracellular (i), extracellular (e), mitochondrial (m) or cytosolic (c) compartment, and F is the fractional volume of that compartment.

Calculation of factors determining homogenate pH_i considers the contribution of mitochondrial and cytosolic volumes, total buffer values and pH differences (ΔpH).

$$pH_i = (F_c \cdot \beta_{c,tot} \cdot pH_c + F_m \cdot \beta_{m,tot} \cdot pH_m)/(F_c + F_m) \tag{e}$$
$$\beta_{tot} = \beta_{NB} + \beta_{CO_2} \ \text{(closed system, at } pH_i) \tag{f}$$

P_{CO_2} values (1 mmHg = 0.1333 kPa; 1 kPa = 7.502 mmHg) are calculated (see text, equation 3) and compared, based on homogenate or weak acid-derived pH_i values and mean intracellular C_{CO_2} evaluated in a toad, *Bufo marinus* (see Pörtner *et al.*, 1990; 1991b). The pH difference between mitochondrial matrix (m) and cytosol (c) and the fraction of mitochrondrial matrix fluid (F_m) in the cell is assumed to be similar in toad ventricle to that described for rat ventricle. With a mitochondrial content of around 35 per cent (Smith & Page, 1976; Hoppeler *et al.*, 1984), 19 per cent of the cell fluid is attributed to the mitochondrial matrix ($F_m = 0.19$, $F_c = 0.81$), with a pH gradient of 0.63 units between cytosol and matrix fluids (Kauppinen, Hiltunen & Hassinen, 1980; Kauppinen, 1983; see Table 2).

Ventricle: $C_{CO_2,i} = 8.45$ mmol l⁻¹ cell water
$\quad\quad\quad\quad \Delta pH_{m-c} = 0.63$
$\quad\quad\quad\quad pH_{i,hom} = 7.13 \quad\quad \rightarrow \quad P_{i}CO_2 = 17.2$ mmHg
$\quad\quad\quad\quad pH_{i,weak\ acid} = 7.35 \quad\quad \rightarrow \quad P_{i}CO_2 = 10.8$ mmHg

Precondition: mitochondrial and cytosolic P_{CO_2} values are more or less equal. Test case: the adequate mean pH_i should give the same intracellular P_{CO_2} as do cytosolic and mitochondrial pH.

$\Delta pH_{m-c} = 0.63 \quad\quad \rightarrow \quad C_c CO_2 = 5.46, \ C_m CO_2 = 21.53$ (mmol l⁻¹)
$pH_{i,weak\ acid} = 7.35 \quad\quad \rightarrow \quad pH_c = 7.15, \ pH_m = 7.78$
$\quad\quad\quad\quad\quad\quad\quad\quad \rightarrow \quad P_c CO_2 = 10.8$ mmHg $= P_m CO_2 = P_i CO_2$

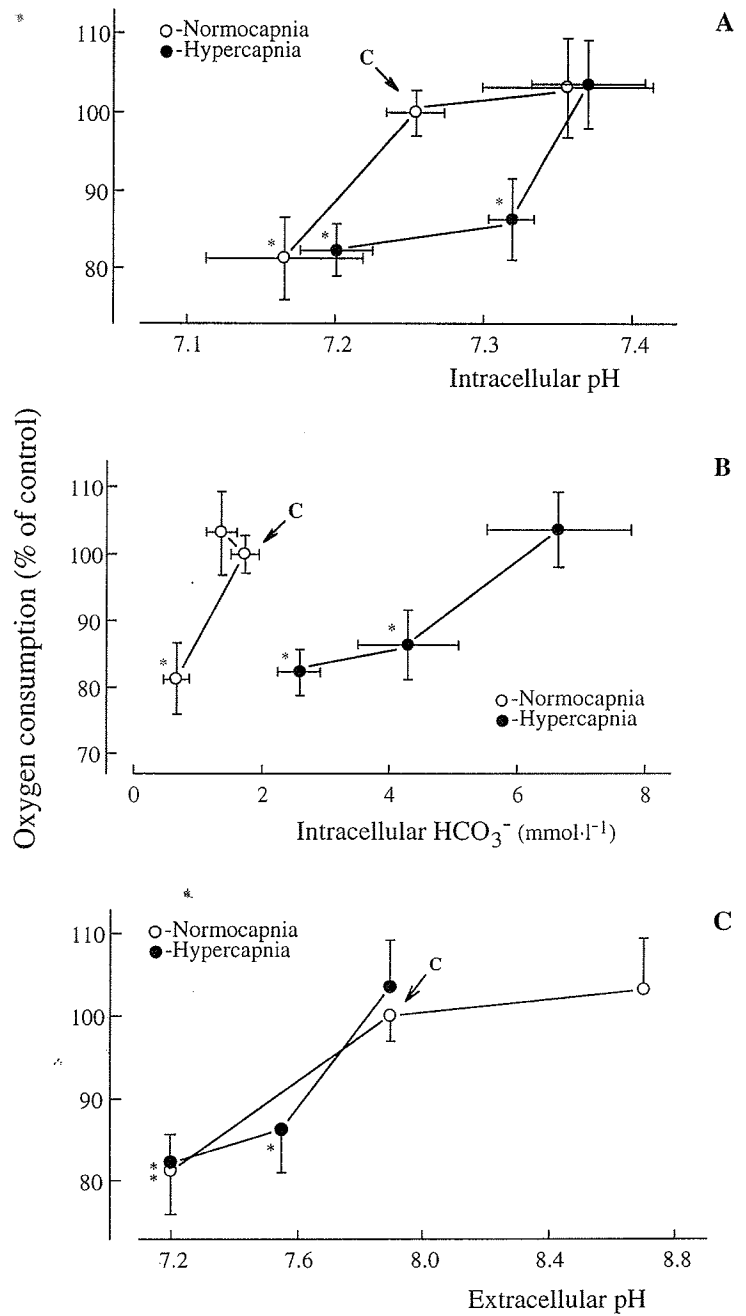Conclusion: $pH_{i,\ weak\ acid}$ is required for $P_{i}CO_2$ calculations.

Fig. 2.

occurs from less ATP-efficient but more flexible acid–base transporters to more ATP-efficient exchange mechanisms (Reipschläger & Pörtner, 1996).

Cellular compartmentalisation

pH values in tissues poor in mitochondria, determined using homogenates, are in good agreement with the mean pH values obtained in DMO studies (cf. Pörtner *et al.*, 1990; Wood & Wang, this volume) and by ^{31}P-NMR (cf. Zange *et al.*, 1990). In cells containing large fractions of mitochondria, these values differ depending on the fraction of mitochondria and the pH gradient maintained between these organelles and the cytoplasm (the contribution of other organelles appears to be less relevant). The contribution of cellular compartments to determining the average pH_i (homogenate) follows their percentage contribution to cellular buffering and their relative volume (mixing two identical volumes of the same buffer values would yield the arithmetic mean of the two pH values). In mitochondria, pH is higher and, in consequence, total CO_2 levels are also higher than in the cytosol owing to pH-dependent distribution (Pörtner *et al.*, 1990; 1991a; 1991b; Tables 1 & 2). During the measurement procedure, all buffers (including total CO_2, which comprises the CO_2/bicarbonate buffers) are mixed in a closed system so that there is no exchange of gases, most importantly CO_2 between the homogenate and the air. This reduces the influence of the CO_2/bicarbonate buffer on homogenate pH. A much larger influence would result under open-system conditions when CO_2 leaves the mixture. (For an analysis of open system characteristics see Burton, 1973; Heisler, 1986a.) The pH dependence of the CO_2/bicarbonate buffer value and the fact that it is rather low at high mitochondrial pH also cause homogenate pH to be closer to cytosolic pH than expected when mean pH only depends upon the mixed volumes (see below).

Differences prevail between techniques for determining mean pH_i in

Fig. 2. O_2 consumption rates of isolated body wall musculature of *Sipunculus nudus* during normocapnia and during hypercapnia (1% CO_2) depicted as a function of pH_i values (**A**), intracellular HCO_3^- concentrations (**B**), and values of pH_e (**C**). Only plot **C** is consistent for both normocapnic and hypercapnic data and demonstrates that the O_2 consumption is significantly depressed (asterisks) below a threshold value of pH_e. Modified after Reipschläger & Pörtner (1996).

Table 2. *An estimate of mitochondrial (m) and cytosolic (c) pH, HCO_3^- levels and buffer values (β_{tot} = total, NB = non-bicarbonate) in the isolated perfused rat heart, based on the pH difference observed between weak acid-derived and homogenate (hom)-derived pH_i values*

Rat heart 37 °C

$pH_{i,hom}$ = 6.90 C_iCO_2 = 11.76 mmol l^{-1}, $P_iCO_2 \approx$ 40 mmHg
$pH_{i,\ weak\ acid}$ = 7.05, ΔpH_{m-c} = 0.63
(pK''' = 6.127, α = 0.03134 mmol/mmHg · l cell water)
Fm = 0.19, Fc = 0.81,
β_{tot} = 44.7 mmol/pH per l cell water (at $pH_{i,\ hom}$)

	[HCO_3^-]	C_{CO_2}	pH	mean pH	β_{tot}	β_{NB}
Cytosol	6.49	7.74	6.841		49.9	47.5
				6.961		
Mitochondria	27.71	28.96	7.471		22.0	19.2

See Pörtner *et al.*, 1990; for comparison and equations, see Table 1.
The accuracy of the calculation largely depends on a correct estimate of the mitochrondrial–cytosolic pH difference and the fraction of the mitochondrial matrix (Kauppinen *et al.*, 1980; see text and legend of Table 1; concentrations given in mmol/l cell or compartmental water). The buffer value of the rat heart was adopted from Hansen and Gesser (1980). The literature value was corrected for the accumulation of inorganic phosphate expected from the degradation of high-energy phosphates (mean pH: volume-weighted mean pH, which would result with identical β_{tot} values in cytosol and mitochondria, equation (e) in Table 1). Total closed system buffer value varies by a factor of 2.27 and non-bicarbonate buffer value by a factor of 2.5 lower in the mitochondria than in the cytosol. Similar conclusions arise from a comparison of homogenate and DMO pH_i values in the cardiac ventricle of a toad, *Bufo marinus* (Pörtner *et al.*, 1990).

their ability to weight all compartmental parameters such as volumes, buffer values and pH values (homogenate technique) or volumes and pH differences only (DMO). The different techniques must therefore lead to different values of pH_i when mitochondrial density is high. Moreover, weak acid distribution characteristics cause the DMO technique not only to ignore compartmental buffer values but also to deviate even further from the arithmetic mean according to volume fractions (see Table 1). The determination of pH_i by DMO distribution relies on a quantification of mean cellular DMO levels. DMO (DMO^-) accumulates at high pH in the mitochondria. A mitochondrial DMO level

approximately ten times higher than in the cytosol is reached with a pH gradient of 1 ($[DMO^-]m/[DMO^-]_c = 10^{\Delta pH}$, see Table 1). Mixing equal volumes of cytosol and mitochondrial matrix assuming equal buffer values, the homogenate pH would be 0.5 pH units above cytosolic pH, whereas the DMO-derived pH would be as much as 0.74 pH units higher. Accordingly, DMO yields high values of mean pH_i in multicompartmental cells (rich in mitochondria; Pörtner *et al.*, 1990; Whiteley, Naylor & Taylor, 1995). However, the comparison of homogenate-derived and DMO-derived pH values yielded even larger differences than could be explained by the characteristics of the DMO technique and, thus, led to the conclusion that non-bicarbonate buffer values within mitochondria must be small (Pörtner *et al.*, 1990; 1991b; see below and Table 2).

In conclusion, homogenate analysis provides an estimate of mean pH_i emphasising cytosolic pH, whereas DMO distribution largely over-estimates mean pH_i. When using ^{31}P-MRS, the localisation of pH values measured in such cells is not yet satisfactorily explained, but is also assumed to reflect cytosolic pH (Gadian *et al.*, 1982). Because the chemical shift of inorganic phosphate is a direct measure of pH (see Kinsey & Moerland, this volume), a mean value of pH should result which is close to the arithmetic mean of the chemical shift values for all NMR-visible phosphate, with the assumption that most mitochondrial phosphate is NMR invisible. In support of this hypothesis, pH values derived from inorganic phosphate and from the cytosolic marker phosphodeoxyglucose were found to be very close in radula protractor muscle of a marine whelk, with a mitochondrial content of about 15 per cent of the cell volume (Wiseman & Ellington, 1989; W.R. Ellington, personal communication). In squid mantle, a tissue with an average mitochondrial content of 14 per cent (see Pörtner *et al.*, 1991b), pH data obtained by homogenate and ^{31}P-NMR techniques were also found to be in close agreement (Pörtner *et al.*, 1996; H.O. Pörtner, D.M. Webber, P.G. Lee & M. Quast, unpublished), in accordance with the conclusion that both techniques emphasise cytosolic pH.

Some complication results from the fact that the various methods yield different mean values of pH_i. Which is the one to use for the calculation of mean intracellular P_{CO_2} (equation 3) in mitochondria-rich cells? Because C_{CO_2} distribution between cytosol and mitochondria (like DMO distribution) follows pH-dependent weak acid distribution characteristics, a mean cellular pH value determined by weak acid distribution analysis is required (see above; Table 1). Again, owing to the high inherent inaccuracy (see above), pH_i values determined in individual samples by the DMO technique are usually not suitable for this

process such that, at present, model considerations of the general difference between homogenate and weak acid-derived mean pH_i values are required to obtain reasonable estimates of mean intracellular P_{CO_2} values in mitochondria-rich tissues (Pörtner et al., 1990; 1991a; 1991b; 1996).

Non-bicarbonate buffer values

As mentioned above, the response of cellular buffers in vivo to disturbances of the acid–base equilibrium comprises closed and open system characteristics. The latter is true for the CO_2/bicarbonate buffer, which reaches high buffer values in the open system (when P_{CO_2} can be adjusted), and is particularly relevant in animals with high internal P_{CO_2} levels such as air-breathing species. Accordingly, it appears appropriate to distinguish bicarbonate from non-bicarbonate (mostly protein and inorganic phosphate) buffers. Determinations of tissue non-bicarbonate buffer values by tonometry (Heisler, 1989a; Pörtner, 1990a) use CO_2 for the titration of buffers and thereby mimic respiratory changes in the acid–base status in vivo. Changes in bicarbonate levels with changes in pH reflect the action of non-bicarbonate buffers (Heisler & Piiper, 1971).

Regardless of whether buffer values are determined in intact tissue or homogenates, metabolic processes may respond to experimental changes in pH and may interfere with the measurements of buffer values in intact tissue and homogenates, leading to erroneous pH values during the titration procedure or the liberation of additional buffers (see Pörtner, 1989). This means that the measured buffer value does not actually correspond to any definite physiological state of the tissue, and definitely not that under control conditions. These effects are now being increasingly considered, and only recently were invasive (homogenate technique) and non-invasive approaches (by ^{31}P-NMR) for the determination of tissue buffer values brought together, yielding similar values in tissue preparations of low P_{CO_2} (Wiseman & Ellington, 1989; Pörtner, 1989; 1990a). The homogenate technique will yield non-bicarbonate buffer values under control conditions as required for quantitative treatments of the acid–base status, that is the determination of the proton quantities responsible for the observed changes in acid–base parameters through pH/bicarbonate analyses (see below; e.g. Pörtner, 1990a; Pörtner et al., 1991a; 1991b; Ferguson et al., 1993).

The concentrations and relative contributions of non-bicarbonate buffer substances are likely to be different in mitochondrial and cytosolic compartments. Cytosolic actomyosin comprises the major protein

fraction in muscle tissue and was found in trout to be two to three times more important in cellular buffering than soluble protein (Abe *et al.*, 1985). Histidine-related compounds (carnosine, anserine, which are predominantly found in white muscle; Abe *et al.*, 1985) may also prevail in the cytosol. Inorganic phosphate levels are similar or lower in mitochondria than in the cytosol (Soboll & Bünger, 1981) but are thought to be bound to Ca^{2+} in the mitochondrial matrix and, therefore, may be inefficient in mitochondrial buffering. A low non-bicarbonate buffer value is expected, a conclusion supported by the observation that the mitochondrial influence on mean pH_i derived from homogenate analyses is small (see Table 2). During anaerobic exercise, cytosolic buffering is increased even further due to the release of inorganic phosphate from phosphagen and ATP.

As emphasised above, mitochondrial bicarbonate buffering may be substantial, even more so since bicarbonate buffering is considerably enhanced in an open system (Burton, 1973; Heisler, 1986a). Therefore, low mitochondrial non-bicarbonate buffer values in the living cell are compensated for to some extent by high bicarbonate levels. A low non-bicarbonate buffer value in mitochondria, however, facilitates the generation of a large pH gradient across the inner mitochondrial membrane because only a small amount of protons is then required for this purpose. The generation of the pH gradient plays an important role in mitochondrial ATP formation. These interrelationships have not been addressed before and call for further investigation of the quantitative contribution of mitochondria to cellular buffering.

pH/bicarbonate analyses

The pH/bicarbonate analysis (Fig. 3) follows these lines of thinking and quantifies respiratory (via changes in P_{CO_2}) and non-respiratory processes contributing to changes in the acid–base status. The pH/bicarbonate diagram illustrates the Henderson–Hasselbalch equation for the CO_2/bicarbonate system, that is the relationships between pH, bicarbonate levels and P_{CO_2}. Non-respiratory changes in a compartment include the net exchange of acid or base equivalents across epithelia or membranes and the influence of metabolic pathways. Also included in the non-respiratory processes are changes in the protonation of proteins which may be connected with the binding or release of ligands (e.g. Pörtner, 1990b). For this type of analysis, control values and associated changes in pH, bicarbonate concentration, P_{CO_2}, and non-bicarbonate buffer value β_{NB} must be known for the respective compartment. The respiratory component of the pH change is derived from the change in
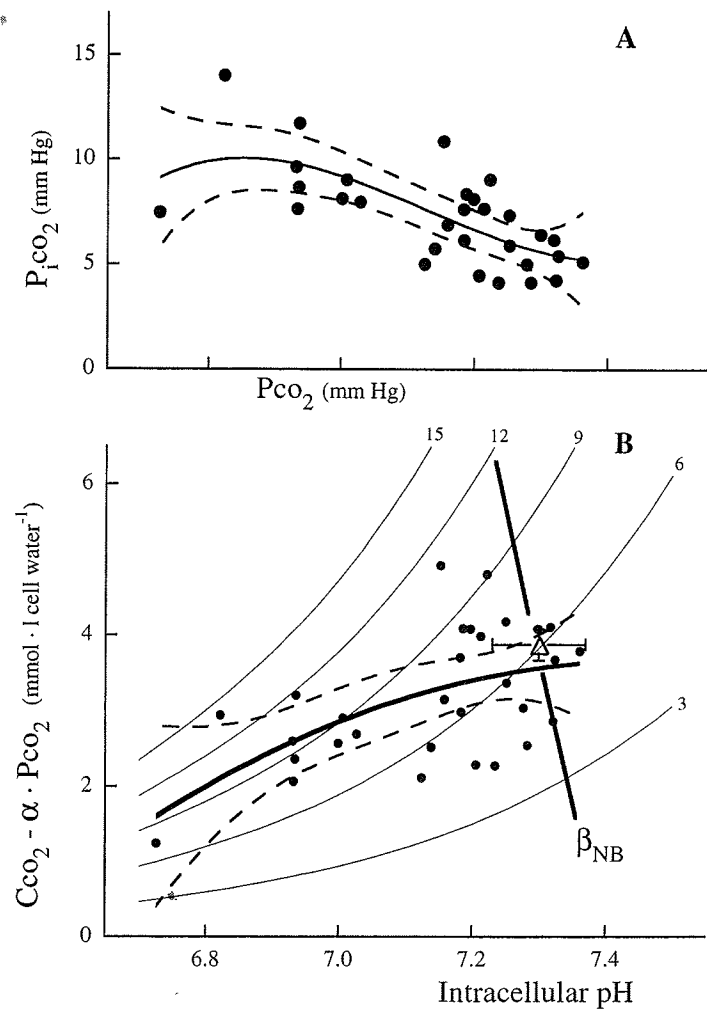
Fig. 3. **A**. Changes in intracellular P_{CO_2} (1 mmHg $=$ 0.1333 kPa; 1 kPa $=$ 7.502 mmHg) of squid (*L. brevis*) mantle musculature with pH_i during progressive fatigue from swimming at different velocities ($r =$ 0.69, third order regression). The data originate from the same set of animals as in Figure 1. The depiction of individual data points for intracellular P_{CO_2} illustrates the variability introduced by the methodological procedure (see text). **B**. Changes in acid–base status of squid (*L. brevis*) mantle musculature during progressive fatigue displayed in a pH/HCO_3^- diagram. The depiction illustrates the predominantly non-respiratory origin of the acidosis (see Fig. 1 and text)

P_{CO_2} along the buffer line, in accordance with the titration of non-bicarbonate buffers by CO_2. This process leads to changes in pH and in bicarbonate levels in opposite directions (pH falls, bicarbonate levels rise). The respiratory (resp.) proton quantities can be derived by pH/bicarbonate analysis as the bicarbonate increment along the buffer line β_{NB}, starting from a control data point specified by a combination of pH, bicarbonate and P_{CO_2} values (see Fig. 3).

$$\Delta pH_{resp.} \cdot -|\beta_{NB}| = \Delta[HCO_3^-]_{resp.} = -\Delta H^+_{resp.} \tag{4}$$

In contrast, non-respiratory (non-resp.) changes in the acid–base status cause a unidirectional change in both pH and bicarbonate levels. Such a process would follow one of the P_{CO_2} isopleths in the diagram, again starting from a control point in the graph defined by pH, bicarbonate and P_{CO_2}. The respective proton quantities are calculated from the pH change and the change in bicarbonate concentration along the P_{CO_2} isopleth considering the non-bicarbonate buffer value (equation 4; tot = total).

$$\Delta H^+_{non-resp.} = (\Delta pH_{tot} \cdot -|\beta_{NB}|) - \Delta[HCO_3^-]_{tot} \tag{5}$$

Equation 5 is still valid when the changes in pH and in bicarbonate are affected by respiratory processes, if a change in P_{CO_2} occurs. Because a respiratory change in pH is associated with a change in bicarbonate levels in the opposite direction (equation 4), respiratory changes always balance to 0 (equation 4 in the format of equation 6). Equation 6 is considered in equation 5 (ΔpH_{tot} includes $\Delta pH_{resp.}$ and $\Delta[HCO_3^-]_{tot}$ includes $\Delta[HCO_3^-]_{resp.}$). Any change in P_{CO_2} will influence ΔpH_{tot} and $\Delta[HCO_3^-]_{tot}$ (again, in opposite directions) but not the resulting $\Delta H^+_{non-resp.}$.

$$(\Delta pH_{resp.} \cdot -|\beta_{NB}|) - \Delta[HCO_3^-]_{resp.} = 0 \tag{6}$$

Prior to the advent of the homogenate technique, the only way to determine pH_i during muscle activity in the whole unrestrained animal was

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Fig. 3 caption contd.

with a small respiratory contribution (r = 0.56, second order regression; Δ = control value, mean ± SD; β_{NB} = non-bicarbonate buffer line of muscle cell water). Solid lines were determined by regression analysis and delineate a significant relationship ($p<0.05$); dashed lines represent 95 per cent confidence intervals. Modified after Pörtner *et al.* (1996).

by calculation. The pH/bicarbonate analysis was used to calculate changes in pH_i from tissue metabolic changes, considering any exchange of acid–base equivalents between intracellular and extracellular space (Pörtner, 1987a; 1987b).

Interstitial pH

Most attention has focused on the properties of intracellular and extracellular bulk fluids when acid–base regulation of an animal is discussed. However, the interstitial fluid is the one in contact with the cell membrane and acts as a mediator for any extracellular signal transferred to the cell. In this context, it is important to note that a Pco_2 gradient may prevail between intracellular and extracellular fluids which is likely to cause a drop in interstitial pH below plasma pH, owing to minimal non-bicarbonate buffering in the interstitial fluid (Pörtner et al., 1991a; Pörtner, 1993). In support of this conclusion, studies in vertebrates have shown that a pH gradient may prevail from the cell surface to the venous blood or ambient medium (De Hemptinne & Huguenin, 1984). The presence of a Pco_2 gradient would explain why pH values are lower on the cell surface than in the surrounding medium or blood. The resulting pH gradient would be most evident with rapid CO_2 hydration, as expected from the action of extracellular, membrane-bound carbonic anhydrase (e.g. Henry, Wang & Wood, 1997).

The Pco_2 gradient and, thus, the difference between plasma and interstitial pH might be characteristic for the tissue in question. However, it was shown in an amphibian, Bufo marinus, that a Pco_2 gradient remains between the intracellular space and the plasma, which is approximately the same in gastrocnemius muscle and in the ventricle of the heart despite the differing metabolic rates. Moreover, intracellular Pco_2 reflects the drop in venous Pco_2 during long-term hyperventilation in hypoxia (Pörtner et al., 1991a). This observation complies with the fact that mitochondrial density and capillarisation of a tissue are interdependent (Weibel, 1984), explaining similar partial pressure gradients under steady state resting conditions in spite of differing rates of CO_2 production.

When taking the steady state Pco_2 gradient between intracellular and extracellular space under resting conditions into account, an interstitial pH can be determined that is between 0.1 and 0.15 pH units lower than that of the venous plasma. Such relationships have been interpreted to explain the deviation from a pH-dependent distribution pattern of lactate which becomes visible during situations of low metabolic rate, e.g. during long-term hypoxia (Pörtner et al., 1991a; Pörtner, 1993). In gen-

eral, they are expected to influence the patterns of pH-dependent distribution between the intracellular and extracellular spaces not only of organic acids *via* pH-dependent carrier mechanisms like the lactate H^+ symporter (cf. Pörtner, 1993), but also of total CO_2 and ammonia, the volatile fractions of both of which are eliminated from the body following the laws of respiratory gas exchange (Heisler, 1995; Henry *et al.*, 1997; see also Wood & Wang, this volume).

Any deviation from steady state will influence such a pH-dependent distribution pattern, for example when net production or removal of the respective metabolite in metabolism occurs at a rate in the same order of magnitude as the transmembrane (facilitated) diffusion process. Some studies have revealed patterns of changes in intracellular and extracellular P_{CO_2} that differ especially during exercise in poorly perfused tissues like white muscle. Larger P_{CO_2} gradients between intracellular and extracellular space are likely to develop with any increase in metabolic rate such as during exercise (Pörtner *et al.*, 1991b; Boutilier *et al.*, 1993). In poorly perfused white muscle, this trend may be exacerbated by the glycolytic acidification and titration of bicarbonate stores, thus leading to even higher intracellular P_{CO_2} levels, steeper P_{CO_2} gradients and lower values of interstitial pH. These considerations emphasise the relevance of compiling P_{CO_2} estimates in various body compartments for a more complete picture, especially during non-steady state metabolic situations.

Temperature and pH$_i$

Reeves (1972) introduced the imidazole alphastat hypothesis, stating that pH regulation in poikilotherms maintains the degree of protonation (α) of imidazole groups in proteins despite changes in body temperature. A pH$_i$ change of around -0.018 pH units /°C is expected to support the alphastat pattern and to ensure protein function at fluctuating temperature. Cameron (1989) refined this approach in proposing a 'Z-stat' model, emphasising that protein net charge Z is maintained rather than α in diverse histidine groups. This may be a more adequate treatment because ΔpK °C^{-1} depends upon local charge configurations in the environment of the imidazole group as well as on ionic strength and, therefore, ranges between -0.016 and -0.024 °C^{-1} for histidine and free imidazole compounds and between -0.0010 and -0.051 °C^{-1} for histidine residues in proteins (Heisler, 1986b).

In a comprehensive paper, Ultsch and Jackson (1996) recently reviewed the literature on the relationship between pH$_i$ and temperature in ectothermic vertebrates, concluding that the data in general support

the concept of alphastat regulation, particularly within the normal temperature range of the species. With some exemptions, the alphastat pattern of pH_i regulation could also be confirmed for marine ectotherms (invertebrates and fish) exposed to various temperatures, both depending on the season and latitude (review by Pörtner et al., 1998). However, alphastat regulation of pH is likely to be restricted to a temperature window between the critical temperature limits of a species (Sommer et al., 1997).

The original hypothesis claimed that the observed changes in pH with temperature are only elicited by passive mechanisms, mostly proton binding or release from intracellular and extracellular buffers owing to the change in dissociation equilibria (pK values) of the buffer components. Later on, the alphastat hypothesis was extended, integrating an active component. In air breathers, active pH regulation is mainly due to the control of P_{CO_2} by means of ventilatory adjustments, whereas active ion exchange predominates in water breathers. The relative contributions of active and passive mechanisms to the adjustment of pH_i have been quantified by model calculations (Reeves & Malan, 1976; Heisler, 1984). However, this analysis is complicated by the variability of the $\Delta pK/\Delta T$ values (see above).

The homogenate technique offers the opportunity to distinguish active and passive elements in temperature-dependent pH regulation by direct analysis. For a quantification of passive mechanisms, tissues collected from animals exposed to control temperatures are analysed in vitro at different temperatures, whereas in vivo $\Delta pH/\Delta T$ values are determined in animals exposed to various temperatures for long enough time periods to allow active mechanisms to achieve and maintain new steady state values of pH_i.

In contrast to the original hypothesis, these authors found that the passive contribution to pH regulation was considerably below the alphastat value in some of the animals investigated (van Dijk et al., 1997; Sartoris & Pörtner, 1997a; Pörtner et al., 1998). The passive contribution accounted for only 35 per cent of the temperature-induced pH shift in white muscle of the eelpout Zoarces viviparus. In contrast, passive pH changes in an isolated mixture of intracellular proteins revealed a slope not significantly different from the in vivo slope. This discrepancy could not be ascribed to low molecular weight components with low $\Delta pK/\Delta T$ values like bicarbonate ($\Delta pK/\Delta T = -0.006\ °C^{-1}$) or phosphate ($\Delta pK/\Delta T = -0.003\ °C^{-1}$; van Dijk et al., 1997). The nature of the buffers responsible for the passive contribution to alphastat and the reasons for the difference between the pH shifts of the protein mix-

ture isolated from white muscle and of the passive components in the KF/NTA homogenate remain unexplained.

First results obtained in comparative studies suggest that the relative contribution of active and passive processes to the pH shift is largely influenced by the temperature regime of the animals' habitat. In all animal species investigated so far, the passive contribution to alphastat regulation was reduced and the active component was higher in eurythermal than in stenothermal species (Fig. 4). Lower passive pH shifts would, on the one hand, lead to more acidic pH values in the cold and leave a larger contribution to ion exchange mechanisms to accomplish alphastat pH regulation. On the other hand, a low passive slope allows flexible adjustments of pH according to metabolic requirements. Large passive slopes would require active pH regulation to compensate for their effect when more acidic pH values are to be maintained. In particular, animals exposed to large seasonal temperature variations exhibit low pH values at low temperatures in the winter (Thebault & Raffin, 1991; Spicer, Morritt & Taylor, 1994). The shrimp *Palaemon* tends to be inactive at temperatures below 10 °C, metabolic depression being reflected by a drop in pH_i below the alphastat pattern (Thebault & Raffin, 1991). Acidic pH_i values were also reported by Whiteley *et al.* (1995) for winter crayfish *Austrapotamobius pallipes*. Low passive slopes may support metabolic depression which should comprise the down-regulation of energy-consuming ion exchange mechanisms otherwise responsible for alphastat pH regulation. One might speculate that a capacity for metabolic depression in eurythermal animals is correlated with a reduced contribution of passive mechanisms to pH adjustment during temperature change.

As a corollary, these interspecies comparisons suggest that the temperature-dependent adjustment of pH_i mostly occurs by active mechanisms in eurythermal animals, whereas in stenothermal animals pH adjustment is mostly achieved by passive processes. A larger active than passive component of alphastat regulation may not only be a prerequisite to colonise shallow coastal waters, but may also allow for a variable adjustment of metabolic activity on a seasonal time scale.

Summary and conclusions

Invasive methodology can be used to study the relationships between acid–base and metabolic regulation in such a way that the role of acid–base parameters in modulating metabolic rate, the mode of catabolism and energetic parameters becomes evident. This includes an analysis of
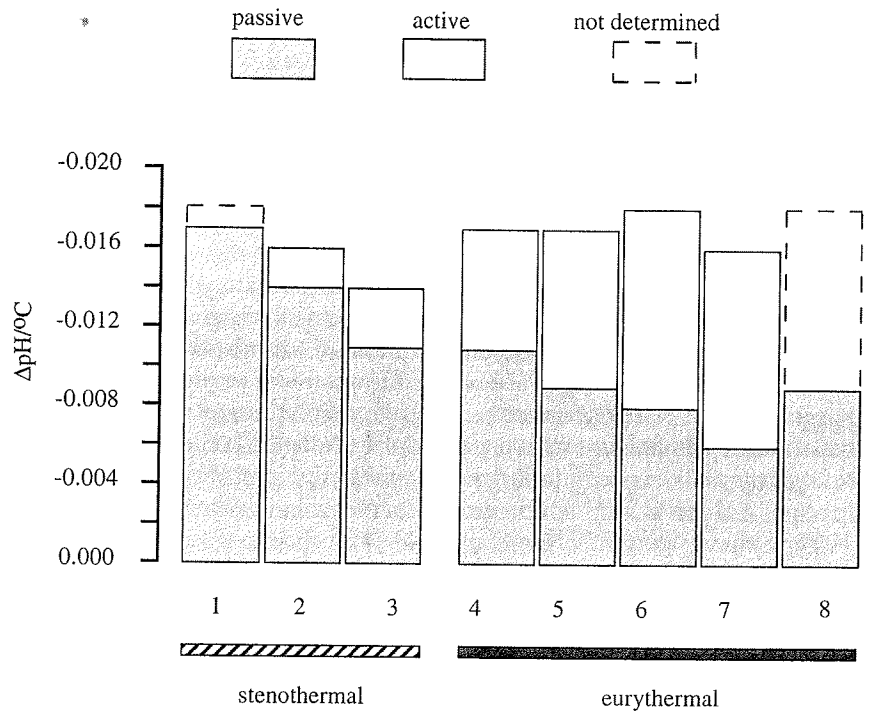
1: *Tryphosella murrayi* (Antarctica)

2: *Pandalus borealis* (Spitzbergen)

3: *Pachycara brachycephalum* (Antarctica)

4: *Arenicola marina* (North Sea)

5: *Crangon crangon* (North Sea)

6: *Crangon crangon* (White Sea)

7: *Zoarces viviparus* (North Sea)

8: pooled shrimps (South America)

Fig. 4.

the full set of acid–base parameters such as pH_i and pH_e (as well as interstitial pH), bicarbonate and P_{CO_2} levels, considering the buffering characteristics of each compartment. The study of these relationships is especially interesting in marine invertebrates and lower vertebrates, many of which experience wide fluctuations in various environmental parameters such as temperature, CO_2 or O_2 levels. Specific patterns of acid–base regulation observed in different groups need to be considered in an attempt to evaluate unifying principles.

The specific features of the homogenate technique allow a precise evaluation of mean pH_i as required for a quantitative picture of the patterns of acid–base regulation in whole animals. It yields cellular P_{CO_2} and bicarbonate levels and quantifies non-bicarbonate buffers, which specifically respond to respiratory, but also to non-respiratory, acidification. The technique allows an estimate of extracellular/intracellular P_{CO_2} gradients and, thereby, interstitial pH. During temperature change, it is possible to distinguish between changes in pH_i elicited by physico-chemical buffering and those caused by a readjustment of the setpoints of proton equivalent ion exchange. The homogenate technique is a useful, easy to use and a readily available alternative to other methods of pH analysis, especially when the experimental design does not allow the use of online techniques. Since tissues can be stored away under

Fig. 4. Relative contributions of passive and active mechanisms to temperature-induced changes in pH_i in stenothermal (1–3) and eurythermal (4–8) marine ectotherms. *Tryphosella murrayi* is an Antarctic amphipod, *Pandalus borealis* is a deep water shrimp caught in Spitzbergen waters, and *Pachycara brachycephalum* is an Antarctic eelpout. These polar species were compared with more temperate zone species such as the lugworm *Arenicola marina*, the sand shrimp *Crangon crangon*, and the eelpout *Zoarces viviparus*. In the polar species, the temperature-induced passive pH change is close to the pH change observed *in vivo* (passive slope = $\Delta pH_i/\Delta T$ = −0.017 in *Tryphosella murrayi*, −0.013 in *Pandalus borealis*, −0.011 in *Pachycara brachycephalum*). In species 1 and 8, only the passive contribution was determined; the dashed lines reflect expected *in vivo* values according to the alphastat pattern. Data originate from Sartoris and Pörtner (1997) for species 2, 5 and 6; from van Dijk *et al.* (1997) for species 7; data for species 1, 3, 4 and 8 are unpublished by F.J. Sartoris, A. Sommer, C. Tesch, I. Hardewig, P. van Dijk and H.O. Pörtner.

liquid nitrogen until analysis, the method is applicable to samples collected in the field. Samples can be collected from free-ranging, unrestrained and even exercising animals. The specific advantages of the homogenate technique are balanced to some extent by its limitations in that it does not allow changes to be recorded online and continuously in one preparation or animal and that it does not access compartmental pH in multicompartmental cells.

Acknowledgements

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