Chapter 10

MULTICOMPARTMENTAL ANALYSES OF ACID-BASE AND METABOLIC HOMEOSTASIS DURING ANAEROBIOSIS: INVERTEBRATE AND LOWER VERTEBRATE EXAMPLES

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ABSTRACT

Based on a quantitative analysis of the interrelations between anaerobic metabolism and acid-base status, linked to multicompartmental considerations, the question can be addressed of how metabolic and acid-base regulation are correlated. The analysis focuses on invertebrate and lower vertebrate examples known to adapt to the hypoxia situation.

Long-term anaerobiosis in facultative anaerobes causes acidotic changes in the intra- and extracellular acid-base status which are quantitatively related to anaerobic metabolism and the respiratory situation of the animals. In one example, the intertidal worm *Sipunculus nudus*, the analysis of proton-equivalent ion transfer between intra- and extracellular compartments and between animals and ambient water demonstrates that a reduction of the metabolic rate during anaerobiosis coincides with a decreased rate and efficiency of ionic acid-base regulation. Nonetheless, intracellular pH is regulated at lower values during anaerobiosis, possibly supporting the reduction in metabolic rate.

With a decrease in ionic acid-base regulation the diffusive movement of undissociated end products becomes more important in acid-base regulation. The distribution characteristics of metabolites and protons between intra- and extracellular compartments in selected invertebrate and vertebrate species reveals that pH influences the movement and release of major anaerobic end products like lactic acid and the volatile fatty acids propionate and acetate.

Based on recent methodological developments for the measurement of intracellular acid-base parameters, correlated changes in energetic parameters and intracellular pH can be elaborated, thus allowing to address the importance of pH in tissue energetics.

I. METABOLIC MECHANISMS AND ACID-BASE STATUS: QUANTITATIVE QUESTIONS

The interrelations between acid-base status and metabolism have gained increasing attention during recent years. Based on the traditional concept of lactic acid formation and dissociation the mechanisms of proton production by metabolism have been rediscussed with the result of controversial viewpoints.[1,2] Special interest was on the mechanisms by which nonsteady state or anaerobic metabolism influences acid-base status. In brief, the final view should be that it is not ATP formation and hydrolysis which always quantitatively mirrors the proton output of anaerobic metabolism (this view had originally been stressed by Gevers and Krebs et al.[3,4] for lactic acidosis). In contrast, the type of substrate and end product, i.e., the net generation of carboxyl groups together with the net metabolization of organic phosphates and the production and fate of ammonium, represent the net proton yield of metabolism.[2,9] Recently, the role of aerobic metabolism has also been reconsidered especially with reference to ammonia and urea metabolism.[5-7]

Understanding the relationships between acid-base status and metabolism means, last not least, an understanding in quantitative terms. When proton quantities are analyzed in intra- and extracellular body compartments and when the net proton transfer to the environment is taken into account, this quantitative approach permits to analyze the production and the fate of metabolic protons. Among others the question can be addressed whether the importance of anaerobiosis for the acid-base status is only reflected by an increase in the net production of protons and the respective influence on the animal's acid-base status, or whether these interrelations also comprise modifications in the animal's strategies and capacities in acid-base regulation. This chapter will discuss these relationships and also the impact that a modified strategy of acid-base regulation may have on the rate of energy turnover or on metabolite fluxes between body compartments.

Capacities for survival in anaerobiosis are large in invertebrate and lower vertebrate species which live in environments where oxygen supply may fluctuate. Among these facultative anaerobes a variety of anaerobic metabolic pathways has evolved, many of which are characterized by a reduction in the extent of proton production.[2] A preliminary conclusion may be that these reduced quantities are easier to be handled by ionic acid-base regulation. However, this conclusion does not consider to what extent ionic acid-base regulation may be affected by the reduction in energy turnover which goes hand-in-hand with the use of these alternative metabolic pathways. In addition, acid-base regulation could also comprise the release of protonated end products (like lactic acid) from the tissue or animal.[2,9] The latter question has been raised for working muscle tissue (e.g., Wiseman et al.[8]). Since there is no cost involved in this mechanism of proton transfer, its use may become important during low energy turnover in environmental hypoxia.

II. ACID-BASE METHODOLOGY FOR QUANTITATIVE ANALYSES

For a quantitative investigation, acid-base parameters (pH, P_{CO_2}, bicarbonate levels, buffer values) need to be analyzed in the intracellular space of relevant organs and in the extracellular fluid compartments. In addition, the acid-base relevant ion exchange between animals and the environment must be monitored by titratable acidity (TA) and ammonia analyses in urine and/or by the same measurements in the water using the "delta bicarbonate system" designed by N. Heisler, or measurements of titratable acidity (TA) or total CO_2 (at a fixed P_{CO_2}) in water samples.[7,10]

Intracellular pH in the intact animal has been analyzed by three different methods, DMO (5,5-dimethyl-2,4-oxazolidinedione) distribution analysis, ^{31}P-NMR (see van den Thillart or van Waarde, this volume), and a new version of the homogenate technique, which eliminates the influence of homogenate metabolism on the measured pH.[11-13] The latter method will also give access to intracellular bicarbonate and P_{CO_2} values. Unlike the DMO method, which exhibits a rather high variability between individual measurements, the new homogenate technique most accurately analyzes pH_i in each individual tissue sample.[13] This technique has, so far, been successfully used to study acid-base disturbances *in vivo* during environmental hypoxia, environmental hypercapnia, or muscular exercise.[14-17]

A large and unknown contribution of metabolism has previously been a serious shortcoming of buffer value estimates in tissue homogenates and, owing to the response of metabolism to pH, may still influence buffer values measured in intact organs or cells by using ^{31}P-NMR or microelectrode techniques.[7,18] Based on the homogenate technique developed by Heisler and Piiper, intracellular buffer values valid for the intact tissue can now be accurately assessed by clearly distinguishing between nonbicarbonate and bicarbonate buffers and by compensating for the influence of homogenate metabolism on the measured buffer values.[19,20]

It may be interesting in this context that the specific properties of the three mentioned techniques for pH_i analysis may not lead to identical intracellular pH values. It has been under dispute whether the different methods measure cytosolic pH.[21,22] In a recent comparison of homogenate and DMO-derived pH_i values, those found with both techniques were similar in white muscle tissues of invertebrates and vertebrates, thus leading to the conclusion that the cytosol determines the measured values in these organs. However, in ventricular muscle of a toad, *Bufo marinus,* and in the isolated perfused rat heart, the homogenate technique yields lower values than obtained with DMO.[13] The DMO technique leads to a mean value of intracellular pH which does not just depend upon the volume fractions exhibiting different pH values in the cell, but heavily weighs the alkaline mitochondrial pH owing to weak acid

distribution characteristics.[21] The homogenate technique records mean intracellular pH considering both the respective volume fractions and the contribution of each cell compartment to cellular buffering. For quantitative estimates of proton movements, the consideration of cellular compartments is complete and, thus, this method appears most adequate to address the quantitative questions mentioned above even in aerobic tissues containing large fractions of mitochondria.

III. QUANTIFICATION OF ACID-BASE METABOLISM DURING ANAEROBIOSIS

Only very few animals would extensively use anaerobic glycolysis during extended environmental hypoxia since high rates of proton formation prevail. Among lower vertebrate anaerobes, turtles overwintering under ice exhibit remarkable capacities of lactate accumulation in the plasma.[25] However, this may only be possible by using the large carbonate stores of the shell as buffers.[26] Other species (among vertebrates fish species like crucian carp and goldfish and among invertebrates insect larvae, annelids, mollusks, and sipunculids) need to reduce the extent of proton formation by metabolism, mostly by the use of "aerobic" mitochondrial mechanisms of proton elimination like oxidative decarboxylations, leading to ethanol production or the formation of volatile fatty acids.[2,7,27] A summary of these strategies and mechanisms just leads to these rather simple points: a minimization of proton accumulation during long-term anaerobic metabolism is due to the depletion of the phosphagen, the accumulation of buffer substances (largely inorganic phosphate), a reduction of H^+ formation, but often an increase in the ATP output of metabolic pathways. Protonated end products may be released by nonionic diffusion (see below). In conjunction with the observation that metabolic rate is reduced during anaerobiosis, this means much less proton formation per time unit than during lactate or opine accumulation.[2]

The number of studies addressing the quantitative relationships between metabolism and acid-base status in the intact facultative anaerobe is small because of limitations usually imposed by the number of tissues and compartments which have to be considered. To this end and in accordance with the August Krogh principle, the intertidal peanut worm, *Sipunculus nudus,* was used as a very adequate model.[9] Only the intracellular space of the body wall musculature and the coelomic plasma including all extracellular space are fluid compartments important enough from a quantitative point of view to be considered. The following section will examine whether the quantitative treatment of the interrelations between cellular energy metabolism and whole organism acid-base status reveals a link between the mode and rate of energy turnover and the pattern of acid-base regulation.

The drop in metabolic rate during anaerobiosis in marine invertebrates occurs slowly linked to a change in the pattern of anaerobic substrate utilization and end product formation (Figure 1).[23] High energy requirements during the early period of anaerobiosis are reflected by the use of cytosolic glycolysis (lactate/opine or alanine formation) and the degradation of the phosphagen, e.g., phospho-L-arginine in sipunculids. Anaerobic mitochondrial metabolism uses malate as a substrate. During early anaerobiosis aspartate degradation replenishes the malate pool, the amino group being transferred to pyruvate. In the mitochondria matrix, malate undergoes disproportionation by being subjected to both oxidation and reduction. Oxidation occurs via citrate formation and delivers reduction equivalents for fumarate reduction. Both oxidation and reduction lead to an increase in the succinate pool. Propionate is formed from succinyl-CoA, and acetate originates from pyruvate decarboxylation.

With the onset of late anaerobiosis, metabolism is shifted to the carboxylation of phosphoenolpyruvate, such that malate can be formed from glycolytic precursors. As indicated

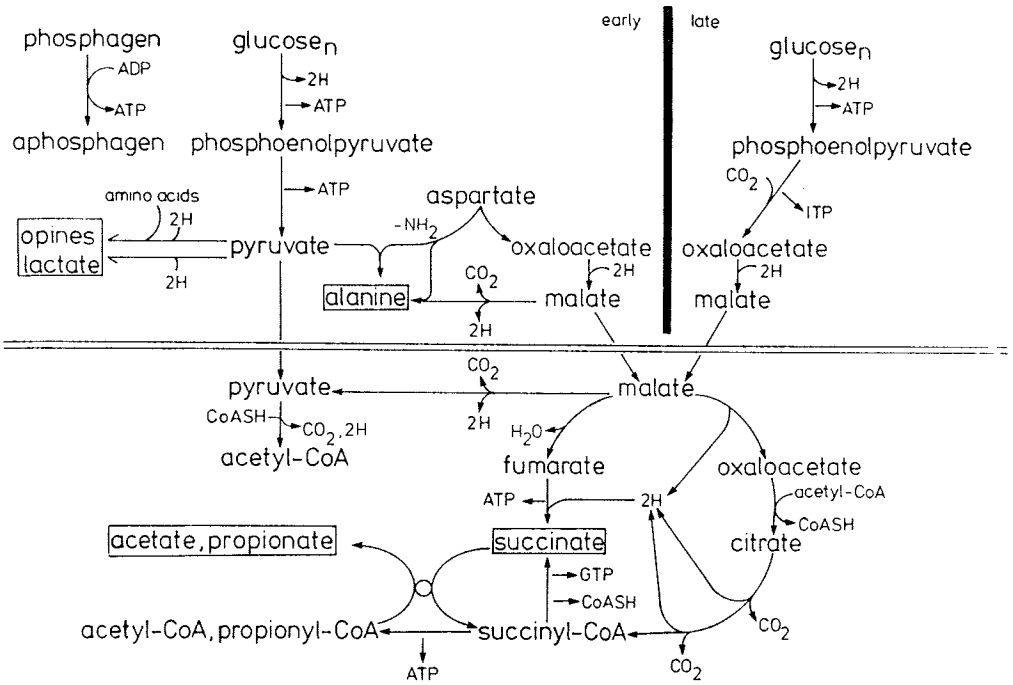

FIGURE 1. Anaerobic catabolism of glycogen (glucose$_n$) and aspartate in marine invertebrates during early and late anaerobiosis (accumulated end and intermediary products outlined in boxes). The contribution of mitochondria to anaerobic ATP generation (lower section) leads to an increase in the ATP yield and a decrease in the proton output of metabolism.[2] (Adapted from Grieshaber et al.[67] For further explanations see text).

by the stop in aspartate mobilization, this shift occurred during a period of intracellular alkalosis.[23,24] These and other findings led to the conclusion that an acidosis, which was assumed to elicit the shift to long-term anaerobic metabolism, cannot be an ultimate requirement for this regulatory change (but may still be useful, see below). Emphasis is now on the covalent modulation of enzymes to allow for the regulatory shift and to adjust metabolism to the reduction in ATP turnover (see Storey, this volume).

To this author's knowledge *Sipunculus nudus* is the only animal species, for which quantitative evidence could so far be provided, that the changes in the anaerobic acid-base status can be explained by the known metabolic end products (Figure 2).[9] This finding is quite opposite to the contention of Graham and Ellington and Gnaiger et al.[28,29] who claimed that there is no quantitative correlation between the degree of acidosis and metabolic proton production. The consideration of anaerobic metabolic processes in this facultative anaerobe is not only complete, as far as the influence on the acid-base status is concerned. By now it is even possible to say that these mechanisms include all those responsible for anaerobic ATP production.[30] These findings are in accordance with theoretical considerations which predict that protons play an important role in anaerobic ATP generation. This is true not only for oxidative phosphorylations but also for all mechanisms of substrate level phosphorylations.[2] Overall, the finding of these quantitative agreements validates the conclusion that, during anaerobiosis, metabolic rate and the rate of proton production are closely correlated, and that an analysis of the transmembrane movements of nonrespiratory proton equivalents permits to assess how the rates of metabolic proton production and ionic acid-base regulation could be coordinated. Unfortunately, such an analysis is not yet available

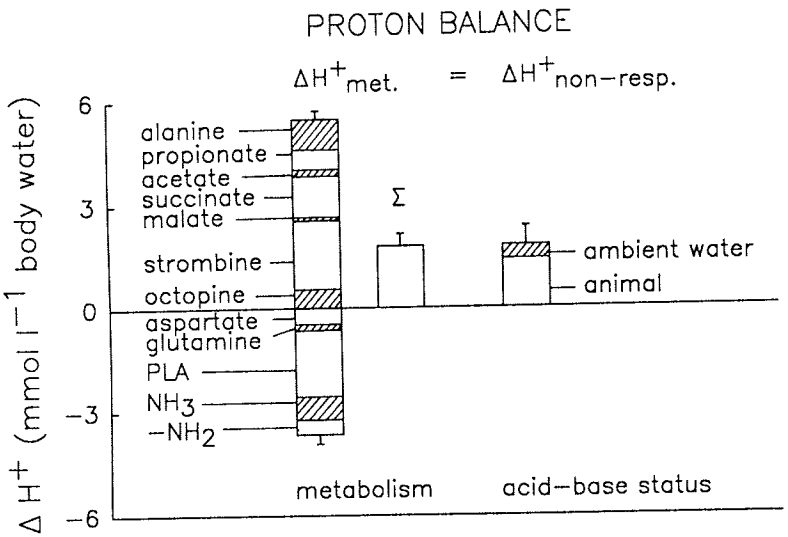

FIGURE 2. Proton consuming and proton producing processes in metabolism (ΔH^+_{met}) lead to net proton production (Σ) during 24 h of anaerobiosis in normocapnic sea water. These quantities are found in the animal's (*Sipunculus nudus*) intra- and extracellular acid-base status and in the ambient seawater ($\Delta H^+_{nonresp}$, based on data by Pörtner and Pörtner et al.[9,32]). The analysis confirms that all processes contributing to proton turnover by anaerobic metabolism have been completely considered (for further explanations see text).

for lower vertebrate anaerobes. Among invertebrates it is, though, for the marine worm *Sipunculus nudus*.

IV. RATES AND SET POINTS OF ACID-BASE REGULATION

The rates of proton equivalent ion exchange between animals (*Sipunculus nudus*) and ambient water were related to the changes in the acid-base status observed during both anaerobiosis and postanaerobic recovery (Figures 3 and 4).[32] The net proton transfer resulting from the changes in water bicarbonate and ammonium levels was slightly negative during the control period (possibly related to the diet of the animals). During anaerobiosis the variations in proton or bicarbonate exchange reflect the extracellular pH changes, a net base release during initial alkalosis turning into a net proton release during the period of progressive metabolic acidosis. However, anaerobic ion exchange was inefficient to maintain aerobic acid-base parameters in intra- and extracellular compartments (Figure 3).[24] This pattern as compared to the drastic increase in net proton release seen during recovery would suggest that the small exchange rates recorded during the period of anaerobiosis could possibly be linked to the observed metabolic rate reduction. That metabolic and ion exchange rates are correlated is also suggested based on a comparison of animals exhibiting differences in aerobic and anaerobic metabolic rates depending on the season.[31-33]

After anaerobiosis, the increased net release of protons into the ambient water may be linked to the extracellular acidosis induced by the replenishment of the phosphagen pool.[31] However, the return of the metabolic rate to prehypoxic values is a prerequisite for the drastic increase in exchange rates. The latter conclusion is supported by the observation that pH_i drops during anaerobiosis but does no longer decrease when the proton load is increased during initial recovery.[31] Intracellular pH is regulated with high priority under these conditions. As a corollary, the increase in net proton transfer to the ambient water reflects an

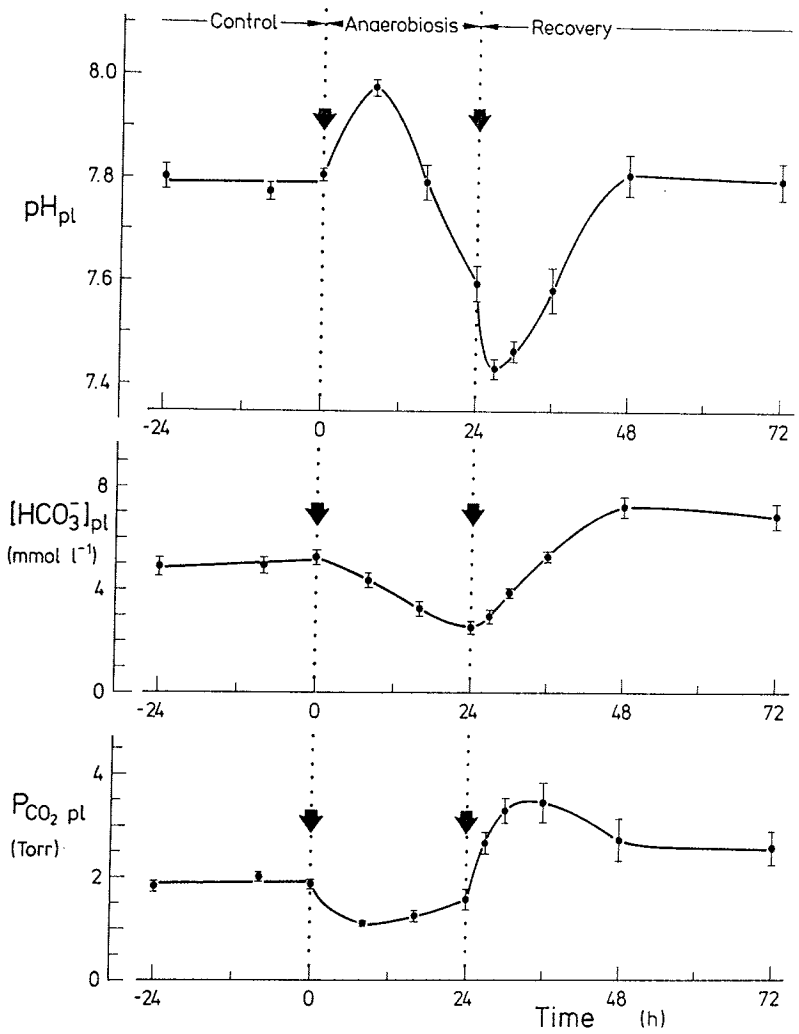

FIGURE 3. Extracellular acid-base parameters in the coelomic plasma (pl.) of *Sipunculus nudus* under control conditions, during 24 h of anaerobiosis and during postanaerobic recovery (adopted from Pörtner et al.[32]). The arrows mark the beginning and end of the anaerobic period. The sequence of events is compared to the concomitant analysis of proton equivalent ion exchange between animals and ambient water (Figure 4).

increased efficiency of acid-base regulation. These mechanisms were not supported by extensive metabolic proton removal during metabolization of the accumulated carbonic acid anions. During long-term recovery the discrepancy between efficient proton removal by acid-base regulation and the retarded proton uptake by metabolism even led to a "proton gap", as evidenced by the observation that the curve of cumulative proton transfer remained displaced from the extrapolated normoxic control rate (Figure 4). This "proton gap" can be related to the slow metabolization of organic acid anions.[32,33] The respective protons are transiently "stored" in the ambient water until they are removed by metabolism.

In summary, the shift to acidosis during anaerobiosis occurs, because the amount of metabolic protons exceeds the quantity that is or can be translocated. The low rates of proton formation by long-term anaerobic metabolism (both by metabolic mechanisms of H^+ removal and the low rate of end product formation) do not change this picture since the energy

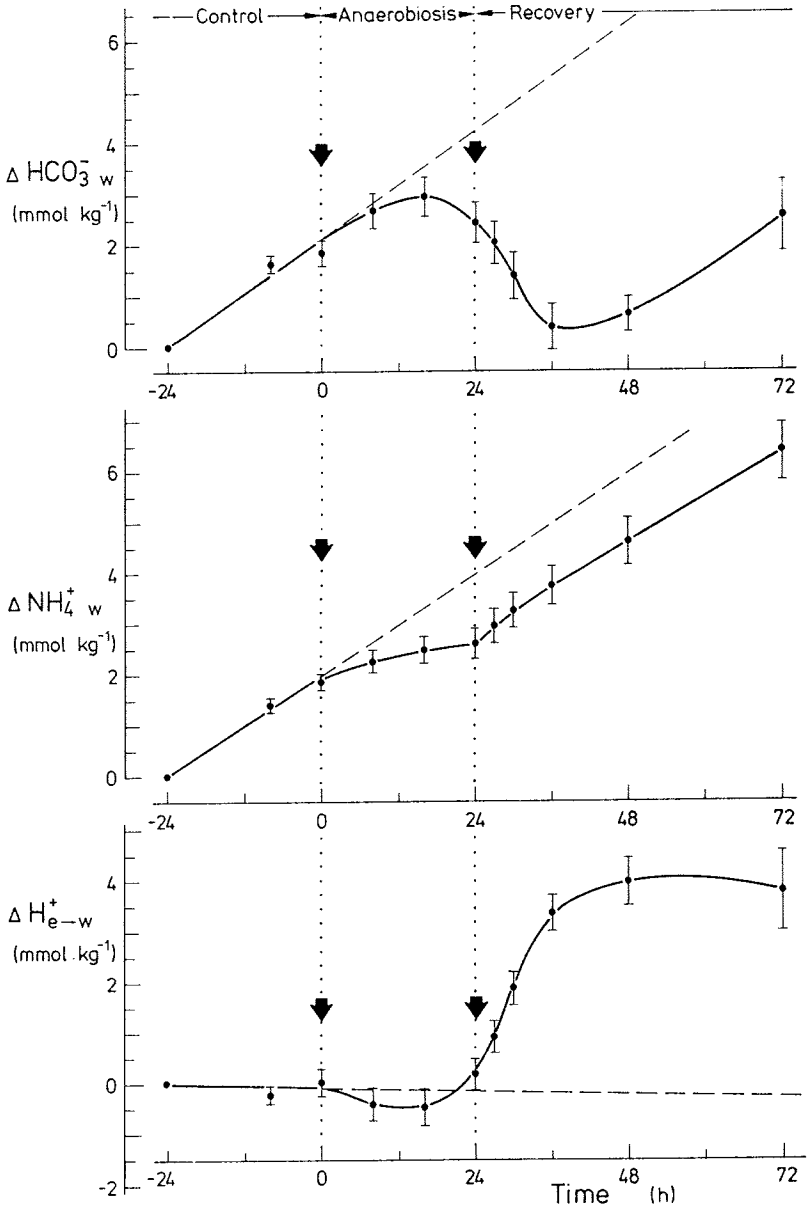

FIGURE 4. Cumulative rates of bicarbonate and ammonium accumulation and the resulting net proton equivalent ion exchange between *Sipunculus nudus* and ambient water (w) under control conditions, during 24 h of anaerobiosis and subsequent recovery. The control rates are extrapolated for the entire experimental period (dashed line) (adapted from Pörtner et al.[32]).

turnover falls linked to a decreasing rate and efficiency of proton equivalent ion exchange. Nonetheless, low rates of proton formation contribute to eliminate the risk of rapid and severe acid-base disturbances. Since acid-base disturbances do occur in both intra- and extracellular compartments, a resulting question is are they just tolerated or are they also part of a changing strategy of acid-base regulation during anaerobiosis?

The pattern of acid-base changes observed during recovery would suggest that the regulation of intracellular pH has the highest priority (see above).[31] Based on a comparison

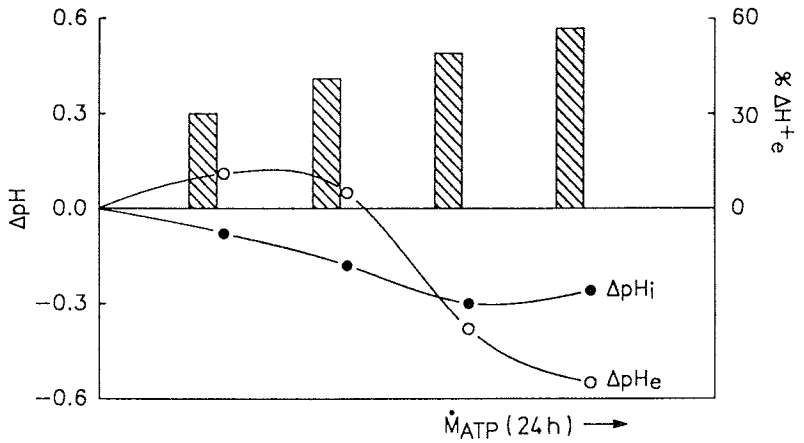

FIGURE 5. Changes in intra- and extracellular pH (ΔpH) and the percentage of metabolic protons (% ΔH_e^+) found in the extracellular fluid of *Sipunculus nudus* during 24 h of anaerobiosis. An enhanced formation of opines and acetate represents an increase in the rate of ATP turnover ($\dot{M}_{ATP}$).[33] The concomitant rise in proton formation leads to an increase in the percentage of protons released into the extracellular space (for further explanations see text, based on data by Pörtner and Pörtner et al.[9,31]).

of animals with different anaerobic metabolic rates, this conclusion remains also valid during the period of anaerobiosis (Figure 5). With low metabolic rates pH_i drops when pH_e increases owing to respiratory CO_2 loss. Under these conditions, the acidosis in the intracellular space is even in excess of the amount of protons produced by metabolism indicating the net loss of base equivalents. A similar conclusion may arise based on the study of Booth et al.[34] and Walsh et al.[35] on hypoxia in *Mytilus edulis*. With increasing rates of ATP turnover, however, the drop in pH_i appears to be limited, whereas pH_e falls to a much larger extent. The percentage of metabolic protons found in the extracellular compartment increases indicating that pH_i is regulated at the expense of extracellular pH during anaerobiosis, even though intracellular buffer values are much higher.[24,68] Based upon that these are long-term changes and may represent quasi steady state pH_i values this comparison leads to the conclusion that the set point for pH_i regulation is shifted to a lower value.

A similar conclusion arises based on an analysis of pH_i in animals dwelling in their preliminary burrows in the sand. When the aerated water was withdrawn, the animals were subjected to a "low tide" situation. pH_i fell under these conditions but only to a limited extent, whereas pH_e continued to fall during the extended low tide period.[68] The defended value of pH_i was about 0.2 pH units below the value valid for normoxic control conditions, which represents a similar shift as observed above (see Figure 5). These two examples strongly suggest that there may be a shift of set points to more acidic pH values during anaerobiosis. It has been assumed early on that an acid shift in pH may contribute to a reduction in glycolytic rate (for review see Busa and Nuccitelli[36]). Anaerobic lugworms (*Arenicola marina*) actually showed decreased rates of propionate formation when pH_i was low but exhibited higher rates of propionate formation when pH_i was kept high by introducing an artificial buffer substance into the experimental system.[37] Thus, the regulation of pH_i at low values may provide the "context" of an efficient reduction in energy requirements. It can, however, not be the exclusively responsible parameter since, during transition from normoxia to anoxia, a large drop in energy requirements occurs already when pH_i is still high or even elevated above control values.[23,24]

V. MEMBRANE TRANSFER OF METABOLITES AND PROTONS: NONIONIC ACID-BASE REGULATION?

A regulatory slow down of ion exchange rates in acid-base regulation is likely to contribute to the observed reduction in anaerobic energy turnover (see above). Under these conditions, the diffusive movements of undissociated organic acids between body compartments and even between animals and ambient water could become important in acid-base regulation, since this mechanism would cost no energy. The effect of such movements is similar to the role of respiratory CO_2 release which is also adequate to compensate for metabolic acidosis. However, the conclusion that metabolite transfer contributes to acid-base regulation can only be supported if the proton equivalents really pass the cell membrane together with the organic acid anions. The pH gradients between body compartments should then cause a weak acid distribution behavior.[44] Alternatively, if anions and protons move independently, pH-independent transfer of the anions would result in an equilibrium distribution according to the membrane potential (Figure 8).

For the whole animal, lactate formation and elimination kinetics have been studied during muscular exercise and recovery in fish.[38-40] The general conclusion from these investigations is that protons are transferred at a much higher velocity than lactate indicating that the transfer of protons and of lactate is largely independent of each other. However, since ionic exchange and metabolite turnover rates are high and difficult to disentangle during exercise, the study of the exercise situation and of postexercise aerobic recovery may not be adequate to give suitable answers (cf. Cameron and Cech).[41] For example, some lactate distribution studies suggest that the pH gradient influences the movement of lactate (see below).[42,43] Other exercise studies were unable to support this finding (see Heisler for review, and Wiseman et al.[8,39]).

The extent and pattern of nonrespiratory proton accumulation seen in the extracellular fluids of *Sipunculus nudus* during environmental anaerobiosis (Figure 6) does also not accord with the proton release expected from the dissociation of organic acids. Only during late anaerobiosis may ionic regulation of intracellular pH be supported to some extent (15%) by the release of organic acids into the extracellular space. However, although proton and metabolite transfer cannot be correlated, metabolites could still move with the respective proton quantities. In this case the ionic mechanisms of pH regulation would cause an apparent discrepancy between proton and metabolite movements.

Under the widespread condition that pH_e is higher than pH_i, both the "weak acid distribution behaviour" and the membrane potential dependent distribution should lead to higher extracellular than intracellular metabolite levels (Figure 8). However, only under conditions when the rate of metabolite turnover is low may the distribution approach the respective equilibrium situation. This may happen during environmental hypoxia, when, in addition, end product levels are high enough to allow for accurate analysis. (The procedure of tissue sampling, for example, may lead to erroneously high tissue lactate levels under control conditions.[45]) In only a few studies have both the transmembrane distribution of lactate and the pH gradients been analyzed during long-term hypoxia *in vivo*. Several other investigations of long-term hypoxia, however, have also recorded higher lactate values in the plasma than in the tissues (Table 1). Although pH has not been measured in most of these studies the pH gradient calculated from the lactate gradient under the assumption of an equilibrium distribution is rather close to the expected value among the invertebrates, which exhibit the lowest rates of lactate formation (and an open circulatory system, see below). Among the vertebrates, the calculated pH difference is always smaller than the one evaluated between plasma and intracellular space.[14,45]

The assumption that pH exerts an influence on the equilibrium distribution of lactic acid would be in accordance with recent studies on lactate transport in human erythrocytes,

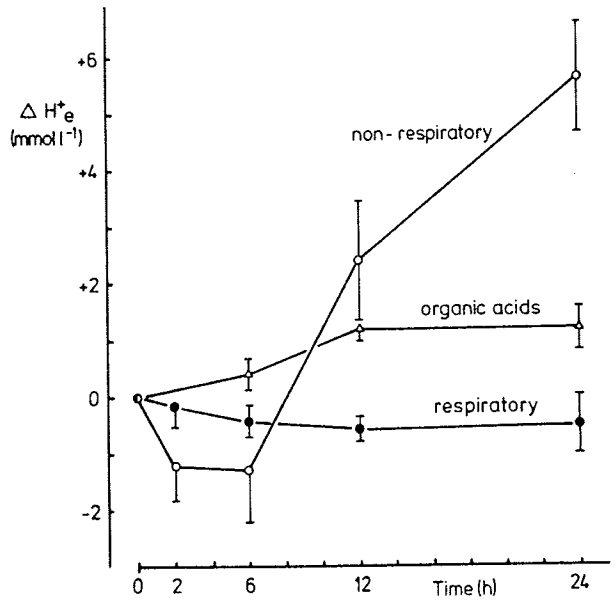

FIGURE 6. Proton quantities (ΔH^+, evaluated from respiratory and nonrespiratory changes in the acid-base status) and metabolite changes in the extracellular (e) fluid compartment of *Sipunculus nudus* during 24 h of anaerobiosis. The contribution of organic acids (succinic, propionic, or acetic acids) to the net proton transfer is assumed to result from the equimolar dissociation of protons. The initial period of (nonrespiratory) proton removal reflects the period of phospho-L-arginine metabolization. Overall, the ionic mechanisms of acid-base regulation are largely independent from the membrane transfer of metabolites (adapted from Pörtner et al.[24]).

mammalian hepatocytes, vertebrate cardiac and skeletal muscles, and in muscular vesicles.[43,46-49] All of these studies suggest that lactate transfer in most tissues does not only occur as a transmembrane diffusion of the lipid-soluble, undissociated weak acid (according to Walsh,[50] this process predominates in fish hepatocytes) but even more so as a carrier-mediated, 1:1 exchange with base or a symport with a proton. It must be emphasized that, although the lactate anion is involved, the transport of lactate via the proposed mechanism is equivalent to the movement of the undissociated acid and represents an apparent increase in the respective permeation coefficient of the undissociated species. A study by Wiseman et al.[8] suggests that a similar facilitated distribution mechanism exists for D-lactate in invertebrate muscle tissues. The independent contribution of a SITS dependent anion exchange mechanism could be more or less ruled out in many of the studies cited above, thus supporting the finding that lactate is far from a membrane potential dependent distribution (Figure 8). Na-cotransport of lactate may be restricted to renal systems.[51]

The question remains whether there are reasons other than a too high rate of lactate production, which could support a discrepancy between lactate distribution and plasma and intracellular pH gradients. One uncertainty inherent to the comparison of measured pH gradients with those calculated based on lactate ratios (Table 1) is the fact that the measured extracellular pH (arterial or venous) may be higher than the actual venous pH valid for the tissue under consideration.[14,45] Another, more important point to note in this context is that the pH in the interstitial fluid may differ from plasma pH especially in those animals with a closed circulatory system where interstitial fluid is characterized by low nonbicarbonate buffering (Figure 7). The carrier is located in the cell membrane and, thus, exposed to interstitial fluid. In resting and working mammalian muscle interstitial or surface pH values 0.1-0.2 pH units below plasma or extracellular pH have been measured.[52,53] The difference

TABLE 1
pH Differences (ΔpH) Calculated From Intra- and Extracellular Lactate Levels and Actual pH Differences Found During Environmental Hypoxia in Resting Muscle or Liver of Selected Invertebrate and Lower Vertebrate Species

				ΔpH	
		Ci	Ce	Calc.	Actual
D-Lactate					
Helix pomatia[57] (snail)	(Liver)	6.3[a]	50	0.9	nd
L-Lactate					
Hirudo medicinalis[58] (leech)	(Muscle)	6.9[a]	13.2	0.28	nd
Orconectes limosus[59] (crayfish)	(Muscle)	10.5[a]	60	0.6	nd
Menippe mercenaria[60] (crab)	(Muscle)	17[a]	50.7	0.5	nd
Platichthys flesus[51] (flounder)	(Muscle)	9.7[a]	15	0.2	nd
Anguilla anguilla[62] (eel)	(Liver)	3.8[a]	7.7	0.3	nd
Chrysemis picta bellii[45] (turtle)	(Muscle)	64.0	109.0	0.23	0.38
	(Liver)	46.0	109.0	0.38	0.60
Bufo marinus[14] (toad)	(Muscle)	3.9	10	0.40	0.75

Note: i: intracellular, e: extracellular; C: mmol l^{-1} cell water or plasma; nd: not determined.

[a] Calculated based on literature data.

will depend upon the P_{CO_2} gradient between intracellular space and venous plasma (cf. Figure 7). Since the buffer value of interstitial fluid is small, this pH gradient may increase during high rates of lactic acid transfer.[52] The gradient between intracellular and interstitial pH represents the actual driving force for the lactate carrier. Since the lactate carrier will correct for any deviation from equilibrium it will even compensate for a potential shift of the equilibrium towards plasma pH (Figure 8) and, thus, support some discrepancy between lactate distribution and the pH gradients observed between intracellular space and the plasma. When plasma pH approaches intracellular pH (e.g., during exercise, cf. Heisler[38,39]) and, in addition, interstitial pH falls far below plasma pH owing to high P_{CO_2} gradients or high rates of acid extrusion during periods of high metabolic rate (e.g., during exercise and postexercise recovery), then the resulting (reversed) pH gradient may actually support a nonrelease of lactate or even the uptake from the plasma into the tissues during recovery. This may explain the otherwise conflicting results reported for postexercise recovery in fish (reviewed by Wood).[40] The fact that interstitial pH has not been considered so far as being the effective pH_e (see above) may also lead to an understanding of the conflicting finding in some exercise studies, where a pH dependence of lactate release could not be established.[39] As a corollary, fluctuations in interstial pH urgently need to be investigated to elucidate its role in the pattern and direction of lactic acid transfer between body compartments.

Based on Figure 8, the general conclusion would be that the rate of lactate formation, the permeability of the weak acid (apparently increased by the lactate carrier), and the difference between intracellular and interstitial pH are responsible for the distribution ratio of lactate between intra- and extracellular space. However, the actual rate of lactate transfer does not only depend upon the pH gradient between intra- and extracellular space. The concentration gradient of undissociated lactic acid is the net driving force which results from both the gradient of total lactate and the pH gradient.

The situation among invertebrates is more complex since lactate is only one possible end product (Figure 1). The distribution behavior of opines indicates that there is only a minor release even when levels remain elevated during long-term incubations (Table 2).[23] Recent findings in fatigued whelk muscles and in exercised squid demonstrate that the release

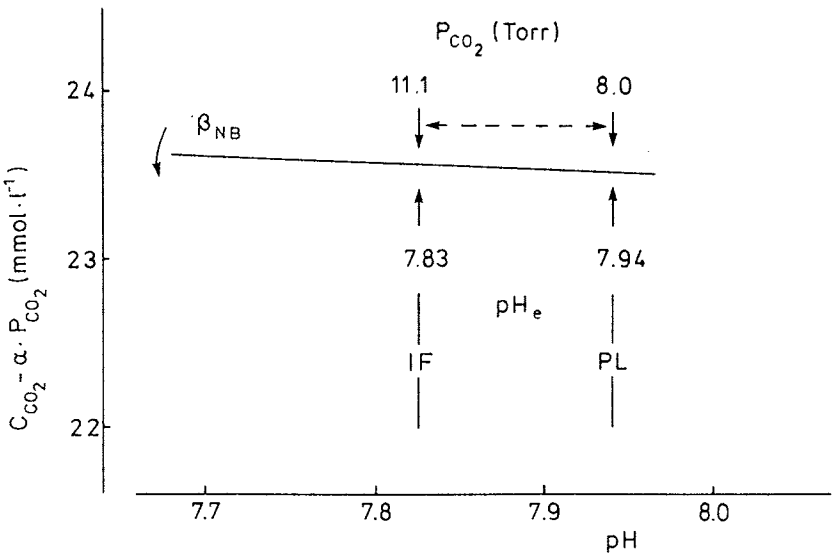

FIGURE 7. An evaluation of interstitial pH in the gastrocnemius muscle of the toad, *Bufo marinus*. Owing to minimal nonbicarbonate buffering (β_{NB}) in the interstitial fluid and rapid equilibration of ions between plasma and interstitial fluid the P_{CO_2} gradient between intra- and extracellular compartments leads to an interstitial (IF) pH 0.11 pH units below plasma (PL) pH under normoxia (based on Pörtner et al.[13]). Under extreme hypoxia (P_{O_2} = 14 torr) the respective pH gradient amounts to 0.2 pH units, possibly being even larger owing to the release of lactic acid.[14]

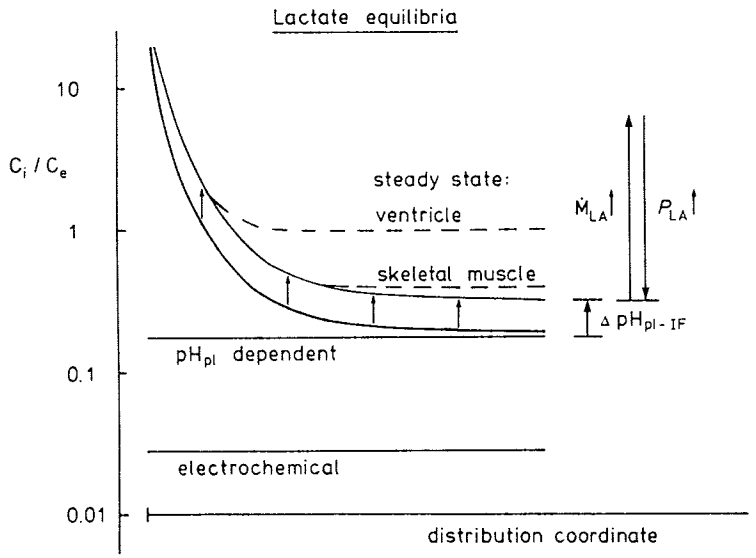

FIGURE 8. A model of lactic acid (LA) distribution equilibria in *Bufo marinus* skeletal (gastrocnemius) and ventricle muscles (based on data by Pörtner et al.[14]). If the distribution is assumed to start from high intracellular levels (not observed *in vivo*) the final concentration ratio (Ci/Ce) in the gastrocnemius muscle approaches a value close to that expected based on pH_i and interstitial (IF) pH (considering ΔpH_{pl-IF}), but above the one expected based on pH_i and plasma pH (pH_{pl} dependent) and far above a membrane potential dependent (electrochemical) distribution. Lactate concentration ratios found in the ventricle can be explained by high rates of lactate formation ($\dot{M}_{LA}$) causing the steady state ratio to deviate from weak acid distribution characteristics. A high apparent permeability of lactic acid (P_{LA}) as caused by the presence (or modulation) of a carrier enables lactate to approach the weak acid distribution equilibrium despite the low dissociation constant of lactic acid.

TABLE 2
pH Differences (ΔpH) Calculated From Intra- and Extracellular Metabolite Levels and Actual pH Differences Found During Normoxia and Up to 48 h of Environmental Hypoxia in Selected Invertebrates

	Ci	Ce	ΔpH Calc.	ΔpH Actual
S. nudus				
Acetate	<0.1	1.3	1.1	1
Propionate	0.5	1.3	0.41	1
Succinate	1.5	0.3	−0.7	1
Opines	11.3	—	—	1
A. marina				
Acetate	2.0[a]	1.9	−0.04	0–0.2
Propionate	2.2[a]	2.4	0.04	0–0.2
	2.9[a]	3.5	0.08	0–0.2
Succinate	5.0[a]	0.9	−0.7	0–0.2
H. medicinalis				
Propionate	8.7[a]	19.6	0.36	nd
Succinate	4.0[a]	6.9	0.23	nd
Malate (normoxia)	8.3[a]	15.2	0.26	nd

Note: i: intracellular, e: extracellular; C: mmol l^{-1} cell water or plasma; nd: not determined.

[a] Calculated from literature data; based on Hardewig et al.; Hildebrandt, Holst and Zebe; Kamp and Juretschke; Pörtner; Reitze; Schöttler et al.; Toulmond.[9,30,55,58,63-66]

of a significant amount of opines is most unlikely.[16,54] However, based on a large difference between intra- and extracellular pH in *Sipunculus nudus,* acetate and propionate are predominantly accumulated in the coelomic fluid. This conclusion is supported by the situation in *Arenicola marina,* where the pH gradient between intra- and extracellular spaces is much smaller, such that intracellular are close to extracellular levels. Evidently, the movement of acetic and propionic acid between intra- and extracellular compartments could contribute to reduce the intracellular proton load. Evidence is scarce whether a contributing carrier exists. The lactate/pyruvate carrier present in vertebrates is obviously not capable to carry end products like propionate.[43,48] In accordance with these considerations, the excretion of volatile fatty acids may occur predominantly via simple diffusion.[9,55] The support of acid-base regulation by the fatty acids is more prominent than by lactic acid since considerable amounts are found to be released into the ambient water. If extracellular pH, as in *Arenicola marina,* is almost as low as intracellular pH (Table 2) emphasis may be on a release of these acids to the environment rather than on the use of the extracellular space as a reservoir as observed in *Sipunculus nudus.*

Succinate also leaves the tissue, but its distribution remains unrelated to the pH gradient in the marine invertebrates. In contrast, pH may be important in the freshwater annelid *Hirudo medicinalis* to maintain high levels of dicarboxylic acids in the blood and low levels in the tissue. This is valid for malic acid which is found in high concentrations under normoxia and for succinic acid under hypoxia when malate is degraded as a substrate of anaerobic metabolism. Similar pH gradients result from the distribution of lactic (Table 1), propionic, and the dicarboxylic acids (Table 2).

As a corollary, the pH gradient usually found between intra- and extracellular compartments is likely to play an important role in the strategy of acid-base regulation during anaerobiosis. When ionic rates of acid-base regulation are reduced, the pH gradient supports the removal of major end products from the intracellular space or the whole animal, thereby

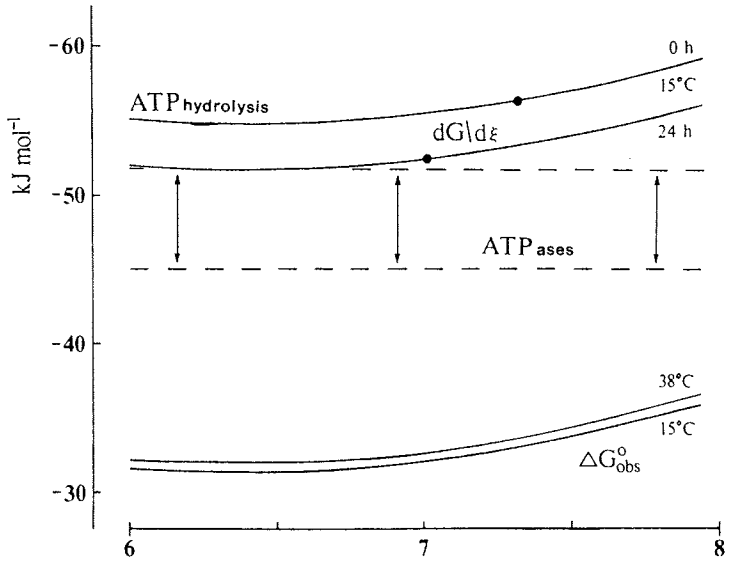

FIGURE 9. Free energy change of ATP hydrolysis calculated based on the analysis of Guynn and Veech and Alberty for different pH values between 6 and 8 (T = 15 or 38° C, respectively).[69,70] ΔG°_{obs} gives the change of ΔG with pH at pMg = 3.3 (cf. Gupta et al.[71]) and at molar concentrations of ATP, ADP, and inorganic phosphate, $dG/d\xi$ has been calculated based on *in vivo* concentrations in *Sipunculus nudus* musculature during aerobiosis and after 24 h of anaerobiosis (Pörtner et al.,[23,24,68] free ADP concentrations were calculated using the equilibrium constant for arginine kinase,[72] corrected for the respective pH value and the temperature). Points indicate the actual pH_i values measured. Neglecting the pH dependence of ATPases, 45 kJ mol^{-1} is given as a (minimum) value for the free energy required for the maintenance of steady state function of some ATPases (like Na^+/K^+ ATPase, myosin-ATPase). However, the value required for the maintenance of function of the Ca^{2+}-ATPase of the sarcoplasmic reticulum is higher and close to 52 kJ mol^{-1} (dashed lines indicate high and low free energy levels required by the respective ATPases).[73]

reducing the risk of severe intracellular acidification. A (moderate) acidosis or a shift of set points to lower pH values increases the fraction of undissociated organic acids and may, thus, emphasize their functional role in nonionic acid-base regulation under these conditions.

VI. PERSPECTIVES: CHANGES IN pH_i AND ENERGY STATUS

Other than balancing protons in metabolism and acid-base status a more direct way can be selected to depict the interrelations between metabolism and pH_i in the intact organism. This is now possible even during muscular exercise *in vivo* since pH_i and metabolic status can be very accurately compared in each individual tissue sample by using the above-mentioned homogenate technique. For example, in exhausted and recovering squid (*Illex illecebrosus*) and in fatigued *Spinculus nudus* (this animal uses its introvert to dig and move on into the sand[56]), we found a linear relationship between pH_i and glycolytic end product (octopine) levels emphasizing the important role of anaerobic glycolysis in provoking pH_i changes.[16,68] This correlation between metabolic proton formation and the degree of acidosis leads to a strong impact on the cellular energy status. The goal of anaerobic metabolism is to provide ATP to an extent sufficient to maintain cellular functions, but the free energy of ATP is affected by the metabolic acidification (Figure 9). In the range of high cellular pH, the free energy change of ATP hydrolysis decreases rapidly with falling pH. With constant levels of the adenylates this decrease does, however, not continue at the same rate when

pH continues to fall but rather reaches a minimum value. An additional drop is only caused by an imbalance between ATP consumption and catabolic ATP regeneration as indicated by the depletion of the phosphagen and/or of ATP. Accordingly, hypoxia or fatiguing exercise may elicit a drop in ATP and an increase in free inorganic phosphate and ADP levels and, thus, a drop in the free energy change. The general and still open question arises whether the free energy change falls below a critical level required for the maintenance of cellular functions. Figure 9 would indicate that one of the first ATPases to be affected is the sarcoplasmic Ca^{2+}-ATPase in muscle tissues. Further investigations are required to elucidate these relationships.

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REFERENCES

1. **Hochachka, P.W. and Mommsen, T.P.,** Protons and anaerobiosis, *Science*, 219, 1391, 1983.
2. **Pörtner, H.O.,** Contributions of anaerobic metabolism to pH regulation in animal tissues: theory, *J. Exp. Biol.*, 131, 69, 1987.
3. **Krebs, H.A., Woods, H.F., and Alberti, K.G.M.M.,** Hyperlactataemia and lactic acidosis, *Essays Med. Biochem.*, 1, 81, 1975.
4. **Gevers, W.,** Generation of protons by metabolic processes in heart cells, *J. Mol. Cell. Cardiol.*, 9, 867, 1977.
5. **Atkinson, D.E. and Camien, M.N.,** The role of urea synthesis in the removal of metabolic bicarbonate and the regulation of blood pH, *Curr. Top. Cell. Regul.*, 21, 261, 1982.
6. **Guder, W.G., Häussinger, D., and Gerok, W.,** Renal and hepatic nitrogen metabolism in systemic acid-base regulation, *J. Clin. Chem. Clin. Biochem.*, 25, 457, 1987.
7. **Pörtner, H.O.,** The importance of metabolism in acid-base regulation and acid-base methodology, *Can. J. Zool.*, 67, 3005, 1989.
8. **Wiseman, R.W., Ellington, W.R., and Rosanske, R.C.,** Effects of extracellular pH and D-lactate efflux on regulation of intracellular pH during isotonic contractions in a molluscan muscle: a ^{31}P-nuclear magnetic resonance study, *J. Exp. Zool.*, 252, 228, 1989.
9. **Pörtner, H.O.,** Anaerobic metabolism and changes in acid-base status: quantitative interrelationships and pH regulation in the marine worm Sipunculus nudus, *J. Exp. Biol.*, 131, 89, 1987.
10. **Heisler, N.,** Parameters and methods in acid-base physiology, in *Techniques in Comparative Respiratory Physiology*, Bridges, C.R. and Butler, P.J., Eds., Cambridge University Press, Cambridge, 1989, 305.
11. **Waddell, W.J. and Butler, T.C.,** Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog, *J. Clin Invest.*, 38, 720, 1959.
12. **Heisler, N., Weitz, H., and Weitz, A.M.,** Extracellular and intracellular pH with changes of temperature in the dogfish, Scyliorhinus stellaris, *Respir. Physiol.*, 26, 249, 1976.
13. **Pörtner, H.O., Boutilier, R.G., Tang, Y., and Toews, D.P.,** Determination of intracellular pH and P_{CO_2}: after metabolic inhibition by fluoride and nitrilotriacetic acid, *Respir. Physiol.*, 81, 255, 1990.
14. **Pörtner, H.O., MacLatchy, L.M., and Toews, D.P.,** Acid-base regulation in the toad Bufo marinus during environmental hypoxia, *Respir. Physiol.*, 85, 217, 1991.
15. **Pörtner, H.O. and Heisler, N.,** in preparation.
16. **Pörtner, H.O., Webber, D.M., Boutilier, R.G., and O'Dor, R.K.,** Acid-base regulation in exercising squid (Illex illecebrosus, Loligo pealei), *Am. J. Physiol.*, 261, R239, 1991.
17. **Tang, Y. and Boutilier, R.G.,** 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, 1991.
18. **Wiseman, R.W. and Ellington, W.R.,** Intracellular buffering capacity in molluscan muscle: superfused muscle versus homogenates, *Physiol. Zool.*, 62, 541, 1989.

19. **Heisler, N. and Piiper, J.,** The buffer value of rat diaphragm muscle determined by P_{CO_2} equilibration of homogenates, *Respir. Physiol.*, 12, 169, 1971.
20. **Pörtner, H.O.,** Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid, *Respir. Physiol.*, 81, 275, 1990.
21. **Roos, A. and Boron, W.F.,** Intracellular pH, *Physiol. Rev.*, 61, 296, 1981.
22. **Gadian, D.G., Radda, G.K., Dawson, M.J., and Wilkie, D.R.,** pHi measurements of cardiac and skeletal muscle using ^{31}P-NMR, in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions,* Alan R. Liss, New York, 1982, 61.
23. **Pörtner, H.O., Kreutzer, U., Siegmund, B., Heisler, N., and Grieshaber, M.K.,** Metabolic adaptation of the intertidal worm Sipunculus nudus to functional and environmental hypoxia, *Mar. Biol.*, 79, 237, 1984.
24. **Pörtner, H.O., Grieshaber, M.K., and Heisler, N.,** Anaerobiosis and acid-base status in marine invertebrates: effect of environmental hypoxia on extracellular and intracellular pH in Sipunculus nudus L., *J. Comp. Physiol.*, 155B, 13, 1984.
25. **Ultsch, G.R. and Jackson, D.C.,** Long term submergence at 3°C of the turtle, Chrysemys picta bellii, in normoxic and severely hypoxic water, *J. Exp. Biol.*, 96, 11, 1982.
26. **Jackson, D.C. and Heisler, N.,** Plasma ion balance of submerged anoxic turtles at 3° C: the role of calcium lactate formation, *Respir. Physiol.*, 49, 159, 1982.
27. **van den Thillart, G. and van Waarde, A.,** pH changes in fish during environmental anoxia and recovery: the advantages of the ethanol pathway, in *Physiological Strategies for Gas Exchange and Metabolism,* Woakes, A.J., Grieshaber, M.K., and Bridges, C.R., Eds., Cambridge University Press, Cambridge, 1991, 173.
28. **Graham, R.A. and Ellington, W.R.,** Phosphorus nuclear magnetic resonance studies of energy metabolism in molluscan tissues: intracellular pH change and the qualitative nature of anaerobic end products, *Physiol. Zool.*, 58, 478, 1985.
29. **Gnaiger, E., Shick, J.M., and Widdows, J.,** Metabolic microcalorimetry and respirometry of aquatic animals, in *Techniques in Comparative Respiratory Physiology. An Experimental Approach,* Bridges, C.R. and Butler, P.J., Eds., Cambridge University Press, Cambridge, 1989, 113.
30. **Hardewig, I., Addink, A.D.F., Grieshaber, M.K., Pörtner, H.O., and van den Thillart, G.,** Metabolic rates at different oxygen levels determined by direct and indirect calorimetry in the oxyconformer Sipunculus nudus, *J. Exp. Biol.*, 157, 143, 1991.
31. **Pörtner, H.O., Vogeler, S., and Grieshaber, M.K.,** Recovery from anaerobiosis in the intertidal worm Sipunculus nudus. II. Gas exchange and changes in the intra- and extracellular acid-base status, *J. Exp. Biol.*, 122, 51, 1986.
32. **Pörtner, H.O., Andersen, N.A., and Heisler, N.,** Proton equivalent ion transfer in Sipunculus nudus as a function of ambient oxygen tension: relationships with energy metabolism, *J. Exp. Biol.*, 156, 21, 1991.
33. **Pörtner, H.O., Vogeler, S., and Grieshaber, M.K.,** Recovery from anaerobiosis in the intertidal worm Sipunculus nudus. I. Restoration of aerobic, steady state energy metabolism, *J. Exp. Biol.*, 122, 37, 1986.
34. **Booth, C.E., McDonald, D.G., and Walsh, P.J.,** Acid-base balance in the sea mussel, Mytilus edulis. I. Effects of hypoxia and air-exposure on hemolymph acid-base status, *Mar. Biol. Lett.*, 5, 347, 1984.
35. **Walsh, P.J., McDonald, D.G., and Booth, C.E.,** Acid-base balance in the sea mussel, Mytilus edulis. II. Effects of hypoxia and air-exposure on intracellular acid-base status, *Mar. Biol. Lett.*, 5, 359, 1984.
36. **Busa, W.B. and Nuccitelli, R.,** Metabolic regulation via intracellular pH, *Am. J. Physiol.*, 246, R409, 1984.
37. **Juretschke, H.P. and Kamp, G.,** Influence of intracellular pH on reduction of energy metabolism during hypoxia in the lugworm Arenicola marina, *J. Exp. Zool.*, 256, 255, 1990.
38. **Heisler, N.,** Acid-base regulation in fishes, in *Acid-Base Regulation in Animals,* Heisler, N., Ed., Elsevier Science, Amsterdam, 1986, 309.
39. **Heisler, N.,** Limiting factors for transient pH regulation in muscle tissues: perfusion and buffering characteristics, *Verh. Dtsch. Zool. Ges.*, 82, 165, 1989.
40. **Wood, C.M.,** Acid-base and ion balance, metabolism, and their interactions after exhaustive exercise in fish, *J. Exp. Biol.*, 160, 285, 1991.
41. **Cameron, J.N. and Cech, J.J., Jr.,** Lactate kinetics in exercised channel catfish, Ictalurus punctatus, *Physiol. Zool.*, 63, 909, 1991.
42. **Seo, Y.,** Effects of extracellular pH on lactate efflux from frog sartorius muscle, *Am. J. Physiol.*, 247, C175, 1984.
43. **Mason, M.J. and Thomas, R.C.,** A microelectrode study of the mechanisms of L-lactate entry into and release from frog sartorius muscle, *J. Physiol.*, 400, 459, 1988.
44. **Roos, A.,** Intracellular pH and distribution of weak acids across cell membranes. A study of D- and L-lactate and of DMO in rat diaphragm, *J. Physiol.*, 249, 1, 1975.
45. **Jackson, D.C. and Heisler, N.,** Intracellular and extracellular acid-base and electrolyte status of submerged anoxic turtles at 3° C, *Respir. Physiol.*, 53, 187, 1983.

46. **Dubinsky, W.P. and Racker, E.,** The mechanism of lactate transport in human erythrocytes, *J. Membr. Biol.*, 44, 25, 1978.
47. **Fafournoux, P., Demigne, C., and Remesy, C.,** Carrier mediated uptake of lactate in rat hepatocytes, *J. Biol. Chem.*, 260, 292, 1985.
48. **de Hemptinne, A., Marannes, R., and Vanheel, B.,** Influence of organic acids on intracellular pH, *Am. J. Physiol.*, 245, C178, 1983.
49. **Roth, D.A. and Brooks, G.A.,** Lactate and pyruvate transport is dominated by a pH gradient-sensitive carrier in rat skeletal muscle sarcolemmal vesicles, *Arch. Biochem. Biophys.*, 279, 386, 1990.
50. **Walsh, P.J.,** Lactate uptake by toadfish hepatocytes: passive diffusion is sufficient, *J. Exp. Biol.*, 130, 295, 1987.
51. **Kinne, R.,** Transport function of renal cell membranes: sodium cotransport systems, in *Renal Biochemistry*, Kinne, R.K.H., Ed., Elsevier Science, Amsterdam, 1985, 99.
52. **Steinhagen, C., Hirche, H.J., Nestle, H.W., Bovenkamp, U., and Hosselmann, I.,** The interstitial pH of the working gastrocnemius muscle of the dog, *Pflügers Arch.*, 367, 151, 1976.
53. **de Hemptinne, A. and Huguenin, F.,** The influence of muscle respiration and glycolysis on surface and intracellular pH in fibres of the rat soleus, *J. Physiol.*, 347, 581, 1984.
54. **Wiseman, R.W. and Ellington, W.R.,** Energetics of contractile activity in isolated radula protractor muscles of the whelk Busycon contrarium: anaerobic end product accumulation and release, *Biol. Bull.*, 173, 277, 1987.
55. **Holst, H. and Zebe, E.,** Volatile fatty acid excretion during anaerobiosis in the lugworm Arenicola marina, *Comp. Biochem. Physiol.*, 83A, 189, 1986.
56. **Trueman, E.R. and Foster-Smith, R.L.,** The mechanism of burrowing of Sipunculus nudus, *J. Zool.*, 179, 372, 1976.
57. **Wieser, W.,** Responses of Helix pomatia to anoxia: changes of solute activity and other properties of the haemolymph, *J. Comp. Physiol.*, 141, 503, 1981.
58. **Hildebrandt, J.-P.,** External CO_2 levels influence energy yielding metabolic pathways under hypoxia in the leech, Hirudo medicinalis, *J. Exp. Zool.*, in press.
59. **Gäde, G.,** Effects of oxygen deprivation during anoxia and muscular work on the energy metabolism of the crayfish, Orconectes limosus, *Comp. Biochem. Physiol.*, 77, 495, 1984.
60. **Albert, J.L. and Ellington, W.R.,** Patterns of energy metabolism in the stone crab, Menippe mercenaria, during severe hypoxia and subsequent recovery, *J. Exp. Zool.*, 234, 175, 1985.
61. **Jorgensen, J.B. and Mustafa, T.,** The effect of hypoxia on carbohydrate metabolism in flounder (Platichthys flesus L.). I. Utilization of glycogen and accumulation of glycolytic end products in various tissues, *Comp. Biochem. Physiol.*, 67B, 243, 1980.
62. **van Waarde, A., van den Thillart, G., and Kesbeke, F.,** Anaerobic energy metabolism of the European eel, Anguilla anguilla, *J. Comp. Physiol.*, 149, 469, 1983.
63. **Kamp, G. and Juretschke, H.P.,** Hypercapnic and hypocapnic hypoxia in the lugworm Arenicola marina: A ^{31}P NMR study, *J. Exp. Zool.*, 252, 219, 1989.
64. **Reitze, M.,** Mechanismen der Osmo- und Volumenregulation: Untersuchungen am Wattwurm Arenicola marina L. (Annelida, Polychaeta), Dissertation, Westfälisch Wilhelms-Universität, Münster, 1987.
65. **Schöttler, U., Wienhausen, G., and Westerman, J.,** Anaerobic metabolism in the lugworm Arenicola marina L.: the transition from aerobic to anaerobic metabolism, *Comp. Biochem. Physiol.*, 79B, 93, 1984.
66. **Toulmond, A.,** Tide-related changes of blood respiratory variables in the lugworm, Arenicola marina (L.), *Respir. Physiol.*, 19, 130, 1973.
67. **Grieshaber, M.K., Kreutzer, U., and Pörtner, H.O.,** Critical P_{O_2} of euryoxic animals, in *Oxygen Sensing in Tissues*, Acker, H., Ed., Springer-Verlag, Berlin, 1988, 37.
68. **Pörtner, H.O.,** unpublished results.
69. **Guynn, R.W. and Veech, R.L.,** The equilibrium constants of the adenosine triphosphate hydrolysis and the adenosine triphosphate-citrate lyase reactions, *J. Biol. Chem.*, 248, 6966, 1973.
70. **Alberty, R.A.,** Calculation of the standard Gibbs free energy, enthalpy and entropy changes for the hydrolysis of ATP at 0°, 25°, 37° and 75°, in *Horizons of Bioenergetics*, San Pietro, A., Ed., Howard Gest, New York, 1972, 135.
71. **Gupta, R.K., Gupta, P., Yushok, W.D., and Rose, Z.B.,** Measurement of the dissociation constant of MgATP at physiological nucleotide levels by a combination of ^{31}P-NMR and optical absorbance spectroscopy, *Biochem. Biophys. Res. Comm.*, 117, 210, 1983.
72. **Ellington, W.R.,** Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens, *J. Exp. Biol.*, 143, 177, 1989.
73. **Kammermeier, H.,** High energy phosphate of the myocardium: concentration versus free energy change, *Basic Res. Cardiol.*, 82 (Suppl. 2), 31, 1987.