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Metabolic adaptation of the intertidal worm *Sipunculus nudus* to functional and environmental hypoxia *

H.-O. Pörtner [1], U. Kreutzer [1], B. Siegmund [1], N. Heisler [2] and M. K. Grieshaber [1]

[1] **Lehrstuhl für Stoffwechselphysiologie, Institut für Zoologie, Universität Düsseldorf; Universitätsstraße 1, D-4000 Düsseldorf 1, Germany (FRG)**

[2] **Abteilung Physiologie, Max-Planck-Institut für experimentelle Medizin; D-3400 Göttingen, Germany (FRG)**

Abstract

The scope of anaerobic metabolism of *Sipunculus nudus* L. was assessed from the maximal activities of some enzymes of the intermediary metabolism and from the concentration of some metabolites accumulated during enhanced muscular activity and during prolonged experimental hypoxia.

(1) Maximal enzyme activities demonstrate that the scope of anaerobic glycolysis, as indicated by maximal activities of glycogen phosphorylase (0.84 U g^{-1} fresh wt), far exceeds the aerobic capacity, which is assumed not to surpass the activity of succinate dehydrogenase (0.09 U g^{-1} fresh wt). Three pyruvate reductase activities (alanopine-, strombine- and octopine dehydrogenase) can possibly terminate anaerobic glycolysis.

(2) During muscular activity, energy is provided by the degradation of phospho-L-arginine and by anaerobic glycolysis. Octopine is the major endproduct during functional anaerobiosis while the formation of strombine is less pronounced.

(3) During exposure to a nitrogen atmosphere, several anaerobic endproducts are found to accumulate. Anaerobic glycolysis is terminated by strombine synthesis. This opine accumulates in concentrations much higher than octopine. In addition the concentrations of succinate, propionate and acetate are found to increase in tissues, and/or in the coelomic fluid and the incubation water.

(4) The relative contribution of energy by the different anaerobic metabolic pathways are estimated during functional and environmental hypoxia.

Introduction

Numerous investigations of invertebrates have revealed two different modes of metabolic adaptation to the various types of hypoxia:

(a) "Functional hypoxia", which originates during excessive muscular activity, exploits energy from the transphosphorylation of a phosphagen as, for example from phosphocreatine or phospho-L-arginine, and from anaerobic glycolysis characterized by the accumulation of lactate and/or opines (Grieshaber, 1982; Livingstone, 1982);

(b) "Environmental hypoxia" can arise in animals that live in habitats which are periodically exposed to hypoxia as, for example in intertidal flats, in the hypolimnion of freshwater lakes or in shallow ponds. During hypoxia most species of these biotops are capable of supplying their own energy requirements from the degradation of a phosphagen, from anaerobic glycolysis, and from anaerobic degradation of glycogen along the succinate-propionate-pathway. The switch to these metabolic pathways leads to an immediate increase in the concentrations of lactate and/or opines as well as of succinate. If environmental hypoxia occurs for long time periods, succinate is further metabolized to propionate. The latter accumulates within the tissues and is also released from the animal into the environment (de Zwaan, 1977; Zebe, 1977; Schöttler, 1980).

The sipunculid worm *Sipunculus nudus* L. provides some interesting features of comparative studies of the anaerobic metabolism of marine invertebrates. In contrast to some other members of the intertidal infauna (e.g. *Arenicola marina* L.), *S. nudus* lies buried in the sand without building an elaborate burrow. A small funnel in the substratum above the anterior part of its body provides access to water for respiration (Hérubel, 1907). When the water recedes during low tides, however, irrigation becomes impossible. Alternative provision of oxygen from the interstitial water is unlikely, due to a low partial pressure of oxygen and negative redox potentials of the

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substratum (Wells and Warren, 1975; Wieser, 1975; Gillandt, 1979). These hypoxic conditions would be detrimental to *S. nudus,* if the worms were incapable of changing to oxygen-independent energy metabolism.

Furthermore, *Sipunculus nudus* shows irregular periods of burrowing activity in the substratum for translocation or feeding. The worm may also be forced to dig rapidly after being flushed out from the sand by wave action. Usually the amount of energy required for such pronounced muscular activities exceeds aerobic energy production, and an alternative anaerobic energy metabolism is required.

In order to evaluate the scope of anaerobic metabolism of *Sipunculus nudus,* relative maximal activities of some enzymes of the intermediary metabolism were estimated, and the concentrations of several metabolites, which are characteristic of certain catabolic pathways, were determined in individuals after muscular exhaustion, and in individuals exposed to environmental hypoxia for various time periods.

Material and methods

Worms

Sipunculus nudus were dug out from the intertidal flats of Brittany, France. Large individuals (20 to 40 g) were found near Loquémeau and small ones (5 to 12 g) were collected near Morgat. They were kept for several weeks in an aquarium containing a bottom layer of sand (10 to 20 cm) from the worm's original habitat. The tanks were circulated with well aerated artificial seawater with a temperature of 15 °C ± 1 C°.

Experimental procedure

Functional hypoxia

This metabolic state was elicited by removing the worms from the substratum and putting them on top of the sand in normoxic sea water. They immediately started to dig into the sand. When they had almost completely disappeared (one digging cycle), the same worms were removed from the sand repeatedly in order to induce as many digging cycles as possible. Usually the worms were exhausted after about 45 min, which is the approximate duration of 15 digging cycles. Controls were taken directly out of the sand.

Environmental hypoxia

Twenty-four hours before an experiment was started, individuals were placed in darkened tanks which contained normoxic, artificial sea water (34‰ S; 15 °C), but no sand. Thereby digging movements of the worms were prevented. Hypoxic incubation (P_{O_2} < 3 Torr) for 2, 6, 12 or 24 h respectively, was performed in darkened bottles containing 500-ml artificial sea water which was flushed with pure nitrogen 30 min prior to and 30 min after inserting the worms. During the incubation period the bottles were completely closed.

Preparation of perchloric acid extracts

At the end of each experiment the worms were dissected as quickly as possible. Body wall tissue and introvert retractor muscle were freeze clamped (Wollenberger *et al.,* 1960). Coelomic fluid was collected in cooled plastic tubes and immediately centrifuged for 1 min at 1 000 × g. The supernatant, which is equivalent to the coelomic plasma, was deproteinized by the addition of a tenth of its volume of 6 N perchloric acid.

After mixing and centrifugation, the extracts were neutralized using 5 M KOH. The resulting precipitate was discarded, and the supernatant used for the determination of metabolites.

The body wall, which consists mainly of muscle tissue, was extracted according to Beis and Newsholme (1975). Small-sized samples, as for example retractor muscle, were extracted following the procedure of Pette and Reichmann (1982).

Determination of metabolite concentrations

The concentrations of the metabolites ATP, ADP, AMP, L-lactate, L-alanine, D-alanine, L-aspartate and malate were estimated enzymatically using standard procedures (Bergmeyer, 1974). The concentration of succinate was measured as described by Michal *et al.* (1976). Determinations of phospho-L-arginine, L-arginine and octopine followed the procedure of Grieshaber *et al.* (1978). Glycine content was measured with an amino acid analyzer (Liquimat III, Kontron, D-8000 München, FRG) using Dionex DC-6A resin and a lithium citrate buffer. The concentrations of strombine and alanopine were estimated by high pressure liquid chromatography (Siegmund and Grieshaber, 1983). Propionate and acetate were first separated from acidified perchloric acid extracts by water vapor destillation, and then quantified by high pressure liquid chromatography. Volatile fatty acids were chromatographed using Bio-Rad Resin Aminex HPX-87 (Bio-Rad, D-8000 München, FRG; column size 300 × 7.8 mm) and 4 m*M* H_2SO_4 as the eluant at a flow rate of 0.6 ml min^{-1}, a column temperature of 20 °C, and a pressure of 50 bar. Propionate and acetate were detected monitoring their absorbance at 210 nm. Their retention times were 16 ± 0.5 min and 19 ± 0.5 min for acetate and propionate respectively.

Extraction of enzymes

Large specimens of *Sipunculus nudus* were used for the preparation of enzyme extracts. Approximately 1 g of

body wall tissue and introvert retractor muscles were cut into small pieces and resuspended in a 5 to 10 fold volume of buffer appropriate for the respective enzymes. Tissues were homogenized for 3×30 s with a mechanical Ultra-Turrax homogenizer (Janke and Kunkel, D-7813 Staufen, FRG) and centrifuged at 48 000×g for 15 min. The supernatant was passed through a Sephadex G-25 column (Deutsche Pharmacia, D-7800 Freiburg, FRG) to remove low molecular weight compounds.

The sediment obtained from the muscle extract was again resuspended in 50 m*M* potassium phosphate buffer (pH 7.2) and used to assay succinate dehydrogenase.

A homogenisation medium containing 50 m*M* triethanolamine (pH 7.4), 1 m*M* EDTA, 2 m*M* $MgCl_2$, 30 m*M* mercaptoethanol (Zammit and Newsholme, 1976) was used to extract the following enzymes: arginine kinase (EC 2.7.3.3), hexokinase (EC 2.7.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.31), lactate dehydrogenase (EC 1.1.1.27/28), octopine dehydrogenase (EC 1.5.1.11), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), succinate dehydrogenase (EC 1.3.99.1), malate dehydrogenase (EC 1.1.1.37), malic enzyme (malate dehydrogenase, oxaloacetate decarboxylating, $NADP^+$; EC 1.1.1.49), glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), strombine dehydrogenase (N-carboxylmethyl-D-alanine: NAD^+ oxidoreductase, EC 1.5.1.?) and alanopine dehydrogenase (meso-N-(1-carboxyethyl) alanine: NAD^+ oxidoreductase, EC 1.5.1.?).

The homogenisation medium for phosphofructokinase (EC 2.7.11) was chosen from Zammit and Newsholme (1976), the one used for pyruvate kinase (EC 2.7.1.40) from Zammit *et al.* (1978), and for citrate synthase (EC 4.1.3.7) as well as for isocitrate dehydrogenase (NAD^+, EC 1.1.1.41; $NADP^+$, EC 1.1.1.42) from Alp *et al.* (1976). Phosphorylase (EC 2.4.1.1) was homogenized in 50 m*M* triethanolamine (pH 7.0) containing 5 m*M* EDTA and 20 m*M* NaF.

Determination of enzymatic activities

The following enzymes were assayed by standard procedures reported in the literature: arginine kinase (Grieshaber *et al.*, 1978), citrate synthase (Alp *et al.*, 1976), glyceraldehyde-3-phosphate dehydrogenase (Bergmeyer *et al.*, 1974 a), glycerol-3-phosphate dehydrogenase, hexokinase, phosphofructokinase, lactate dehydrogenase (Zammit and Newsholme, 1976), malate dehydrogenase (Mehler *et al.*, 1948), malic enzyme (carboxylating) (de Zwaan and van Marrewijk, 1973), phosphoenolpyruvate carboxykinase (carboxylating) (Utter and Kurahashi, 1954), pyruvate kinase (Zammit *et al.*, 1978), octopine dehydrogenase (Haas *et al.*, 1973) and succinate dehydrogenase (Brdiczka *et al.*, 1968).

NADP-dependent isocitrate dehydrogenase was assayed according to Alp et al. (1976), modified as follows: 70 m*M* tris-HCl pH 7.5, containing 1.13 m*M* DL-isocitrate, 7.5 m*M* citrate, 0.5 m*M* NADP, 2 m*M* $MnCl_2$, and 8 m*M* $MgCl_2$.

The reaction mixture of glycogen phosphorylase contained 40 m*M* potassium phosphate buffer pH 6.8, 0.1 m*M* EDTA, 0.3 m*M* NADP, 4 μM glucose-1,6-bisphosphate, 25 m*M* $MgCl_2$, 0.1 U ml^{-1} phosphoglucomutase, 0.1 Uml^{-1} glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 2 mg ml^{-1} glycogen. Glycogen phosphorylase was activated by adding 0.5 m*M* 5′-AMP (Bergmeyer *et al.*, 1974 b).

Alanopine dehydrogenase as well as strombine dehydrogenase activities were determined using a buffer which contained 100 m*M* imidazole/HCl (pH 7.0), 10 m*M* pyruvate, and 0.25 m*M* NADH. The reaction was started by the addition of the amino acid substrate which was 200 m*M* L-alanine or 200 m*M* glycine for alanopine or strombine dehydrogenase respectively.

Results

Enzyme activities

The activities of some enzymes catalyzing the energy metabolism in the body wall and the introvert retractor of *Sipunculus nudus* are compiled in Table 1. Glycogen phosphorylase extracted from the body wall could be activated about 6-fold to 0.84 U g^{-1} fresh wt by the addition of AMP, while the same enzyme from the introvert retractor showed only a small and insignificant increase in the presence of 5′-AMP. Hexokinase had low activities in both tissues. Comparing the enzymes of the Embden-Meyerhof pathway, phosphofructokinase demonstrated an approximately 8-fold greater activity than activated phosphorylase. As expected, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase showed the highest activity of this group, the enzymes of the body wall musculature being twice as active as those of the introvert retractor.

The reduction of pyruvate could be catalyzed by three enzymatic activities (Table 1): firstly, by octopine dehydrogenase, which showed the highest activity; secondly, by alanopine dehydrogenase whose activity was estimated to be 49 and 30 U g^{-1} fresh wt in body wall and introvert retractor respectively; thirdly, the lowest activity was determined for strombine dehydrogenase, which was also the only pyruvate reductase found to be more active in the introvert retractor than in body wall tissue.

Arginine kinase activities were high, showing values of 437 and 687 U g^{-1} fresh wt from body wall and introvert retractor respectively.

Malate dehydrogenase had approximately the same activity as glyceraldehyde-3-phosphate dehydrogenase while glycerol-3-phosphate dehydrogenase was very low.

Carboxylation of phosphoenolpyruvate to oxaloacetate by phosphoenolpyruvate carboxykinase was extremely low. The enzymatic activity could hardly be measured. In contrast, the second enzyme catalyzing the fixation of CO_2 without concomitant consumption of ATP, malic enzyme, could readily be assayed in the direction of malate

Table 1. *Sipunculus nudus.* Activities of some enzymes catalyzing the energy metabolism in muscle tissues. A unit of activity is defined as the disappearance or formation of 1 μmol NAD(P)H in 1 min at 25 °C. Specific activity (mean values ± SD; $n = 4$) is expressed as enzyme units per g fresh weight

Enzyme	Enzyme activities		Metabolic function
	Body wall musculature	Introvert retractor muscle	
Phosphorylase	0.14 ± 0.04	0.23 ± 0.20	Mobilization of glycogen
Phosphorylase (AMP activated)	0.84 ± 0.11	0.37 ± 0.14	
Hexokinase	0.08 ± 0.04	0.04 ± 0.02	Phosphorylation of glucose
Phosphofructokinase	5.95 ± 1.44	6.09 ± 1.25	Embden-Meyerhof pathway
Glyceraldehyde-3-phosphate dehydrogenase	30.2 ± 6.7	13.3 ± 1.9	
Pyruvate kinase	58.6 ± 11.8	23.8 ± 2.3	
Lactate dehydrogenase	< 0.05	< 0.05	Terminal reactions of anaerobic glycolysis (reduction of pyruvate)
Alanopine dehydrogenase	49.3 ± 15.9	29.9 ± 4.1	
Octopine dehydrogenase	445.4 ± 63.3	228.2 ± 58.9	
Strombine dehydrogenase	9.20 ± 2.28	12.9 ± 1.5	
Arginine kinase	436.7 ± 55.6	687.1 ± 77.6	Phosphagen utilization
Malate dehydrogenase	30.0 ± 3.3	23.4 ± 5.4	Enzymes possibly involved in hydrogen transport from the cytosol to the mitochondria
Glycerol-3-phosphate dehydrogenase	0.75 ± 0.07	0.73 ± 0.24	
Phosphoenolpyruvate carboxykinase	< 0.05	< 0.05	Fixation of CO_2 upon phosphoenol-pyruvate or pyruvate rendering oxaloacetate or malate
Malic enzyme (carboxylating)	0.48 ± 0.06	0.26 ± 0.03	
Citrate synthase	0.53 ± 0.06	0.46 ± 0.01	Enzymes of the tricarbonic acid cycle
Isocitrate dehydrogenase (NADP-dependent)	0.20 ± 0.01	0.13 ± 0.02	
Succinate dehydrogenase	0.09 ± 0.01	0.04 ± 0.01	

formation. Its activity was 0.48 and 0.26 U g^{-1} fresh wt in body wall tissue and introvert retractor, respectively.

Citrate synthase and isocitrate dehydrogenase had less than 1 U g^{-1} fresh wt and succinate dehydrogenase activity was usually lower than 0.1 U g^{-1} fresh wt in both tissues.

Functional hypoxia during digging movements

Sipunculus nudus was forced to dig by removing the animal from the sand. During this enhanced locomotory activity the concentrations of adenylates and the energy charge changed only slightly (Table 2a). Phospho-L-arginine, however, was reduced from 30 to 17 μmol g^{-1} fresh wt and from 12 to 7 μmol g^{-1} fresh wt in body wall tissue and introvert retractor respectively.

The concentrations of the endproducts of anaerobic glycolysis (Table 2b) were found to be increased in the case of octopine by 8 μmol g^{-1} fresh wt in body wall musculature and 1 μmol g^{-1} fresh wt in introvert retractor muscles. Strombine seemed to accumulate in both tissues but the concentration within the body wall was highly variable. Neither alanopine nor (D + L)-lactate could be demonstrated in muscle tissue of fatigued *Sipunculus nudus.*

Of the amino acids investigated (Table 2c), L-arginine concentration changed from 9 to 16 μmol g^{-1} fresh wt in body wall tissue and L-aspartate decreased from 1.7 to 0.9 μmol g^{-1} fresh wt in the introvert retractor.

Estimation of carbonic acids (Table 2d) demonstrated a small increase of succinate and malate concentrations.

Environmental hypoxia during N_2-application

Individuals of *Sipunculus nudus* were subjected to extreme hypoxia by incubation in sea water with an oxygen partial pressure close to zero ($P_{O_2} < 3$ torr) for various periods of time. Within 24 h this treatment did not result in a significant change of the adenylate concentrations and energy charge. Only the phosphagen content decreased from 34 to 21 μmol g^{-1} fresh wt within the body wall tissue (Table 3a).

The endproducts of anaerobic glycolysis accumulated in the body wall from 1 to 8 μmol g^{-1} fresh wt and from 0.1 to 0.6 μmol g^{-1} fresh wt in the case of strombine and octopine respectively (Table 3b). No D- or L-lactate could be found and only a very small increase of alanopine was substantiated after 12 and 24 h of anoxia.

The concentration of aspartate (Table 3c) decreased by 1.1 and 1.4 μmol g^{-1} fresh wt in the body wall tissue and in the introvert retractor respectively. The alanine concentration rose almost stoichiometrically, however, the increase was statistically insignificant. No changes of glycine concentrations could be found. In contrast, L-arginine rose from 6 to 17 μmol g^{-1} fresh wt in the body wall and from 10 to 12 μmol g^{-1} fresh wt in the introvert retractor.

Table 2. *Sipunculus nudus.* The concentrations of metabolites extracted from body wall musculature (BWM) and introvert retractor muscle (IRM) after extensive digging movements lasting for approximately 45 min. Concentrations are given in μmol g^{-1} fresh weight as mean values $\pm$ SD ($n=5$)

				Phospho-L-Arginine	ATP	ADP	AMP	Energy charge
(a)	Phosphagen and adenosine phosphates	control	BWM	30.58±2.28	2.42±0.51	0.41±0.15	0.05±0.03	0.91±0.01
			IRM	12.20±1.34	2.29±0.62	0.37±0.18	0.08±0.06	0.90±0.05
		working	BWM	17.22±6.42	2.60±0.77	0.23±0.04	0.04±0.01	0.95±0.02
			IRM	7.18±2.01	2.24±0.56	0.46±0.18	0.23±0.28	0.84±0.08
				D-and L-Lactate	Strombine	Alanopine	Octopine	
(b)	End products of anaerobic glycolysis	control	BWM	<0.05	1.57±1.07	<0.05	0.97±0.71	
			IRM	<0.05	1.05±0.70	<0.05	0.11±0.09	
		working	BWM	<0.05	4.96±4.78	<0.05	9.07±2.55	
			IRM	<0.05	3.61±0.07	<0.05	1.09±0.39	
				L-Aspartate	Glycine	(L+D)-Alanine	L-Arginine	
(c)	Amino acids involved in energy metabolism	control	BWM	1.35±0.18	151±29	6.71±3.64	9.14±1.09	
			IRM	1.66±0.26	211±12	5.31±1.08	9.91±0.39	
		working	BWM	1.18±0.19	159± 9	7.49±2.09	15.88±2.71	
			IRM	0.91±0.39	195±24	5.11±1.56	12.10±2.37	
				Succinate	Acetate	Propionate	Malate	
(d)	Carbonic acids involved in environmental hypoxia	control	BWM	0.11±0.02	0.66±0.17	0.16±0.07	0.15±0.03	
			IRM	0.08±0.03	2.97±0.83	0.48±0.10	0.05±0.02	
		working	BWM	0.29±0.03	0.59±0.18	0.17±0.06	0.46±0.18	
			IRM	0.30±0.13	2.18±0.51	0.42±0.22	0.19±0.02	

The carbonic acid anions succinate and propionate accumulated continuously from the onset of anaerobiosis within body wall tissue and introvert retractor (Table 3 d). After 6 h of anaerobiosis, propionate was released into the coelomic plasma and into the ambient sea water (Table 4). Acetate concentrations remained virtually unchanged in muscle tissues but increased in the coelomic plasma and in the incubation medium. Malate rose during the first two hours of anaerobiosis from 0.1 to 0.3 μmol g^{-1} fresh wt within the body wall.

Discussion

Scope of aerobic and anaerobic energy metabolism as estimated by oxygen consumption and maximal enzyme activities

The energy metabolism of body wall musculature and introvert retractor muscles of *Sipunculus nudus* was first assessed by the determination of relative maximal enzyme activities of the intermediary metabolism. Table 1 shows that the degradation of glycogen in the body wall can be increased at least 6-fold by the AMP-dependent activation of glycogen phosphorylase. The capacity of AMP-activated phosphorylase is about 10-fold higher than hexokinase activity indicating that during enhanced energy demand more glycosyl-residues can be contributed by glycogenolysis than by the phosphorylation of glucose. Maximal activities of phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase are much greater than the activity of AMP-activated phosphorylase. This last enzyme, therefore, can be considered to catalyze the flux limiting reaction of the Embden-Meyerhof-pathway.

It is important to note that maximal activities of the three assayed enzymes of the Krebs-cycle are lower than the value estimated for AMP-activated phosphorylase. Succinate dehydrogenase (0.09 U g^{-1} fresh wt) possibly catalyzes the flux limiting reaction. The capacity of the tricarbonic-acid-cycle could even be lower assuming 2-oxoglutarate dehydrogenase as the control element (Read *et al.*, 1977; Cooney *et al.*, 1981). Unfortunately the determination of this enzyme's activity proved to be impossible.

Maximal enzymatic activities determined *in vitro* and, therefore, in the absence of cellular control elements

Table 3. *Sipunculus nudus.* The concentrations of metabolites extracted from body wall musculature (BWM) and introvert retractor muscle (IRM) after various periods of hypoxia. Concentrations are given in μmol g^{-1} fresh weight as mean values ± SD ($n=3$)

(a) Phosphagen and adenosine nucleotides	Time of hypoxia (h)	Phospho-L-arginine		ATP		ADP		AMP		Energy charge	
		BWM	IRM	BWM	IRM	BWM	IRM	BWM	IRM	BWM	IRM
	0	34.49±6.87	12.30±1.52	3.11±0.86	2.64±0.26	0.41±0.08	0.35±0.20	0.05±0.01	0.07±0.03	0.93±0.02	0.92±0.04
	2	27.24±0.71	10.27±2.03	3.26±0.19	2.83±0.17	0.42±0.13	0.37±0.03	0.07±0.02	0.07±0.01	0.92±0.01	0.92±0.01
	6	27.16±1.59	10.03±1.17	3.14±0.18	3.25±0.52	0.33±0.06	0.41±0.01	0.07±0.01	0.07±0.01	0.93±0.01	0.93±0.01
	12	20.39±5.92	9.10±2.21	2.90±0.18	2.87±0.40	0.36±0.07	0.56±0.06	0.07±0.02	0.07±0.01	0.93±0.01	0.90±0.01
	24	21.36±1.51	11.08±1.83	3.04±0.16	3.07±0.51	0.45±0.05	0.49±0.08	0.03±0.03	0.14±0.12	0.93±0.01	0.90±0.04

(b) End products of anaerobic glycolysis	Time of hypoxia (h)	D- and L-Lactate		Strombine		Alanopine		Octopine	
		BWM	IRM	BWM	IRM	BWM	IRM	BWM	IRM
	0	<0.05	<0.05	1.22±0.46	0.92±0.73	<0.05	<0.05	0.09±0.04	0.10±0.03
	2	<0.05	<0.05	6.62±3.46	3.51±0.62	<0.05	<0.05	0.48±0.33	0.25±0.02
	6	<0.05	<0.05	4.82±2.29	3.16±1.05	<0.05	<0.05	0.51±0.26	0.23±0.14
	12	<0.05	<0.05	3.86±0.84	1.53±0.92	0.12±0.08	<0.05	0.68±0.31	0.47±0.10
	24	<0.05	<0.05	7.99±1.03	–	0.19±0.05	–	0.60±0.26	0.26±0.02

(c) Amino acids involved in energy metabolism	Time of hypoxia (h)	L-Aspartate		Glycine		(D+L)-Alanine		L-Arginine	
		BWM	IRM	BWM	IRM	BWM	IRM	BWM	IRM
	0	1.74±0.27	1.87±0.28	181±16	–	6.93±3.29	6.47±1.38	6.42±1.79	9.80±0.34
	2	1.41±0.03	1.55±0.39	205±49	–	7.65±1.25	6.12±1.51	8.03±0.94	9.71±0.86
	6	0.71±0.23	0.86±0.13	213±18	–	7.53±2.62	6.40±1.93	8.56±0.05	10.80±0.51
	12	0.70±0.16	0.73±0.14	185±13	–	7.62±2.00	7.82±1.59	16.00±3.21	10.98±0.75
	24	0.64±0.02	0.51±0.12	176±20	–	7.95±2.90	7.26±1.97	16.70±1.52	12.05±0.22

(d) Carbonic acids involved in environmental hypoxia	Time of hypoxia (h)	Succinate		Acetate		Propionate		Malate	
		BWM	IRM	BWM	IRM	BWM	IRM	BWM	IRM
	0	0.10±0.01	0.17±0.03	0.59±0.02	2.30±0.54	0.06±0.02	0.43±0.02	0.08±0.03	–
	2	0.34±0.03	0.40±0.01	0.49±0.17	1.88±0.39	0.11±0.08	0.48±0.06	0.32±0.01	–
	6	0.40±0.04	0.49±0.03	0.87±0.25	1.89±0.02	0.37±0.17	0.41±0.11	0.21±0.02	–
	12	0.67±0.10	0.70±0.04	0.77±0.20	2.41±0.37	0.56±0.23	0.62±0.01	0.30±0.06	–
	24	1.46±0.08	1.52±0.14	0.60±0.03	2.67±0.74	1.10±0.06	0.95±0.25	0.23±0.04	–

Table 4. The concentrations of carbonic acids within the coelomic plasma (CP) and the incubation water (IW). Concentrations are given in μmol ml^{-1} plasma and in μmol g^{-1} fresh weight for the incubation water as mean values ± SD ($n=4$)

Time of hypoxic incubation (h)	Succinate		Acetate		Propionate	
	CP	IW	CP	IW	CP	IW
0	0.06±0.02	0	0.24±0.11	0	0.03±0.02	0
2	0.09±0.01	0	0.17±0.04	0.05	0.05±0.03	0.02
6	0.11±0.02	0	0.20±0.09	0.11	0.10±0.05	0.02
12	0.19±0.07	0	0.66±0.37	0.64	0.73±0.22	0.14
24	0.22±0.04	0	0.77±0.19	0.49	0.92±0.12	0.40

usually cannot be expected to reflect actual cellular flux rates. The consumption of oxygen ($\dot{M}_{O_2}$), however, is closely correlated to actual aerobic metabolic rates.

Isolated body wall musculature of *Sipunculus nudus* consumes 0.86 μmol O_2 h^{-1} g^{-1} fresh wt at 15 °C (Pörtner, 1982), which corresponds to a glycolytic flux rate of 0.14 μmol glycosyl-residues h^{-1} g^{-1} fresh wt with glycogen as the only substrate. For comparison, enzyme activities measured at 25 °C had to be calculated for 15 °C applying a value of 2 for Q_{10} (Zammit and Newsholme, 1976). A metabolic rate of 0.14 μmol glycosyl-residues h^{-1} g^{-1} fresh wt equals 3.4% of the glycolytic flux as controlled by non-activated phosphorylase (4.2 μmol h^{-1} g^{-1} fresh wt) and 0.6% of the maximal glycolytic flux as controlled by AMP-activated phosphorylase activity (25.2 μmol h^{-1} g^{-1} fresh wt). When $\dot{M}_{O_2}$ of the body wall is compared to the maximal activity of succinate dehydrogenase (2.7 μmol h^{-1} g^{-1} fresh wt), it is obvious that the Krebs cycle spins with 11% of its capacity. Thus, with regard to the Krebs cycle, the aerobic energy metabolism could be increased at most tenfold, but the anaerobic rate could be raised by a factor of 178. The low scope of aerobic metabolism is in accordance with a low number of mitochondria in the muscle tissues of *S. nudus* (de Eguileor and Valvassori, 1977).

The glycolytic flux may increase considerably during enhanced energy demand. If more than 1.35 μmol glycosyl-residues h^{-1} g^{-1} fresh wt are supplied through the Embden-Meyerhof pathway, the maximal flux of the Krebs cycle is achieved as indicated by the maximal activity of succinate dehydrogenase. This rate, however, corresponds to only 5.4% of the maximal glycolytic flux as calculated from AMP-activated phosphorylase. Therefore, an increase of the flux of the Embden-Meyerhof pathway above 5.4% of its maximal rate would lead to an increase in the concentration of NADH and to an accumulation of pyruvate.

Reduction of pyruvate by opine dehydrogenase

Pyruvate can be metabolized via the Krebs cycle, or it is substrate for anaplerotic reactions. During hypoxia, lactate dehydrogenases catalyze the reduction of pyruvate to L- or D-lactate. In many molluscs and annelids, several opine dehydrogenases catalyze the reductive condensation of pyruvate with various amino acids. Glycolytically formed NADH serves as cosubstrate for lactate dehydrogenases as well as opine dehydrogenases, thereby replenishing NAD^+ for the continuous oxidation of glyceraldehyde-3-phosphate.

In body wall tissue as well as in introvert retractor muscles of *Sipunculus nudus* three opine dehydrogenases were found. Octopine dehydrogenase (having L-arginine as second substrate) shows the highest activity and has been known in this worm for a long time (Haas *et al.*, 1973). Surprisingly, *S. nudus* also possesses alanopine dehydrogenase (with L-alanine serving as amino acid substrate) and strombine dehydrogenase (reacting with glycine). The latter enzymes, however, have considerably lower activities than octopine dehydrogenase. Since all enzymes were assayed from extracts which had only their low molecular compounds removed, it cannot be decided yet, whether these opine dehydrogenases are three distinct proteins of different activities or one single polypeptide unspecific with regard to its amino acid substrate.

In the presence of CO_2 and NADPH, cytosolic pyruvate can also be reduced to malate by the malic enzyme. This enzyme has a much lower activity than the other pyruvate reductases. Finally, in *Sipunculus nudus* lactate dehydrogenase was beyond detectable limits.

Energy metabolism during functional hypoxia

The significance of this enzymatic pattern for the anaerobic provision of energy was demonstrated in individuals of *Sipunculus nudus* which were forced to dig until exhaustion, thereby provoking functional hypoxia. Subsequent analysis of the introvert retractor and the body wall musculature revealed that the energy charge remained virtually unchanged (Table 2). In contrast to other species (e.g. *Chlamys opercularis;* Grieshaber, 1978), sufficient energy is provided to balance the expenditure of ATP during enhanced muscular activity.

The contribution of aerobic metabolism during multiple digging cycles is probably limited (Pörtner, 1982), but there is a pronounced anaerobic synthesis of ATP in worms digging for approximately 45 min. Phospho-L-arginine provides 44 and 49% of the total amount of

anaerobic ATP in the body wall and introvert retractor respectively (cf. Table 2a). The rate of transphosphorylation (approximately 17.8 μmol ATP h^{-1} g^{-1} fresh wt) can be catalyzed by arginine kinase, whose maximal activity is about 740-fold greater than the rate of ATP synthesis.

More than half of the total amount of ATP is provided by anaerobic glycolysis (cf. Table 2b). Octopine, which is the major endproduct of this pathway in body wall musculature, increased by 8.1 μmol g^{-1} fresh wt corresponding to 12.2 μmol ATP g^{-1} fresh wt (39.7%). Strombine accumulated by 3.4 μmol g^{-1} fresh wt contributing 5.1 μmol ATP g^{-1} fresh wt (16.6%). Both compounds were never found to be released into the coelomic plasma.

Introvert retractor muscles show a much lower anaerobic generation of ATP than body wall musculature. There is also more strombine accumulated than octopine. These data, however, vary between different experiments. *In-vitro* studies using isolated introvert retractor muscles, which were contracting isometrically, always revealed a higher accumulation of octopine than of strombine (Kreutzer, Pörtner and Grieshaber, unpublished results).

As expected, maximal activities of the three opine dehydrogenases are far in excess when compared to the rate of opine synthesis. Kinetic parameters of octopine dehydrogenase suggest (Haas *et al.*, 1973) that an increase of the concentrations of either pyruvate or NADH promote synthesis of octopine.

An increase of the contents of pyruvate or NADH probably also promotes the catalytic activity of both strombine and alanopine dehydrogenase. Assuming that the apparent k_m values for the respective amino acids are as high as those derived from these enzymes of other species (Fields and Hochachka, 1981; Baldwin and England, 1982), then the concentration of glycine (Table 2c), which is about 20-fold higher than the alanine content, would favour synthesis of strombine.

It should be mentioned that aspartate is also metabolized and that succinate as well as malate accumulate in both tissues during functional hypoxia, indicating either an incomplete spinning of the Krebs cycle due to lack of oxygen or the reduction of fumarate by fumarate reductase. Their contribution of ATP, however, is negligible. Despite the increase of succinate concentrations, an accumulation of acetate or propionate was never found during functional hypoxia.

Energy metabolism during environmental hypoxia

In a second set of experiments, *Sipunculus nudus* was placed in N_2-equilibrated water in order to simulate environmental hypoxia. During 24 h of anaerobiosis the adenylate pool did not change significantly and the energy charge remained virtually unchanged in body wall musculature and introvert retractor muscle (Table 3a). The anaerobic metabolism of *Sipunculus nudus* is obviously efficient enough to cover energy requirements during hypoxia periods continuing much longer than those usually occurring in the habitat which last between 2 to 6 h.

In muscle tissue of *Sipunculus nudus,* at least four different metabolic sources could replenish ATP during environmental hypoxia (Table 5):

Table 5. *Sipunculus nudus.* Anaerobic generation of ATP from different metabolic sources after 24 h of experimental hypoxia. In a simplified model the body wall musculature (BWM) was assumed to comprise the total tissue (instead of 75%) corresponding to 48% of the total body weight and the coelomic plasma (CP) representing the remaining 52% (Pörtner, 1982). In order to calculate the total changes of metabolites (μmol g^{-1} fresh wt of body wall musculature), metabolites released into the coelomic plasma and to the incubation water (IW) become diluted and must, therefore, be corrected by the factors 1.08 (52%:48%) and 2.08 (100%:48%) respectively

Metabolic source of energy	Concentration changes of metabolites			Total changes of metabolites	Equivalents of ATP	
	BWM (μmol g^{-1} fresh wt)	CP (μmol ml^{-1})	IW (μmol g^{-1} body wt)	(μmol g^{-1} fresh wt of BWM)	(μmol g^{-1} fresh wt of BWM)	% of total ATP generation
Transphosphorylation of the phosphagen						
$-\Delta$ [phospho-L-arginine]	13.13	0	0	13.13	13.13	30.4
Anaerobic glycolysis						
$+\Delta$ [strombine]	6.77	0	0	6.77	10.16	25.3
$+\Delta$ [octopine]	0.51	0	0	0.51	0.77	
Succinate-propionate pathway						
$+\Delta$ [malate]	0.15	0	0	0.15	0.23	34.9
$+\Delta$ [succinate]	1.36	0.16	0	1.53	3.83	
$+\Delta$ [propionate]	1.04	0.89	0.40	2.83	11.00	
Formation of acetate						
$+\Delta$ [acetate]	0	0.53	0.49	1.60	4.00	9.3
Total					43.12	99.9

Firstly, the transphosphorylation of phospho-L-arginine provided 13.6 μmol ATP g^{-1} fresh wt during 24 h of anaerobiosis. This amount, which was turned over during the first 12 h of anaerobiosis, accounted for 30.4% of the total yield of anaerobic energy.

Secondly, anaerobic glycolysis delivered 10.9 μmol ATP g^{-1} fresh wt (25.3%), as calculated from the amount of strombine and octopine synthesized during 24 h of anaerobiosis. The accumulation of the opines seems to be more pronounced during the beginning of anaerobiosis. This indicates a high energy demand during the first few hours of anaerobiosis as can be derived from the initial conversion of the phosphagen as well.

The accumulation of octopine was minor in most of the experiments contributing no more than 1 to 2 μmol ATP g^{-1} fresh wt. It can be generalized, therefore, that during environmental anaerobiosis strombine, and not octopine, is the major endproduct of anaerobic glycolysis.

The third source of anaerobic energy is the succinate-propionate pathway. Its contribution of ATP is indicated by an increase of the cellular concentrations of succinate and propionate which commenced right from the beginning of anaerobiosis (Table 3 d). The carbonic acids were also released into the coelomic plasma, but only propionate was released into the ambient sea water (Table 4). During the beginning of anaerobiosis, aspartate is degraded, its carbon chain possibly being channelled into succinate and its amino group into alanine (Schöttler, 1980; Zandee *et al.*, 1980).

Fourthly, ATP can be provided from the formation of acetate via acetyl-CoA (Wienhausen, 1981). The concentration of this volatile fatty acid increases within the coelomic plasma and it is also released into the ambient sea water (Table 4).

The latter two metabolic routes, which are located within the mitochondria, synthesize ATP more efficiently than anaerobic glycolysis. For reasons of redox balance, ⅙ of the total amount of both succinate and propionate synthesized must originate from the citric acid cycle via citrate, isocitrate and 2-oxoglutarate, the typical reaction sequence during aerobic metabolism (Schöttler, 1980; Zandee *et al.*, 1980). The remaining ⅚ are formed via the fumarate reductase reaction. Both pathways join into succinyl-CoA, the first intermediate of propionate formation (Schroff and Zebe, 1980; Schulz and Kluytmans, 1983). Since propionate is already formed at the very beginning of anaerobiosis, it is unlikely that succinyl-CoA synthesized via 2-oxoglutarate is converted first into succinate instead of being metabolized directly into propionate.

On the basis of these assumptions, the yield of ATP was calculated from concentrations of the respective

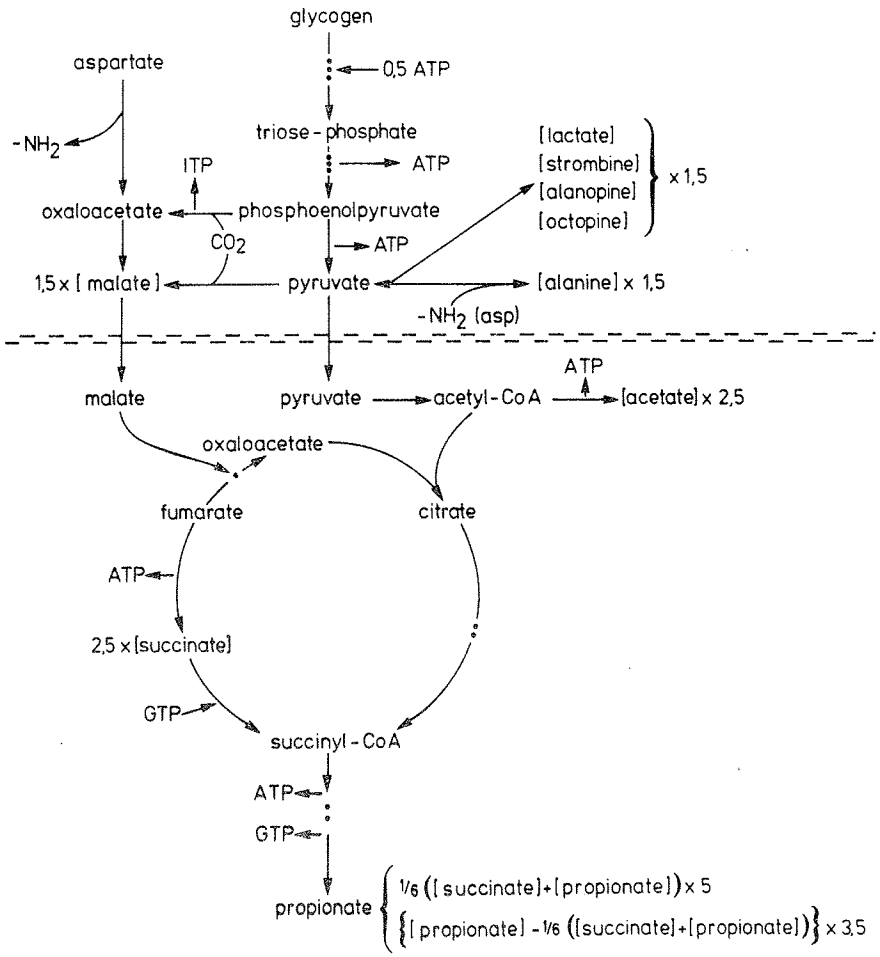

Fig. 1. *Sipunculus nudus.* Scheme of anaerobic energy metabolism starting from aspartate and glycogen. The quantitative formation of ATP, which accompanies the accumulation of the respective intermediary products and endproducts, is indicated by numbers associated with the respective metabolites. For propionate, the yield of ATP is given assuming redox balance during synthesis of succinate and propionate. The dots indicate metabolites which are not shown. (For details see text)

metabolites as follows (μmol ATP per μmol metabolite): acetate ≙ 2.5; succinate ≙ 2.5; propionate formed via 2-oxoglutarate ≙ 5; propionate originating from succinate ≙ 3.5. Metabolisation of aspartate into succinate will not diminish the yield of ATP if there is a concomitant net synthesis of alanine which derives its carbon chain from glycolysis (Fig. 1).

From the amounts of malate, succinate, propionate and acetate accumulated, a total yield of 19.1 μmol ATP g^{-1} fresh wt was calculated which corresponds to 44% of anaerobically provided energy (Fig. 1 and Table 5).

The analysis of the energy metabolism during 24 h of anaerobiosis clearly demonstrates that like *Cardium tuberculatum* (Gäde, 1980), *Sipunculus nudus* exploits both anaerobic glycolysis and the succinate-propionate pathway to produce ATP. The contribution of energy via anaerobic glycolysis is clearly indicated by the pronounced accumulation of strombine, which occurs at the beginning of anaerobiosis.

Previously, synthesis of ATP via anaerobic glycolysis may have been underestimated during environmental hypoxia, since the concentrations of the endproducts alanopine and strombine could not be determined. Only de Zwaan and Zurburg (1981), and Zurburg *et al.* (1982) quantified the concentration of strombine which they found to accumulate in the adductor muscle of *Mytilus edulis* during the initial phase of hypoxia and during postanaerobic recovery (de Zwaan *et al.*, 1983).

From Gäde's (1980) data, and those reported here, it might be predicted that during the first 2 to 6 h of environmental hypoxia, several invertebrate species may supply their energy requirements mainly from anaerobic glycolysis, leading to the accumulation of lactate and/or a specific opine. This time period of anaerobiosis is similar to that normally occurring in the intertidal habitat. It can be concluded, therefore, that during low tide, at least in some species, anaerobic glycolysis might be more important for the provision of energy than the succinate-propionate pathway.

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