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Recovery from Anaerobiosis of the Lugworm, *Arenicola marina* L.: Changes of Metabolite Concentrations in the Body-Wall Musculature

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Summary. 1. In the body-wall of *Arenicola marina* the changes of concentrations of ATP, ADP and AMP, phosphotaurocyamine, succinate, acetate and propionate, alanine and aspartate were estimated during recovery following 24 h of anoxia. The energy charge was calculated from the adenylates.

2. After 24 h of anoxia the energy status which comprises the energy charge and the concentration of phosphotaurocyamine had decreased; the concentrations of succinate, acetate, propionate and alanine were elevated, thus indicating an anaerobic state of the energy metabolism (Table 1).

3. Within 40 min after the onset of aerobic conditions the adenylates showed concentrations typical of an aerobic state (Fig. 1 A); phosphotaurocyamine was replenished by 84% within 1 h (Fig. 1 C); about half of the succinate pool was metabolized within the first 20 min and reached control levels after 1 to 2 h (Fig. 2 B).

4. The accumulation of acetate and propionate continued during the first 30 to 60 min of recovery, but after this the concentrations of these metabolites decreased slowly (Fig. 2 C).

5. Alanine and aspartate concentrations decreased during recovery to approach control levels within 2 h (Fig. 2 A).

Introduction

Arenicola marina is found in huge numbers in the intertidal zones of the northern hemisphere. The lugworms dwell in U-shaped burrows which conceal the animals from enemies, protect them from wave action and prevent loss of water during periods of low tide. During high tide *Arenicola marina* maintains a current of water through its burrow thus aerating the gills (Krüger, 1971). At low tide, however, when irrigation becomes impossible, the animal could suffer from reduced concentrations, or even the complete absence of oxygen, unless some provisions are made.

Previous investigations using experimental anoxia suggest that *Arenicola marina* is well adapted to its habitat, not only by its mode of life but also by its ability to survive without oxygen for several days. Zebe (1975) and Surholt (1977a, b) demonstrated that the lugworm possesses an anaerobic energy metabolism characterized by the formation of succinate, acetate and propionate, a decrease of the energy charge, and depletion of phosphotaurocyamine and aspartate. Succinate is accumulated and metabolized within the animal. If anoxia continues for more than 24 h, acetate and propionate are excreted into the medium. Toulmond (1975a) showed anaerobic metabolism to commence at a partial pressure of oxygen (P_{O_2}) lower than 50 Torr. Simultaneously blood pH begins to decrease possibly due to the appearance of acidic metabolites. Decreased pH values of the blood (Toulmond, 1973) and elevated concentrations of acetate and propionate within the blood (Toulmond, personal communication) were also found in animals taken from their burrows after different periods of emersion.

By these experiments it was established that *Arenicola marina* can switch from aerobic to anaerobic energy metabolism during low tide. However, almost nothing is known about the reverse transition from anaerobic to aerobic metabolism. There are only limited data on oxygen consumption in the very early phase of recovery following anaerobiosis. Borden (1931) and Toulmond (1975b) found only a minor

Abbreviations: *Glu-6-PDH*, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); *LDH*, lactate dehydrogenase (EC 1.1.1.27); *HK*, hexokinase (EC 2.7.1.1); *PK*, pyruvate kinase (EC 2.7.1.40); *TCK*, taurocyamine kinase (EC 2.7.3.4); *E.C.*, energy charge or $\frac{[ATP]+0.5\,[ADP]}{[ATP]+[ADP]+[AMP]}$ (Atkinson, 1968)

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oxygen debt, but Wells (1949) found an increased frequency and a greater amplitude of ventilation movements. Van Dam (1937) estimated an increase in the volume of water pumped by the worm after anoxia.

In view of these somewhat contradictory reports it seemed necessary to investigate the recovery metabolism by monitoring the levels of characteristic metabolites involved in anaerobiosis. In particular we were interested (1) in the time needed by *Arenicola marina* to regain the energy status typical for aerobic metabolism and (2) in the time dependent changes of the concentrations of various compounds related to anaerobic energy metabolism, thereby revealing information on the duration of recovery from prolonged anoxia in *Arenicola marina*.

Materials and Methods

Animals

The lugworms (*Arenicola marina*) were collected from the intertidal zone of the East Frisian coast near Carolinensiel, and kept in sea-water at 7 °C for at least 24 h prior to the experiments. In one experiment the worms were used 3 weeks after collection.

Enzymes and Chemicals

The enzymes, coenzymes and substrates were obtained from Boehringer (Mannheim). Taurocyamine and phosphotaurocyamine were synthesized as described previously (Surholt, 1977b). Taurocyamine kinase was isolated from body-wall musculature of *Arenicola marina* (Surholt, 1979). The enzyme preparation was further purified by preparative isoelectric focusing using the LKB electrofocusing kit for granulated gels. A total amount of 150 units of taurocyamine kinase was applied to the middle of a pH gradient ranging from pH 4.5 to pH 9.0. After 24 h the gel plate was divided into 30 fractions and taurocyamine kinase eluted with 3 ml buffer. The main fraction of the enzyme recovered by this treatment contained approximately 90% of the total activity applied. It was homogeneous with regard to protein staining and demonstration of enzymatic activity following analytical isoelectric focusing on polyacrylamide gels. The isoelectric point was estimated at pH 7.4. The final preparation of taurocyamine kinase which was to be used for the estimation of the phosphagen was stabilized by addition of 60 mM mercaptoethanol.

Sephadex G-25 was obtained from Pharmazia (Uppsala), DEAE-Cellex-D from Bio-Rad (Richmond), "Ampholine", "Ultrodex" and polyacrylamide gel plates from LKB (Stockholm), Coomassie Brilliant Blue, phenazine methosulfate and nitro blue tetrazolium from Serva (Heidelberg), and all other chemicals from Merck (Darmstadt).

Incubations

Prior to each experiment the lugworms were randomly sampled from the tanks and adapted to 12 °C in the presence of air. Then the animals were incubated in oxygen deficient sea water for 24 h at 12 °C. Immediately afterwards the worms were transferred into fresh, well aerated sea water. After various periods of recuperation, samples of 5 worms each were removed, wrapped in aluminium foil, frozen in liquid nitrogen and stored at −70 °C. The body wall musculature was later dissected from the still frozen body of the worm and the metabolites extracted as described previously (Surholt, 1977b).

Estimation of Metabolites

Determinations of metabolites from perchloric acid extracts were performed by enzymatic assays (Warburg and Christian, 1936). ATP, ADP and AMP were estimated according to the procedure of Jaworek et al. (1974a, b), succinate to that of Michal et al. (1976), alanine to that of Williamson (1974) and aspartate to that of Bergmeyer et al. (1974).

Phosphotaurocyamine was estimated using the following coupled assay:

$$\text{phosphotaurocyamine} + \text{ADP} \xleftrightarrow{\text{TCK}} \text{taurocyamine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xleftrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NADP}^- \xleftrightarrow{\text{Glu-6-PDH}} \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+$$

$$\text{phosphotaurocyamine} + \text{glucose} + \text{NADP}^+ \leftrightarrow \text{taurocyamine} + \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+.$$

The assay mixture (0.825 ml) contained: 0.1 M Tris-HCl, pH 7.2; 20 mM glucose; 2.5 mM ADP; 1 mM NADP; 15 mM Mg^{2+} (acetate); 0.7 U hexokinase; 0.7 U glucose-6-phosphate dehydrogenase, and tissue extract. The reaction was started by addition of taurocyamine kinase (0.5 U).

The concentration of taurocyamine was estimated in the following system:

$$\text{taurocyamine} + \text{ATP} \xleftrightarrow{\text{TCK}} \text{phosphotaurocyamine} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xleftrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xleftrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

$$\text{taurocyamine} + \text{phosphoenolpyruvate} + \text{NADH} + \text{H}^+ \leftrightarrow \text{phosphotaurocyamine} + \text{lactate} + \text{NAD}^+.$$

The assay mixture (1.05 ml) contained: 0.2 M Tris-HCl, pH 8.0; 0.5 mM phosphoenolpyruvate; 6 mM ATP; 0.3 mM NADH; 15 mM Mg^{2+} (acetate); 3 U pyruvate kinase; 3 U lactate dehydrogenase, and tissue extract. The reaction was started by the addition of 0.5 U taurocyamine kinase.

Acetate and propionate were determined, after steam distillation, by gas chromatography as described previously (Surholt, 1977a).

Results

Anoxia in *Arenicola marina* resulted in anaerobic metabolism typical of a variety of invertebrates of the intertidal zone (de Zwaan, 1977; Zebe, 1977). In the body-wall musculature, 24 h of anoxia caused the ATP level to drop from 1.33 to 0.90 μmol/g wet weight with a concomitant increase of ADP and AMP concentrations. Accordingly the energy charge fell from 0.85 to 0.68. In addition, the phosphagen, phos-

Table 1. The effect of prolonged anoxia and of aerobic recovery on the metabolite content of the body-wall tissue of *Arenicola marina*. Concentrations are expressed in μmol/g wet wt. Means ± S.D., $n=3$, with the exception of fatty acids where $n=2$. The energy charge is calculated according to the equation $E.C. = \frac{[ATP]+0.5[ADP]}{[ATP]+[ADP]+[AMP]}$

Treatment of *Arenicola marina*	ATP	ADP	AMP	Adenylates	E.C.	Taurocyamine-phosphate	Taurocyamine	Succinate	Acetate	Propionate	Ala	Asp
Control	1.33 ±0.16	0.44 ±0.03	0.07 ±0.01	1.84 ±0.15	0.85 ±0.02	8.92 ±1.25	4.43 ± 1.39	0.24 ±0.25	0.5	0.2	9.80 ± 1.40	7.51 ±0.37
24 h of anoxia	0.90 ±0.31	0.58 ±0.04	0.26 ±0.09	1.73 ±0.25	0.68 ±0.10	1.82 ±0.77	11.87 ± 1.73	1.97 ±0.23	0.9	1.6	15.66 ± 6.79	2.39 ±0.43
1 h of aerobic recovery	1.41 ±0.15	0.37 ±0.09	0.09 ±0.01	1.87 ±0.24	0.85 ±0.01	7.51 ±1.70	6.23 ± 0.46	0.46 ±0.14	2.1	2.1	14.02 ± 2.66	4.10 ±0.90

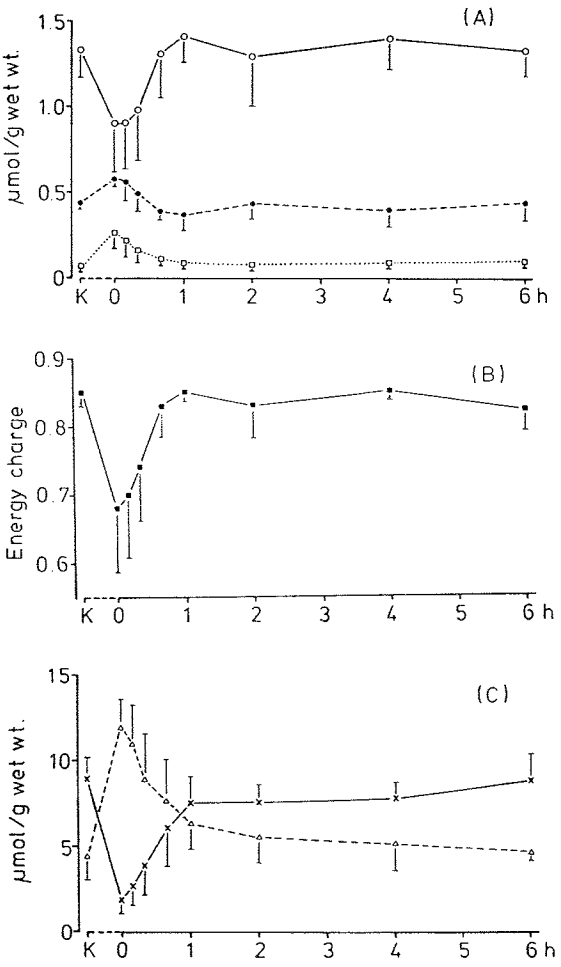

Fig. 1A–C. Energy charge and metabolite content of the body wall musculature of *Arenicola marina* during recovery following 24 h of anoxia. The period of anoxia is indicated by the dashed abscissa between K and O. A ATP o—o; ADP •---•; AMP □·····□. B Energy charge ■ —■. C Phosphotaurocyamine × — ×; taurocyamine △---△

photaurocyamine, was depleted by 80%. Generally, anaerobic metabolism is characterized by an accumulation of succinate and the subsequent synthesis of acetate and propionate. In the lugworm succinate rose from 0.24 to 1.97 μmol/g fresh weight, acetate from 0.6 to 0.9, and propionate from 0.1 to 1.9 μmol/g fresh weight within 24 h of the onset of anoxia. The involvement of amino acids in anaerobiosis was shown by the decrease of the content of aspartate from 7.51 to 2.39 μmol/g fresh weight and an increase of alanine from 9.80 to 15.7 μmol/g fresh weight (Table 1).

Recovery from anaerobiosis is reflected by the changes of the concentrations of the following metabolites: (1) the adenylates and phosphagen, (2) succinate and volatile fatty acids, and (3) alanine and aspartate.

From Fig. 1A it is evident that the ATP concentration started to increase after 10 to 20 min and reached a normal level for aerobic animals within 40 min. The contents of AMP and ADP began to decrease within the first 10 min of aerobiosis. After 1 h all the adenylates showed concentrations typical of an aerobic state. The co-ordinated change of the high energy phosphates was reflected by the energy charge (Fig. 1B) which reached control values within 1 h. Phosphotaurocyamine was replenished by 84% within 1 h and continued to rise for 5 h (Fig. 1C).

Accumulated succinate started to disappear immediately after the onset of aerobic conditions. About half of it was metabolized within the first 20 min (Fig. 2B). In contrast to succinate, acetate and propionate continued to rise for 30 to 40 min following aeration (Fig. 2C) and returned to control levels after 5 to 6 h. The concentration of aspartate remained low for 30 min, rose to about 50% of the control

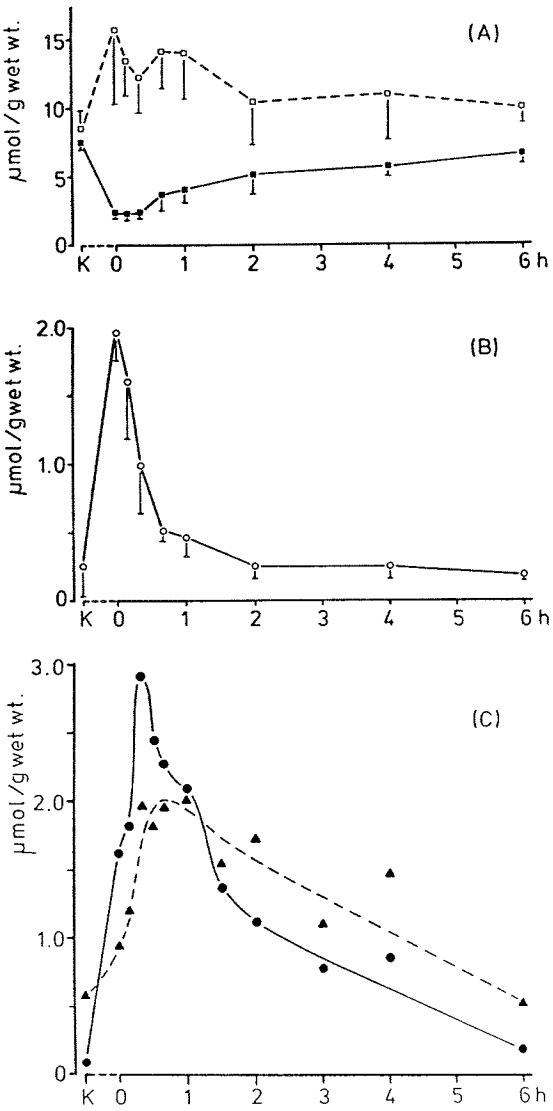

Fig. 2A–C. Metabolite content of the body-wall musculature of *Arenicola marina* during recovery following 24 h of anoxia. A Alanine □---□; aspartate ■—■. B Succinate ○—○ C Acetate ▲----- ▲; propionate ●—●

values after 40 to 60 min and then continued to increase steadily over a period of 6 h (Fig. 2A). Due to great individual variations in alanine content, the duration of the degradation of this amino acid cannot be accurately stated but from Fig. 2A it is obvious that at least after 6 h the concentration of alanine approximated that of controls.

Discussion

Even under natural conditions *Arenicola marina* may suffer from prolonged anoxia since its habitat may be emersed for up to 8–10 h during each tidal cycle; under extreme circumstances an emersion of 12 to 24 h may even be possible. Since blood P_{O_2} and percentage oxygen saturation, S_{O_2}, dropped to 1.2 Torr and 12%, respectively, after 1 h of emersion (Toulmond, 1973), the survival of the lugworm during prolonged anoxia can only be ensured by anaerobic energy production and the reduction of activity. The periods mentioned are short, however, when compared to the lugworm's ability to withstand 5–6 days of complete anoxia without apparent harm.

As soon as the burrows are immersed again by the rising tide the lugworm has access to oxygen-rich water and can switch back to aerobic metabolism. The data presented here show that the energy status, which comprises the adenosinephosphates and the phosphagen, increases rapidly during the first 40 min of anaerobiosis. After a maximum of 1 h of recovery an energy status is reached which allows the animal to perform its normal aerobic activity.

The rapid decrease of succinate within 30 min of recovery indicates, firstly, an immediate cessation of fumarate reduction, oxidizing NADH during anaerobiosis (Schroff and Schöttler, 1977), and secondly, the transformation of this carbonic acid into other compounds. For example the continued synthesis of propionate during the first 30 min of recovery could utilize the remaining succinate; in addition the aspartate pool could also be replenished by succinate. Acetate also accumulates during the first hour of aerobiosis but as yet this cannot be explained. Both volatile fatty acids disappear rather slowly from the tissue reaching normal values after 5 to 6 h of recovery. This delay in the decrease of fatty acids corresponds to the slow recovery (2–3 h) of normal blood pH after anaerobic acidosis (Toulmond, 1973).

The fate of acetate and propionate during recovery is not known yet. They could either be metabolized or excreted into the incubation medium. In the latter case the fatty acids would be unlikely to influence the recovery metabolism since they would be extremely diluted.

Synthesis of aspartate and normalization of the energy status do not begin simultaneously during recovery, the aspartate level remaining low for about 30 min after transfer from anoxic conditions. The concentration of this amino acid then increases gradually over a period of 6 h.

Although alanine concentration appears to fluctuate, after 2 h the levels recorded are similar to those of the control. One should, however, keep in mind that the analysis deals only with the body wall musculature and it is possible that the other organs of the lugworm may respond differently.

From the change of the energy status and the

quick cessation of fumarate reduction one can conclude that *Arenicola marina* switches back to aerobic metabolism very rapidly. The lugworm is therefore able to utilize oxygen as soon as it is available even during short periods of immersion. This fast resumption of aerobic energy metabolism is certainly facilitated by the irrigation behaviour and the respiratory system of the lugworm. At high tide *Arenicola marina* irrigates the burrow by means of approximately 5 peristaltic waves of the body wall per minute and extracts about 60% of the available oxygen by successive shrinking and swelling of the gills (Milne-Edwards, 1838). The respiratory movements and a total blood flow of 14 ml/h (the blood volume of a 10 g animal is about 0.5 ml) through the branchial system at 150 Torr causes haemoglobin S_{O_2} to increase from 7 to 75% within 30 min (Toulmond, 1973; 1975). In the muscle cells the presence of two myoglobins, with a P_{50} lower than that of the lugworm's haemoglobin, ensure quick delivery of O_2 making aerobic metabolism possible (Weber and Pauptit, 1972).

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