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Anaerobiosis and acid-base status in marine invertebrates: effect of environmental hypoxia on extracellular and intracellular pH in *Sipunculus nudus* L.

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Summary. Intra- and extracellular acid-base status was investigated during prolonged experimental anaerobiosis in *Sipunculus nudus* L. An acidosis could not be observed during the first 6–12 h of anaerobiosis, in contrast, a slight alkalosis developed in both extra- and intracellular body compartments. Extra- and intracellular pH only started to decrease gradually after 12 h of environmental hypoxia as an expression of a non-compensated non-respiratory acidosis.

The initial alkalosis associated with a positive base excess is interpreted as being due to the concomitant degradation of phospho-L-arginine (Pörtner et al. 1984a). The amount of succinate, propionate, and acetate accumulated in the extracellular fluid (coelomic plasma) could not be correlated quantitatively with a concomitant negative base excess. This discrepancy suggests that protons and anionic metabolites are distributed between various body compartments according to different equilibria and kinetics.

Comparison of the changes in the acid-base status with the concentration changes of characteristic anaerobic metabolites (Pörtner et al. 1984a) indicates that (at least in *Sipunculus nudus*) pH_i is not the crucial factor initiating the observed shift of the metabolite flux from the Embden-Meyerhof-pathway towards the succinate-propionate pathway.

Introduction

Many invertebrate species of the intertidal zone are well adapted to survive regular periods of hypoxia or even anoxia during tidal cycles. Lack of ambient oxygen is compensated for by anaerobic metabolism characterized, (1) by partial degradation of a phosphagen, (2) by the accumulation of lactate, and/or opines during the beginning of hypoxia, and (3) by the formation of succinate, propionate, and acetate (Zebe 1977; de Zwaan 1977; Schöttler 1980; Zandee et al. 1980). Marine invertebrates may also use aspartate besides glycogen as an additional substrate, in particular during early anaerobiosis (Felbeck and Grieshaber 1980; Felbeck 1980; Schöttler 1980; Zandee et al. 1980). The amino group of aspartate is transferred to pyruvate resulting in the formation of alanine, and the carbon skeleton is channelled into succinate (Zebe 1975). During prolonged anoxia energy is derived from break-down of glycogen only, which is metabolized via the succinate-propionate pathway.

Formation of succinate from glycogen requires the carboxylation of a three-carbon molecule, that is of either phosphoenolpyruvate or pyruvate, originating from the reaction sequence of the Embden-Meyerhof pathway. On the basis of in vitro investigations, Hochachka and Mustafa (1972) and de Zwaan and van Marrewijk (1973a) proposed that phosphoenolpyruvate is carboxylated to oxaloacetate which is converted via malate and fumarate into succinate (Fig. 1). A possible involvement of phosphoenolpyruvate carboxykinase in anaerobic carboxylation was deduced by Schöttler and Wienhausen (1981) and de Zwaan et al. (1983) who demonstrated a lower rate of succinate formation after inhibition of phosphoenolpyruvate carboxykinase with mercapto-picolinic acid in *Arenicola marina* L., *Nereis virens* L., and *Mytilus edulis* L.

The main hypothesis concerning the regulatory shift of metabolism towards the succinate-propionate pathway is based on the in vitro characteristics of the enzymes phosphoenolpyruvate carboxy-

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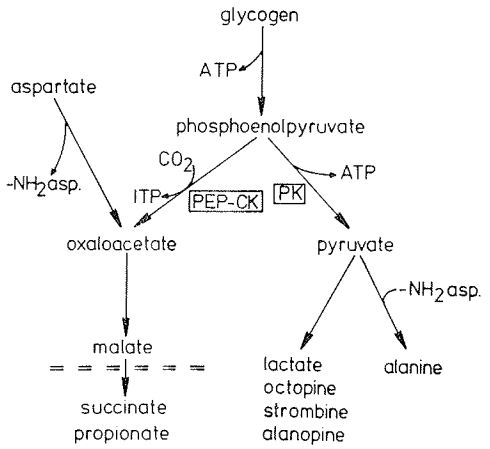

Fig. 1. Simplified scheme of the anaerobic metabolism of marine annelids and molluscs. A pivotal role is assigned to the phosphoenolpyruvate branchpoint where the regulatory shift of the metabolite flux should occur (see text, PEP-CK: phosphoenolpyruvate carboxykinase; PK: pyruvate kinase)

kinase and pyruvate kinase, which compete for phosphoenolpyruvate as a substrate. The in vitro pH-optimum of phosphoenolpyruvate carboxykinase isolated from *Crassostrea gigas* (Hochachka and Mustafa 1972) and *Mytilus edulis* (Livingstone and Bayne 1974; de Zwaan and de Bont 1975) is fairly acidic (pH 5–6.7) whereas the optimum for pyruvate kinase is positioned in the more alkaline range (pH 7.5 to 8.5). An accumulation of acidic metabolites or of CO_2 during early anaerobiosis would be expected to result in a drop of intracellular pH and, thereby, favour formation of oxaloacetate above synthesis of pyruvate (Hochachka and Mustafa 1972; Livingstone 1982).

Direct support for this hypothesis, however, can only be provided by measurements of intracellular pH (pH_i) after various periods of anaerobiosis. The present study was designed to investigate changes in the acid-base status of *Sipunculus nudus* during lack of ambient oxygen. *Sipunculus nudus* is especially suited for these experiments because of its morphological simplicity and its resemblance to other intertidal infauna species (e.g. *Arenicola marina*) in terms of anaerobic energy metabolism (Pörtner et al. 1984a).

Materials and methods

Animals

Specimens of *Sipunculus nudus* were collected from the intertidal flats of Brittany, France. Large animals (25 to 35 g) were dug out near Loquémeau, small specimens (5 to 12 g) near Morgat. The animals were kept for several weeks in an aquarium containing a bottom layer of sand (10–20 cm) from the worm's original habitat. The tanks were circulated with well aerated artificial seawater with a temperature of 15 ± 1 °C.

Experimental procedure

Prior to the experiments the animals were catheterized by introducing a PE 60 tubing (total length approx. 75 cm) into the body cavity at the posterior pole of the body. The tubing was secured by Bostik Nr. 7432 cyanoacrylate glue (Bostik GmbH, Oberursel, Federal Republic of Germany). Samples of coelomic fluid were withdrawn from animals fitted with indwelling catheters. After the implantation procedure the animals were adapted to the experimental conditions for 24 h in a darkened tank containing about 14 l of aerated artificial sea water thermostatted to 15 °C. Hypoxic conditions ($P_{O_2} < 3$ Torr) were induced by continuous bubbling of pure nitrogen through the water. These conditions are similar to those applied in previous studies (Schöttler 1979; Schöttler and Wienhausen 1981; de Zwaan and van Marrewijk 1973b; Kluytmans et al. 1978; Pörtner et al. 1984a). Handling of the animals, which was found to affect coelomic pH, was absolutely avoided throughout the adaptation and incubation periods. After various periods of anaerobiosis intracellular pH (pH_i), and pH (pH_e), P_{CO_2}, P_{O_2}, and metabolite concentrations in coelomic fluid samples were determined.

Determination of pH, P_{CO_2} and P_{O_2}

Coelomic fluid (0,3–0,6 ml) was sampled anaerobically via the indwelling catheter during the normoxic control period and after 2, 6, 12 and 24 h of hypoxia. Samples were analyzed for pH_e, P_{CO_2}, and P_{O_2} using a thermostatted (15 ± 0.1 °C) microelectrode assembly (BMS 3, Radiometer, Copenhagen). The electrodes were calibrated with precision phosphate buffers (Radiometer, Copenhagen) or humidified gas mixtures of N_2, CO_2, and O_2, provided by gas mixing pumps (Type M 303/a-F; Wösthoff, Bochum, FRG).

Intracellular pH was determined in body wall musculature and introvert retractors of small animals by application of the DMO-distribution method (Waddell and Butler 1959). Three hours prior to the end of the incubation, the animals were injected with 0.05 µCi ^{14}C-DMO (5,5-dimethyloxazolidine-2,4-dione) and 0.1 µCi ^{3}H-inulin (as marker for the extracellular space) via the indwelling catheter. Both compounds were dissolved in seawater and applied in a total volume of 0.1 ml. After 2, (6), 12 and 24 h of hypoxia the animals were sacrificed and tissues separated from coelomic fluid which was immediately centrifuged. The plasma was decanted and stored in sealed caps at 4 °C. Six random samples of the body wall and two samples of the introvert retractors of each individual specimen were excised, blotted dry, placed on filter paper and dried for 24 h at 110 °C. Tissue water content was determined from the weight difference before and after drying.

Plasma samples which were absorbed by filter paper pills, and dried muscle tissue samples pressed into filter paper pills were oxidized in an automatic sample oxidizer (Packard, model 306) for subsequent analysis of ^{14}C- and ^{3}H-activity by liquid scintillation counting. Details of the procedure and calculations have been described previously (Heisler 1975; Heisler et al. 1976). The values of the water content and of the fractional extracellular space of the tissues are summarized in Table 1.

Analysis of metabolites

Succinate, propionate, and acetate concentrations were determined in coelomic plasma obtained by coelomic fluid centrifugation. The plasma was deproteinated by addition of a tenth

Table 1. Fractional water content and extracellular space Q in body wall musculature and introvert retractor muscles of *Sipunculus nudus* under normoxia (air) and after 24 h of anaerobiosis (N_2), the animals lying in artificial seawater ($\bar{x} \pm SD$, $n = 5$)

Tissue	Incubation	Q	Water content
Body wall	air	0.273 ±0.058	
			0.792 ±0.018
	N_2	0.256 ±0.046	
Introvert retractors	air	0.271 ±0.093	
			0.820 ±0.020
	N_2	0.248 ±0.072	

of its volume of 6 N perchloric acid and was centrifuged and neutralized with 5 M KOH. Succinate was analyzed according to Michal et al. (1976). Acetate and propionate concentrations were determined by high pressure liquid chromatography after steam destillation (Pörtner et al. 1984a).

Determination of buffer values

Non-bicarbonate buffer values $\left(\beta_{NB} = -\frac{\Delta[HCO_3^-]}{\Delta\ pH}\right)$ of muscle homogenates and body fluid samples were determined by CO_2 equilibration (Heisler and Piiper 1971; Heisler and Neumann 1980) in an intermittently rotating cuvette (tonometer model 273, Instrumentation Laboratory, Padorno Dugano, Italy) which was flushed with humidified gas mixtures provided by gas mixing pumps (Wösthoff, Bochum, FRG) at 15 °C.

Coelomic fluid with an average haematocrit of 7% was pooled from 5 animals. Muscle tissue homogenates were prepared after powdering the muscles with mortar and pestle under liquid nitrogen. The powder was suspended in an isotonic medium containing (in meq l^{-1}): K^+, 53; Ca^{2+}, 10.8; Na^+, 519.6; SO_4^{2-}, 32.9; Cl^-, 597.9; $H_2PO_4^-$, 1.2; HCO_3^-, 2.3. The bicarbonate concentration was adjusted to the values calculated from pH_i and P_{CO_2} found in vivo during normoxia. Other components of the medium were adopted from determinations of the muscle fluid composition of *Sipunculus natans* and *Sipunculus multisulcatus* (De Jorge et al. 1970). The mixture of medium and muscle powder was homogenized for 3×30 s by means of an Ultra-Turrax homogenizer (Janke und Kunkel, Staufen, Federal Republic of Germany).

Coelomic fluid was equilibrated for 20 min at each gas mixture with P_{CO_2}'s in the range from 0.7 to 11 Torr (P_{O_2} similar as in vivo: 22 Torr). The muscle homogenate was equilibrated for 30 min with mixtures containing CO_2 in the range from $P_{CO_2} = 0.3$ to 3 Torr ($P_{O_2} \sim 700$ Torr). The sequence of equilibration for tissue homogenates was: medium CO_2, high (low) CO_2, medium CO_2, low (high) CO_2 and medium CO_2 (for details of the methodology see Heisler and Piiper 1971; Heisler and Neumann 1980). Total CO_2 content of samples taken after equilibration was measured according to Cameron (1971) using standard solutions containing 5 or 10 mM $NaHCO_3$.

The average apparent pK''' values of the H_2CO_3/HCO_3^- buffer system applicable to the present method of pH determination were calculated for coelomic fluid and tissues by application of the Henderson-Hasselbalch equation and a solubility coefficient for CO_2 (α) of 0.049 µmol ml^{-1} $Torr^{-1}$ (cf. Harvey 1974; α was corrected for the volume of the non-water components of tissues and coelomic cells). This coefficient was chosen because of the isoosmolarity of sipunculid body fluids with sea water (Oglesby 1969, 1982). Measurements of the Cl^- contents of coelomic fluid and of sea water (Radiometer CMT 10) also yielded similar values. The resulting pK''' values were utilized to construct pH-bicarbonate diagrams and to calculate intra- and extracellular bicarbonate concentrations from measured pH and P_{CO_2} by application of the Henderson-Hasselbalch equation. The buffer values of coelomic fluid and muscle homogenates were determined by linear regression analysis. For the muscle homogenates the buffer value of the inorganic phosphate which had been added to the medium was taken into account (see Heisler and Piiper 1971; pK'_2 of phosphate derived from Netter 1959; Barrow et al. 1980). The buffer value of the intracellular fluid was determined from the homogenate buffer value, the fractional water content and the fractional extracellular space (for details see Heisler and Piiper 1971; Heisler and Neumann 1980).

Quantitative analysis of changes in the acid-base status

Changes in the acid-base status were analyzed quantitatively on the basis of the in vitro non-bicarbonate buffer values. The amount of protons which caused the observed non-respiratory[1] alterations was calculated as follows (cf. Heisler 1975):

$$\Delta H^+_{\text{non-resp.}} = -\beta_{NB} \times \Delta pH - \Delta HCO_3^- \ (\text{mmol } l^{-1}).$$

The contribution of respiratory processes to the changes in the acid-base status can be estimated graphically from the intersection of the respective CO_2 isobar and the buffer line (cf. Heisler 1975). The quantity of protons bound by non-bicarbonate buffers was calculated as:

$$\Delta H^+_{\text{resp.}} = -\beta_{NB} \times \Delta pH_{\text{resp.}} \ (\text{mmol } l^{-1}).$$

Results

During the first 12 h of experimental anaerobiosis, no acidosis occurred in small specimens of *Sipunculus nudus* (Fig. 2). In contrast, a slight extracellular and also intracellular (in the body wall musculature) alkalosis was observed during the first hours of hypoxia. Control values were not restored until after 12 h. During 24 h of anaerobiosis both intra- and extracellular pH were reduced as expression of a non-compensated acidosis (Fig. 2). By then pH dropped in both compartments by about 0.3 to 0.4 pH-units compared to control values (7.32 ± 0.14 to 7.02 ± 0.12 in the body wall; 7.40 ± 0.11 to 7.09 ± 0.16 in the introvert retractors; 8.09 ± 0.15 to 7.71 ± 0.09 in the coelomic fluid). This pH reduction was accompanied by a decrease of intra- and extracellular bicarbonate concentrations. P_{CO_2} was reduced during the first 12 h of anaerobiosis but was restored to control values after 24 h.

1 The term 'non-respiratory' includes changes in metabolite concentrations and transmembrane ion transfer processes

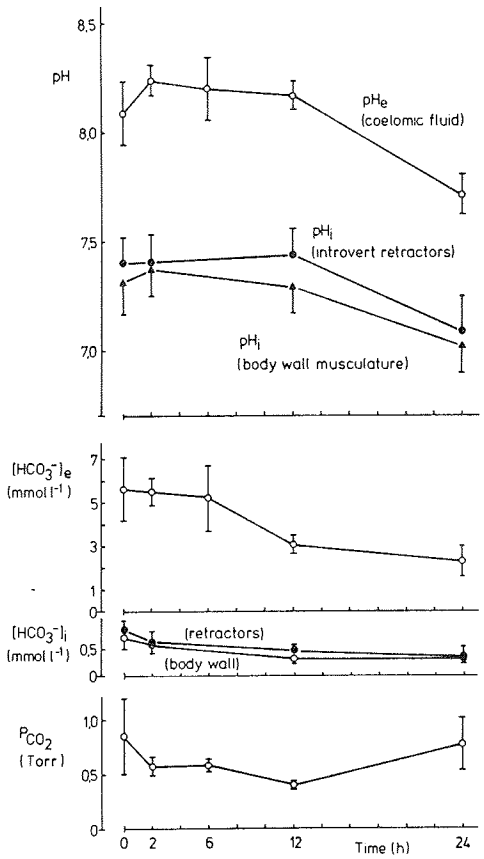

Fig. 2. Extra- and intracellular pH, bicarbonate concentrations and P_{CO_2} during 24 h of experimental anaerobiosis in small specimens of *Sipunculus nudus* ($\bar{x} \pm SD$, $n = 5$)

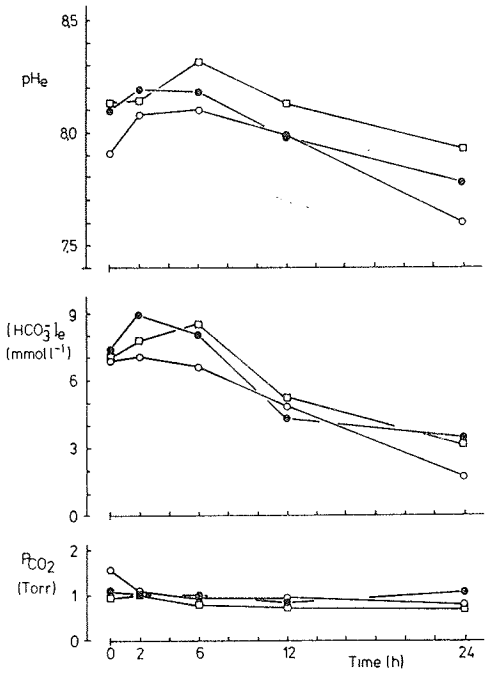

Fig. 3. Coelomic pH (pH_e), bicarbonate concentration and P_{CO_2} after different periods of experimental anaerobiosis, monitored in 3 large *Sipunculus nudus* to demonstrate the reproducibility of the pattern of changes in the acid-base status

It is obvious that under these conditions both intra- and extracellular pH are similarly affected by hypoxia. In order to verify the results obtained by using small animals and to exclude the effect of non-comparable animal groups, the influence of anaerobiosis on extracellular pH was monitored individually in three large specimens of *Sipunculus nudus* during the whole period of 24 h of hypoxia (Fig. 3), which is impossible in small specimens because of the limited volume of coelomic fluid.

The pattern of results was essentially the same as in the small animals; pH increased during the first 6 h of anaerobiosis and was more or less restored to control values after 12 h. Bicarbonate concentration rose during the first 6 h in two of the three specimens whereas in the third P_{CO_2} dropped instead. After 24 h the bicarbonate content was considerably reduced as compared to the controls (Fig. 3). The pH had decreased in the coelomic fluid to the same extent as described above for small specimens (8.04 ± 0.12 to 7.76 ± 0.17, cf. Fig. 2).

Measurements of the oxygen partial pressure revealed that the coelomic fluid of the large animals investigated was almost totally depleted of oxygen 2 h after the onset of hypoxic exposure (Fig. 4). Anaerobiosis led to an accumulation of succinate, propionate, and acetate, in the coelomic plasma during the first 12 h of anaerobiosis. Later on the concentrations remained virtually constant. After 24 h of anaerobiosis succinate had been accumulated by 0.11 ± 0.02 µmol (to 0.16 ± 0.01 µmol), propionate by 0.74 ± 0.27 µmol (to 0.77 ± 0.28 µmol), and acetate by 0.26 ± 0.08 µmol (to 0.63 ± 0.14 µmol) ml^{-1} plasma.

Presentation of pH, P_{CO_2} and bicarbonate concentration in a pH-bicarbonate diagram allows to assess the non-respiratory or respiratory origin of the alterations. Therefore, non-bicarbonate buffer values were determined in coelomic fluid and muscle tissue. The buffer value β_{NB} of 'true' coelomic fluid was found to be 4.6 mmol $pH^{-1}\,l^{-1}$. The average apparent $pK'_1{}''$ of the CO_2/bicarbonate buffer system was 5.92 ± 0.04. For the body wall musculature β_{NB} was calculated as 20.2 ± 0.9 µmol $pH^{-1}\,g^{-1}$ fresh weight, corresponding to $\beta_{NB} = 25.5 \pm 1.1$ mmol $pH^{-1}\,l^{-1}$ muscle water and $\beta_{NB} = 35.1 \pm 1.5$ mmol $pH^{-1}\,l^{-1}$ cell water. The last value was applied for the graphic representation of the buffer line in Fig. 5. For the introvert retractor β_{NB} was evaluated as 32.9 ± 2.5 mmol $pH^{-1}\,l^{-1}$

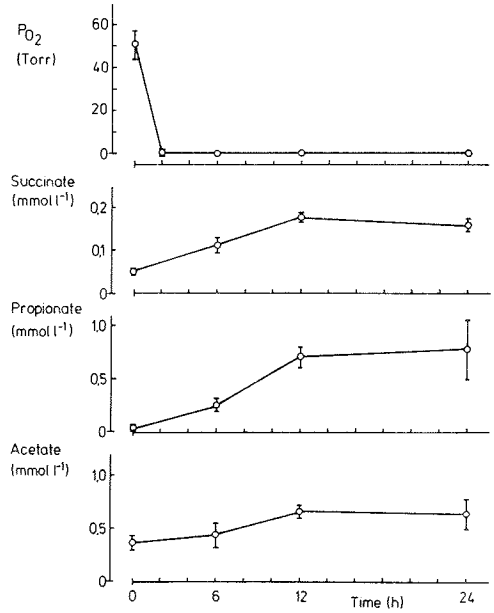

Fig. 4. Coelomic fluid P_{O_2} and the concentrations of succinate, propionate and acetate determined in the coelomic plasma of 3 large *Sipunculus nudus* after different periods of experimental anaerobiosis. The respective acid-base data are presented in Fig. 3

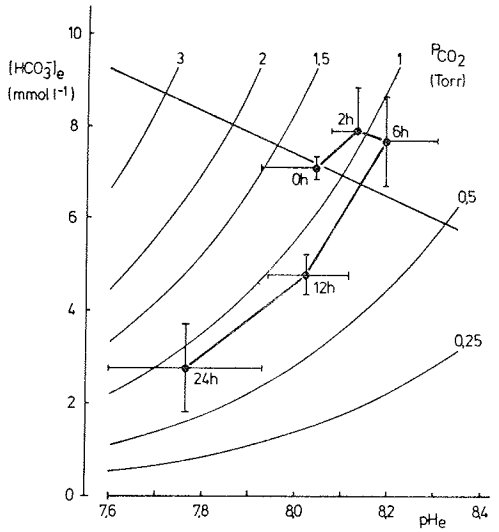

Fig. 5. Changes in the extracellular (coelomic-fluid) acid-base status during experimental anaerobiosis, presented in a pH-bicarbonate diagram with the buffer line as evaluated in vitro

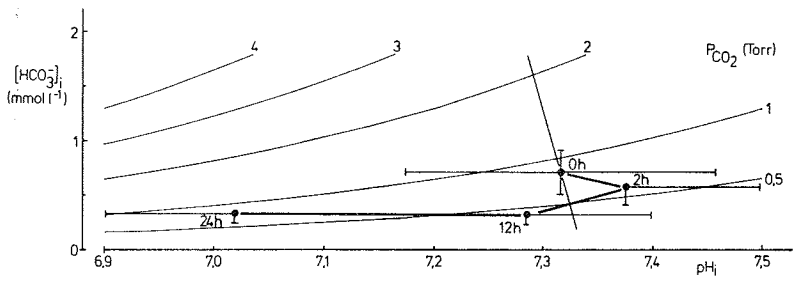

Fig. 6. Changes in the intracellular acid-base status of the body-wall musculature during experimental anaerobiosis, presented in a pH-bicarbonate diagram with the buffer-line drawn through the pH- and bicarbonate-value found at normoxia

cell water. The average apparent pK‴ of H_2CO_3 in muscle homogenates was 6.07 ± 0.02. It must be emphasized that the apparent pK_1''' values presented here have to be taken as experimental values which are likely different from the actual pK_1' in the body fluids of *Sipunculus nudus*.[2] Nevertheless they are the best possible approximation under the experimental conditions applied.

Plotting of the measured pH and P_{CO_2} values in a pH-bicarbonate diagram demonstrated that the initial alkalosis in both the intracellular as well as the extracellular compartment was mainly of non-respiratory origin with a much smaller respiratory component (Figs. 5 and 6). The initial alkalosis was compensated by H^+ ions of non-respiratory origin after 12 h and then turned into a severe non-respiratory acidosis after 24 h of anaerobiosis.

The changes in the extracellular acid-base status (coelomic fluid) were analyzed quantitatively in large animals (Fig. 7). Respiratory processes are of minor importance for the changes in the acid-base status, exhibiting a slightly positive base excess during initial anaerobiosis. Non-respiratory changes quantitatively reflect the qualitative descriptions given above (Figs. 5 and 6), a positive base excess during the first 6 h turning into a negative base excess during long term anaerobiosis. The accumulation of organic acid anions in the extra-

2 The relatively large difference between the apparent pK_1''' values in coelomic fluid and the homogenates is inconsistent with theoretical considerations (cf. Heisler 1984). It is mostly due to inherent properties of the used measurement devices and could, for instance, be caused by differing reactions of the pH-electrode in the respective media. The small scatter indicates that this unidirectional systematic error is rather constant and does accordingly not significantly affect the conclusions of the present paper. However, since this error may well affect significance of data calculated based on measurements with other electrodes and species, estimation of pK_1''' in each single species and set-up is, as always, strongly recommended (cf. Heisler 1984)

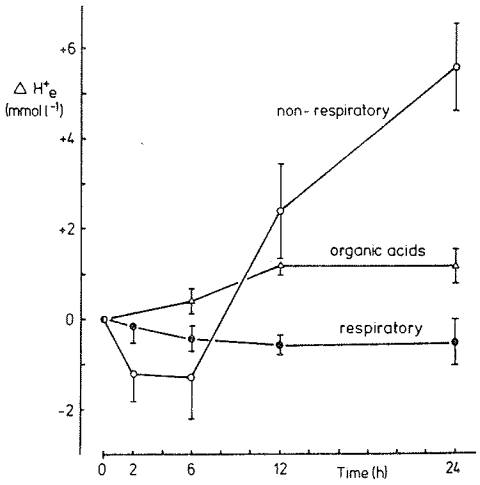

Fig. 7. Respiratory and non-respiratory changes in the extracellular acid-base status of *Sipunculus nudus* during experimental anaerobiosis compared to the amount of protons calculated from the stoichiometric dissociation of accumulated organic acids (succinic, propionic and acetic acid, cf. Fig. 4). The initial positive base excess followed by a negative base excess after 6 h of anaerobiosis is not reflected by concomitant changes in the concentration of organic acid anions

cellular compartment is not in kinetic and quantitative accordance with the amount of surplus non-respiratory H^+ ions (cf. Fig. 4, dissociation of 1 mol H^+ per mol propionate or acetate and 2 mol H^+ per mol succinate was assumed). The initial increase in organic acid anions is not accompanied by an appropriate negative base excess but after 6 h of anaerobiosis. Later on the amount of protons surpasses the amount of metabolites found, resulting in a considerable discrepancy of 4.4 mmol l^{-1} after 24 h of anaerobiosis.

Discussion

During experimental anaerobiosis intra- and extracellular acidosis does not occur in *Sipunculus nudus* but after 12 h of hypoxic exposure. Instead, an alkalosis was observed during the first 6 h of hypoxia. These results are rather unexpected with regard to the hypothesis of Hochachka and Mustafa (1972), de Zwaan (1977) and Livingstone (1982) assuming an acidosis during initial anaerobiosis which should be caused by accumulation of acidic metabolic end products or of CO_2. Theoretical analysis of the proton generation by anaerobic metabolism, however, reveals that during the initial stage of anaerobiosis (when aspartate is metabolized) anaerobic glycolysis with the formation of opines and alanine is the only process in energy metabolism which generates protons. Formation of succinate from aspartate causes no acidification, and production of propionate from the same substrate even results in proton consumption (Pörtner 1982; Pörtner et al. 1984b). Additionally a phosphagen is cleaved during the first hours of anaerobiosis (Schöttler 1980; Pörtner et al. 1984a) mopping up extra protons (Lipmann and Meyerhof 1930; Pörtner et al. 1984b), so that in contrast to the above hypothesis even a net consumption of protons may occur during initial anaerobiosis. Correspondingly, Figs. 5, 6 and 7 reveal that the observed alkalosis is partly non-respiratory, very likely due to consumption of protons by phosphagen degradation. A fraction of the respiratory component of the alkalosis may be caused by the experimental conditions, since P_{CO_2} in the ambient sea water was reduced from 0.2 Torr during the control period (gassing with air) to about 0 Torr during gassing with pure nitrogen.

In contrast to these results data obtained from isolated catch muscles of *Mytilus edulis* and *Geukensia demissa* have indicated an initial intracellular acidosis during the first hours of anaerobiosis (Ellington 1983). This, however, is not in contradiction to the above conclusion, since at least for *Mytilus edulis* it is well known, that the phospho-L-arginine content is much lower in the posterior adductor muscle (2.3 μmol g^{-1} fresh weight; Ebberink and de Zwaan 1980) than in the body wall musculature of *Sipunculus nudus* (34.5 μmol g^{-1} fresh weight; Pörtner et al. 1984a). Consequently the results of Ellington (1983) support the hypothesis, that the degradation of the phosphagen plays a pivotal role in intracellular pH regulation during the initial stages of anaerobiosis (Pörtner 1982; Pörtner et al. 1984b).

From the results of Barrow et al. (1980, Fig. 3), who measured pH_i in isolated anaerobic foot muscle of *Tapes watlingi*, a delayed decrease of pH_i after 6 to 10 h can be deduced. This fits well with a phospho-L-arginine content of 9.2 μmol g^{-1} fresh weight being hydrolyzed almost completely during the first 10 h of anaerobiosis. According to the results of Barrow et al. (1980) long term monitoring of pH_i in isolated organs requires that muscle deterioration should be considered. They found that the pH_i in isolated foot muscle of *Tapes watlingi* decreased anaerobically from 7.2 to 6.8 (20 h) but aerobically from 7.2 to 6.6 (26 h). In conclusion the observed changes under anaerobic conditions may not necessarily occur in the living animal as well and have therefore to be verified by measuring pH_i in intact specimens.

In intact *Sipunculus nudus* intracellular and extracellular pH were similarly affected by hypoxia indicating a close interrelation of acid-base regula-

tion in both compartments. If the observed changes in the acid-base status of the intact animal are mainly caused by anaerobic energy metabolism (a question which still remains to be answered) the changes of pH in the extracellular compartment (coelomic fluid) could be caused by the accumulation or consumption of organic acids. Quantitative analysis of the amount of H^+ ions and organic acid anions present in the extracellular compartment of *Sipunculus nudus*, however, reveals that the changes in the concentrations of succinate, propionate, and acetate, in the coelomic fluid are not correlated to the movement of protons. Protons therefore very likely move between intra- and extracellular spaces independently of the metabolite distribution. Some metabolites whose formation or consumption would affect acid-base balance (e.g. octopine, strombine, phospho-L-arginine, cf. Pörtner 1982; Pörtner et al. 1984b) are not released from the tissues at all (Pörtner et al. 1984a). Figure 7 strongly suggests that hydrolysis of phospho-L-arginine during initial anaerobiosis (Pörtner et al. 1984a) consumes protons not only from the intracellular compartment, but also from the coelomic fluid, and that during prolonged hypoxia protons are released to the extracellular compartment, the associated anions remaining within the tissues. Obviously the coelomic fluid which comprises 52% of the total animal weight (Pörtner 1982) is utilized to only a small extent as a sink for metabolites compared to its function as a sink for protons.

In *Sipunculus nudus* aspartate is also utilized as an additional substrate during early anaerobiosis before metabolism shifts to the carboxylation reaction. This shift, however, must be complete after about 6 h of anaerobiosis since aspartate is then nearly depleted. The changes of metabolite concentrations in the coelomic plasma previously observed in *Sipunculus nudus* (Pörtner et al. 1984a) are very similar to those reported by the present study (Fig. 4). Therefore, similar metabolic rates can be claimed for both experiments.

Alkalosis attains its maximum exactly when the shift of the metabolic flux is assumed to occur (cf. Fig. 3). This observation strongly suggests that in *Sipunculus nudus* the regulatory shift to the carboxylation reaction can operate without any acidic deflection of the intracellular pH. Vice versa the reduction of pH observed after 24 h has no decisive effect on metabolic regulation. Accordingly the regulatory shift appears to be independent of the intracellular pH regulation.

If in *Sipunculus nudus* a phosphoenolpyruvate branchpoint does exist, one has to turn to other mechanisms which could also be responsible for the shift of metabolic flux. Changing concentrations of ITP, GTP, ATP, alanine, phosphoenolpyruvate, and fructose-1,6-bisphosphate, have been discussed to be effective (Mustafa and Hochachka 1971, 1973; de Zwaan and Holwerda 1972; Livingstone and Bayne 1974; de Zwaan 1977). Recently phosphorylation and, thereby, inhibition of pyruvate kinase was postulated to be involved in the regulation of anaerobic metabolism (Siebenaller 1979; Holwerda et al. 1981, 1983).

Although intracellular pH has now been demonstrated not to be the crucial regulatory factor which initiates the carboxylation reaction in *Sipunculus nudus*, it might, however, support the shift to the succinate-propionate pathway if, under conditions others than reported here, an accumulation of CO_2 would cause a decrease of pH during initial anaerobiosis. Then a synergistic effect of pH cannot be excluded totally, but only if phosphoenolpyruvate carboxykinase is stimulated in vivo by low pH as has been demonstrated in vitro (Holwerda et al. 1981; de Zwaan and de Bont 1975). In the natural habitat *Sipunculus nudus* as well as some other facultative anaerobes experience hypoxia because of obstructed respiratory gas exchange. Accordingly during simulation of low tide, an accumulation of CO_2 could be demonstrated in *Sipunculus nudus* eliciting an extracellular acidosis within 1 to 3 h, pH could contribute to bring about an early shift of the metabolite flux to the succinate-propionate pathway if an intracellular acidosis should occur (Pörtner, Heisler and Grieshaber, unpubl.), but, as shown by the present data, it is certainly not the decisive factor.

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References

Barrow KD, Jamieson DD, Norton RS (1980) ^{31}P nuclear-magnetic-resonance studies of energy metabolism in tissue from the marine invertebrate *Tapes watilingi*. Eur J Biochem 103:289–297

Cameron JN (1971) Rapid method for determination of total carbon dioxide in small blood samples. J Appl Physiol 31:632–634

Ebberink RHM, de Zwaan A (1980) Control of glycolysis in the posterior adductor muscle of the sea mussel *Mytilus edulis*. J Comp Physiol 137:165–171

Ellington WR (1983) The extent of intracellular acidification during anoxia in the catch muscles of two bivalve molluscs. J Exp Zool 227:313–317

Felbeck H (1980) Investigations on the role of the amino acids in anaerobic metabolism of the lugworm *Arenicola marina* L. J Comp Physiol 137:183–192

Felbeck H, Grieshaber M (1980) Investigations on some enzymes involved in the anaerobic metabolism of amino acids of *Arenicola marina* L. Comp Biochem Physiol 66B:205–213

Harvey HW (1974) The chemistry and fertility of sea waters. Cambridge Univ Press, Cambridge

Heisler N (1975) Intracellular pH of isolated rat diaphragm muscle with metabolic and respiratory changes of extracellular pH. Respir Physiol 23:243–255

Heisler N (1984) Acid-base regulation in fishes. In: Fish physiology, vol X. Academic Press, New York in press

Heisler N, Neumann P (1980) The role of physico-chemical buffering and of bicarbonate transfer processes in intracellular pH regulation in response to changes of temperature in the larger spotted dogfish (*Scyliorhinus stellaris*). J Exp Biol 85:99–110

Heisler N, Piiper J (1971) The buffer value of rat diaphragm muscle tissue determined by P_{CO_2} equilibration of homogenates. Respir Physiol 12:169–178

Heisler N, Weitz H, Weitz AM (1976) Extracellular and intracellular pH with changes of temperature in the dogfish *Scyliorhinus stellaris*. Respir Physiol 26:249–263

Hochachka PW, Mustafa T (1972) Invertebrate facultative anaerobiosis. Science 178:1056–1060

Holwerda DA, Kruitwagen ECJ, de Bont AMT (1981) Regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase activity during anaerobiosis in *Mytilus edulis* L. Mol Physiol 1:165–171

Holwerda DA, Veenhof PR, van Heugten HAA, Zandee DJ (1983) Modification of mussel pyruvate kinase during anaerobiosis and after temperature acclimation. Mol Physiol 3:225–234

Jorge FB de, Petersen JA, Ditadi ASF (1970) Comparative biochemical studies in *Sipunculus natans* and *Sipunculus multisulcatus* (Sipuncula). Comp Biochem Physiol 35:163–177

Kluytmans JH, van Graft M, Janus J, Pieters H (1978) Production and excretion of volatile fatty acids in the sea mussel *Mytilus edulis* L. J Comp Physiol 123:163–167

Lipmann F, Meyerhof O (1930) Über die Reaktionsänderung des tätigen Muskels. Biochem Z 227:84–109

Livingstone DR (1982) Energy production in the muscle tissues of different kinds of molluscs. In: Addink ADF, Spronk N (eds) Exogenous and endogenous influences on metabolic and neural control. Pergamon Press, Oxford New York, pp 257–274

Livingstone DR, Bayne BL (1974) Pyruvate kinase from the mantle tissue of *Mytilus edulis* L. Comp Biochem Physiol 48B:481–497

Michal G, Beutler HO, Lang G, Guenter U (1976) Enzymatic determination of succinic acid in foodstuffs. Z Anal Chem 279:137–138

Mustafa T, Hochachka PW (1971) Catalytic and regulatory properties of pyruvate kinases in tissues of a marine bivalve. J Biol Chem 246:3196–3203

Mustafa T, Hochachka PW (1973) Enzymes in facultative anaerobiosis of molluscs – III. Phosphoenolpyruvate carboxykinase and its role in aerobic-anaerobic transition. Comp Biochem Physiol 45B:657–667

Netter H (1959) Theoretische Biochemie. Physikalisch-chemische Grundlagen der Lebensvorgänge. Springer, Berlin Heidelberg New York

Oglesby LC (1969) Inorganic components and metabolism; ionic and osmotic regulation: Annelida, Sipuncula and Echiura. In: Florkin M, Scheer BT (eds) Annelida, Echiura and Sipuncula (Chemical Zoology, vol IV) Academic Press, New York, pp 211–310

Oglesby LC (1982) Salt and water balance in the sipunculan *Phascolopsis gouldi*: Is any animal a 'simple osmometer'? Comp Biochem Physiol 71A:363–368

Pörtner HO (1982) Biochemische und physiologische Anpassungen an das Leben im marinen Sediment: Untersuchungen am Spritzwurm *Sipunculus nudus* L. Dissertation, Universität Düsseldorf

Pörtner HO, Kreutzer U, Siegmund B, Heisler N, Grieshaber MK (1984a) Metabolic adaptation of the intertidal worm *Sipunculus nudus* to functional and environmental hypoxia. Mar Biol 79:237–247

Pörtner HO, Heisler N, Grieshaber MK (1984b) Anaerobiosis and acid-base status in marine invertebrates: Atheoretical analysis of proton generation by anaerobic metabolism. J Comp Physiol B 155:1–12

Schöttler U (1979) On the anaerobic metabolism of three species of *Nereis* (Annelida). Mar Ecol Prog Ser 1:249–254

Schöttler U (1980) Der Energiestoffwechsel bei biotopbedingter Anaerobiose: Untersuchungen an Anneliden. Verh Dtsch Zool Ges 1980:228–240

Schöttler U, Wienhausen G (1981) The importance of the phosphoenolpyruvate carboxykinase in the anaerobic metabolism of two marine polychaetes. In vivo investigations on *Nereis virens* and *Arenicola marina*. Comp Biochem Physiol 68B:41–48

Siebenaller JF (1979) Regulation of pyruvate kinase in *Mytilus edulis* by phosphorylation – dephosphorylation. Mar Biol Lett 1:105–110

Waddell WJ, Butler TC (1959) 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 38:720–729

Zandee DI, Holwerda DA, de Zwaan A (1980) Energy metabolism in bivalves and cephalopods. In: Gilles R (ed) Animals and environmental fitness, vol 1. Pergamon Press, Oxford New York, pp 185–206

Zebe E (1975) In vivo-Untersuchungen über den Glucose-Abbau bei *Arenicola marina* (Annelida, Polychaeta). J Comp Physiol 101:133–145

Zebe E (1977) Anaerober Stoffwechsel bei wirbellosen Tieren. Rheinisch-Westf Akademie Wissensch, Vorträge N 269:51–73

Zwaan A de (1977) Anaerobic energy metabolism in bivalve molluscs. Oceanogr Mar Biol Annu Rev 15:103–187

Zwaan A de, de Bont AMT (1975) Phosphoenolpyruvate carboxykinase from adductor muscle tissue of the sea mussel *Mytilus edulis* L. J Comp Physiol 96:85–94

Zwaan A de, Holwerda DA (1972) The effect of phosphoenolpyruvate, fructose 1,6-diphosphate and pH on allosteric pyruvate kinase in muscle tissue of the bivalve *Mytilus edulis* L. Biochim Biophys Acta 276:430–433

Zwaan A de, van Marrewijk WJA (1973a) Intracellular localization of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and 'malic enzyme' and the absence of glyoxylate cycle enzymes in the sea mussel (*Mytilus edulis* L.). Comp Biochem Physilol 44B:1057–1066

Zwaan A de, van Marrewijk WJA (1973b) Anaerobic glucose degradation in the sea mussel *Mytilus edulis* L. Comp Biochem Physiol 44B:429–439

Zwaan A de, de Bont AMT, Hemelraad J (1983) The role of phosphoenolpyruvate carboxykinase in the anaerobic metabolism of the sea mussel *Mytilus edulis* L. J Comp Physiol 153:267–274