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Kerstin Hauschild · Manfred K. Grieshaber

Oxygen consumption and sulfide detoxification in the lugworm *Arenicola marina* (L.) at different ambient oxygen partial pressures and sulfide concentrations

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Abstract The lugworm *Arenicola marina* is a typical inhabitant of intertidal flats. In its L-shaped burrow the animal is exposed to varying concentrations of O_2 and toxic sulfide depending on the tides. The lugworm is able to detoxify sulfide through its oxidation to thiosulfate. When exposed to declining O_2 tensions *Arenicola marina* reacted as an oxyconformer. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide the respiration was not affected. In contrast, the lugworm consumed significantly less O_2 at any Po_2 in the presence of 200 $\mu mol \cdot l^{-1}$ sulfide. Without sulfide anaerobic metabolism started at a Po_2 of approximatedly 10 kPa. Even at high O_2 tensions animals exposed to sulfide produced significantly more anaerobic metabolites compared with the controls. Accordingly the critical value Pc_M, the ambient Po_2 below which anaerobic metabolism starts, was shifted towards normoxia. Since O_2 supply was sufficient for aerobic metabolism, anaerobiosis was induced by sulfide. An influx of sulfide was observed at 25 as well as at 200 $\mu mol \cdot l^{-1}$ sulfide. The main product of sulfide detoxification in the lugworm was thiosulfate. Its synthesis increased with ambient Po_2 and depended on the sulfide concentration. Sulfide and thiosulfate were detected in the coelomic fluid, the blood, and the body wall of *Arenicola marina*. Only about 2% of the ambient O_2 was used for sulfide detoxification at 25 $\mu mol \cdot l^{-1}$ sulfide and about 50% at 200 $\mu mol \cdot l^{-1}$ sulfide, respectively. Even at the low sulfide concentration *Arenicola marina*'s capacity to detoxify sulfide was too low to maintain a complete aerobic metabolism.

Key words *Arenicola marina* · Oxygen consumption · Anaerobic metabolism · Sulfide detoxification · Thiosulfate production

K. Hauschild · M.K. Grieshaber (✉)
Institut für Zoophysiologie,
Lehrstuhl für Stoffwechselphysiologie,
Heinrich-Heine-Universität, Universitätsstrasse 1/26.12,
D-40225 Düsseldorf, Germany,
Tel.: +49-211/811-4990, Fax: +49-211/811-3897,
e-mail: griesha@uni-duesseldorf.de

Abbreviations *HEPES N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid · *HPIC* high-performance ion chromatography · *HPLC* high-performance liquid chromatography · $\dot{M}O_2$ rate of O_2 consumption ($\mu mol \cdot h^{-1} \cdot g^{-1}$wwt) · *Pc* critical Po_2, Pc_M critical Po_2 below which anaerobiosis starts · Pc_R critical Po_2 below which O_2 consumption decreases · *wwt* wet weight

Introduction

Hydrogen sulfide is a highly toxic substance reversibly inhibiting aerobic respiration of organisms at nanomolar to low micromolar concentrations primarily by binding to the cytochrome *c* oxidase (National Research Council 1979; Bagarinao 1992). Nevertheless, sulfidic environments such as the marine sediment of the benthos or intertidal flats are inhabited by a rich fauna (Fenchel and Riedel 1970; Völkel and Grieshaber 1995) that possess several mechanisms for surviving in the presence of sulfide (reviews: Vetter et al. 1991; Vismann 1991a; Bagarinao 1992; Völkel and Grieshaber 1995). A widespread adaptation to this detrimental ambient condition resides in the oxidation of sulfide to less toxic sulfur compounds, mainly to thiosulfate. Sulfide is oxidized either by the animal's tissue itself (Powell and Somero 1985; Vetter et al. 1987; Powell and Arp 1989; Bagarinao and Vetter 1989, 1990; Vismann 1990, 1993; Völkel and Grieshaber 1992) or by bacterial symbionts (Felbeck et al. 1981; Vetter 1991; Childress and Fisher 1992; Nelson and Fisher 1995). For this detoxification process O_2 is as necessary as for the maintenance of an aerobic metabolism. In sulfidic habitats, however, the availability of O_2 is often limited. Thus, the question arises to what extent sulfide influences O_2 consumption ($\dot{M}O_2$), the O_2-dependent mechanism of sulfide detoxification of mitochondria, and the mode of energy metabolism employed by sulfide-tolerant species.

Hitherto the effect of sulfide exposure on $\dot{M}O_2$ has mainly been determined in endosymbiont-containing

animals. All investigated chemoautotrophic species increased their $\dot{M}O_2$ under sulfidic conditions due to a rise in bacterial sulfur oxidation. In the presence of 150 $\mu mol \cdot l^{-1}$ sulfide the respiration of the hydrothermal vent clam *Calyptogena pacifica* was doubled (Childress and Mickel 1982). Up to 100 $\mu mol \cdot l^{-1}$ sulfide, *Solemya reidi* also increased its $\dot{M}O_2$ and allocated its energy demand from an aerobic metabolism. At higher sulfide concentrations respiration decreased and anaerobic end products were accumulated in the symbiont-free tissue of the clam (Anderson et al. 1987, 1990). The respiration of the related species *Solemya velum* increased even in the presence of 500–800 $\mu mol \cdot l^{-1}$ sulfide (Chen et al. 1987).

Animals that lack chemoautotrophic bacteria reacted to sulfide in various ways. The $\dot{M}O_2$ of the fat innkeeper worm, *Urechis caupo*, tended to increase up to an ambient sulfide concentration of 30 $\mu mol \cdot l^{-1}$, but declined with further increasing sulfide (Eaton and Arp 1993). Johns et al. (1997) reported a slight increase in $\dot{M}O_2$ in the calocaridid mud shrimp *Calocaris macandreae* when exposed to sulfide concentrations in excess of 150 $\mu mol \cdot l^{-1}$ under normoxia and in excess of 100 $\mu mol \cdot l^{-1}$ under hypoxia. The hydrothermal vent crab *Bythograea thermydron* has been shown to maintain the heart and scaphognathite rates even during exposure to high ambient concentrations of 1.4 $mmol \cdot l^{-1}$ sulfide (Vetter et al. 1987). This has been interpreted as indication of continued O_2 consumption under these conditions (Eaton and Arp 1993), and Vetter et al. (1987) attributed it to an efficient sulfide detoxification system in the hepatopancreas of the crab. In the same species the detoxification product thiosulfate accumulating in the hemolymph during sulfide exposure increased the O_2 affinity of hemocyanin (Sanders and Childress 1992) which may augment in vivo the ability to regulate $\dot{M}O_2$ at low Po_2 values and result in a decrease of the critical Po_2 (Gorodezky and Childress 1994).

Another animal well suited to studies of the effect of sulfide on $\dot{M}O_2$ is the lugworm *Arenicola marina*. The symbiont-free polychaete lives in L-shaped burrows in the reduced sediment of the intertidal zone (Krüger 1971). During high tide the burrows are irrigated with oxygenated seawater by the animals (Wells 1945) allowing them to maintain an aerobic metabolism. Any sulfide which may diffuse or may be washed into the burrow will be oxidized or flushed out. In contrast, during low tide irrigation stops, and the O_2 content in the stagnant burrow water decreases rapidly (Watling 1991). Under these conditions the lugworm switches to an anaerobic metabolism (Schöttler et al. 1984; Zebe and Schiedek 1996). Sulfide diffusing into the burrow cannot be removed or detoxified and the lugworm is therefore exposed to increasing concentrations of sulfide (Völkel et al. 1995).

Arenicola marina is highly insensitive to sulfide and survives sulfide exposure for several days (Groenendaal 1980). Its tolerance is based on various strategies, namely, (1) maintaining internal sulfide concentration below ambient by acidification of the coelomic fluid (Groenendaal 1981; Völkel and Grieshaber 1992), (2) switching to an anaerobic metabolism in the presence of sulfide (Völkel and Grieshaber 1992, 1994), and (3) detoxifying sulfide via mitochondrial mechanisms resulting in the production of non-toxic thiosulfate (Völkel and Grieshaber 1994, 1996).

In this study $\dot{M}O_2$ of *Arenicola marina* was monitored at various Po_2 in the presence of 25 $\mu mol \cdot l^{-1}$ sulfide, a concentration which occurs in the burrow water during low tide (Völkel et al. 1995), and in the presence of 200 $\mu mol \cdot l^{-1}$ sulfide which is hardly ever reached in the field, but was applied to see how the lugworm reacts to a high sulfide exposure. From these experiments we wanted to obtain information on (1) the effect of sulfide on the Po_{2-} dependent $\dot{M}O_2$, (2) the influence of sulfide on the transition from an aerobic to an anaerobic energy metabolism, and (3) on the correlation between $\dot{M}O_2$ and sulfide detoxification.

Materials and methods

Animals

Arenicola marina were collected in spring and early summer 1992, in November 1993, and during the winter season 1994 from an intertidal flat at Texel, The Netherlands. Specimens were kept in the laboratory in darkened tanks with aerated artificial seawater containing ($mmol \cdot l^{-1}$): 435 NaCl, 28 $MgSO_4$, 24 $MgCl_2$, 10 $CaCl_2$, 10 KCl and 2 $NaHCO_3$ at 15 ± 1 °C prior to the experiments which were performed within 6 weeks after collection.

Flow-through setup

To keep the experimental conditions (sulfide[1] concentration, Po_2, and pH) constant, a flow-through system was used to determine $\dot{M}O_2$. In a 10 l reservoir containing artificial seawater a constant Po_2 was adjusted using a Woesthoff gas mixing pump (Typ 1H20/a-F, Bochum, Germany). Seawater from the reservoir was pumped with a peristaltic pump into a mixing chamber (volume 15 ml) into which sulfide was introduced from a stock solution via a second peristaltic pump and thoroughly mixed. Employing a system of three-way valves the incubation medium was pumped either directly or via the animal chamber into the electrode chamber, where the Po_2 was monitored using a Po_2 electrode (Radiometer E 504610, Copenhagen, Denmark). By switching the three-way valves, $\dot{M}O_2$ could be determined from the difference between the Po_2 before and after the animal chamber. Another three-way valve was situated immediately before the animal chamber and was used to withdraw water samples every hour to determine the sulfide concentration.

Determination of $\dot{M}O_2$

$\dot{M}O_2$ of *Arenicola marina* was measured at 15 ± 1 °C. Since the animals live in complete darkness under natural conditions, the animal chamber was darkened with aluminium foil. The weight of the specimens used was 2.8 ± 0.7 g (n = 55). Artificial seawater

[1] In this paper the term sulfide refers to total dissolved sulfide, i.e., the undissociated H_2S, HS^- and S^{2-}. At physiological pH the concentration of S^{2-} can be neglected (Goldhaber and Kaplan 1975)

(salinity 32 $mg \cdot ml^{-1}$) was prepared at least 12 h before the beginning of the experiment. It was buffered with 20 $mmol \cdot l^{-1}$ HEPES. The pH was adjusted to 8.00 with 5 $mmol \cdot l^{-1}$ KOH. The seawater was equilibrated 3 h before and during the experiment with a mixture of air and O_2 or N_2 to obtain the desired O_2 partial pressures. Calibration of the O_2 electrode was carried out with air-saturated distilled water and a saturated solution of sodium dithionite prior to and after the experiment. During the incubation period of 7 h $\dot{M}O_2$ was continuously monitored. Through switching the three-way valves, the Po_2 in the offered seawater was determined before and after the incubation. The flow rates were chosen to ensure that the difference in Po_2 between the bypass flow and the flow via the animal chamber was at least 0.7 kPa and not over 2.7 kPa. Flow rates ranged between 470 $ml \cdot h^{-1}$ at normoxia and 170 $ml \cdot h^{-1}$ at anoxia. $\dot{M}O_2$ was measured in the presence of 25 and 200 $\mu mol \cdot l^{-1}$ sulfide. Since the chemical oxidation of sulfide in the presence of O_2 leads to a declining Po_2 (Cline and Richards 1969), a higher Po_2 was maintained in the seawater reservoir to obtain the desired Po_2 in the animal chamber. After filling the flow-through system with seawater, stock solution of sulfide (1–3 $mmol \cdot l^{-1}$) was added, depending on the desired sulfide concentration in the incubation vessel. The solution was prepared by adding washed crystals of $Na_2S \cdot 9H_2O$ to N_2 saturated distilled water and adjusting to pH 8.00 with 1 $mmol \cdot l^{-1}$ KOH. Two worms were placed in the animal chamber after all parameters were constant.

Calculation of $\dot{M}O_2$

To calculate $\dot{M}O_2$ the following equation was used:

$$\dot{M}O_2 = \frac{\Delta P_{O_2} \cdot \alpha \cdot V_W}{G}$$

where $\dot{M}O_2 = O_2$ consumption ($\mu mol \cdot h^{-1} \cdot g^{-1}$wwt), ΔPo_2 = O_2 partial pressure difference between inlet and outlet of the animal chamber (torr), α = solubility coefficient of O_2 at 15 °C and 32 $mg \cdot ml^{-1}$ salinity corresponding to 1.644 $\mu mol \cdot l^{-1} \cdot torr^{-1}$ (Carpenter 1966), V_W = flow rate ($l \cdot h^{-1}$) and G = mass of the worms (g).

In the experiments without sulfide $\dot{M}O_2$ was calculated by taking the difference between the inflowing Po_2 and the outflowing Po_2. In the presence of sulfide the Po_2 also decreased due to the spontaneous chemical oxidation of sulfide while flowing through the animal chamber. This amount of consumed O_2 was determined before and after the introduction of the animals and subtracted from the total Po_2 difference.

Preparation of tissue samples

At the end of each experiment the coelomic fluid of the worms was quickly collected from a dorsal incision of the body wall. The blood vessels were then exposed by an extended dorsal incision along the abdominal region. Blood was withdrawn from the main vessel using a glass micropipette. To measure sulfur compounds, body fluids and tissues were derivatized with monobromobimane according to a method described by Vetter et al. (1989) modified as follows: for the determination of thiols in the body fluids, 50 µl coelomic fluid or an equivalent volume of blood, the latter of which was determined exactly by weighing, were mixed with a buffer solution consisting of 10 µl monobromobimane (48 $mmol \cdot l^{-1}$, Sigma), 50 µl acetonitrile and 50 µl HEPES (160 $mmol \cdot l^{-1}$ containing 16 $mmol \cdot l^{-1}$ EDTA, pH 8.00). To determine the contents of thiols in the tissue, freshly dissected pieces of body wall tissue (10–30 mg) were homogenized in buffer solution containing 10 µl monobromobimane, 75 µl acetonitrile and 75 µl HEPES using a glass homogenizer. The samples were incubated for 30 min in the dark at room temperature. The fluorescent derivatization products were stabilized by the addition of 100 µl methanosulfonic acid (65 $mmol \cdot l^{-1}$) and stored at −80 °C until measuring. The remaining body wall tissue was freeze clamped (Wollenberger et al. 1960) and stored in liquid N_2 for the determination of anaerobic metabolites.

Determination of sulfur compounds

The sulfide concentration of the incubation medium was determined spectrophotometrically using the methylene blue method (Gilboa-Garber 1971). Coelomic fluid, blood, and muscle tissue were analysed for sulfide and thiosulfate via high-performance liquid chromatography (HPLC) after derivatization with monobromobimane (Fahey et al. 1981; Newton et al. 1981; Vetter et al. 1989). A Merck/Hitachi L-6200 Intelligent Pump (Merck, Darmstadt, Germany) combined with a Merck LiChrospher 60 RP-select B (5 µm) column was used. The thiols were separated with an increasing hydrophobic gradient of 0.25% acetic acid and methanol and detected with a Merck/Hitachi F-1050 fluorescence spectrophotometer with an excitation filter of 380 nm and an emission wavelength of 480 nm.

Determination of anaerobic end products

For determination of anaerobic end products the body wall tissue was extracted according to Beis and Newsholme (1975). Succinate was measured spectrophotometrically as described by Beutler (1985). Alanopine and strombine were determined by high-performance ion chromatography (HPIC) using a DX-100 Ion Chromatograph (Dionex, Idstein, Germany). The opines were separated isocratically at 45 °C using a Polyspher AR AC ion exclusion column (Merck, Darmstadt, Germany) with $3.75 \cdot 10^{-5}$ $mol \cdot l^{-1}$ H_2SO_4 as solvent. The flow rate was 0.6 $ml \cdot min^{-1}$ and the pressure 82 bar. Opines were detected by conductivity.

Data treatment

Data are given as means ± standard deviations. Differences between the data were evaluated with a statistical software package (Sigma Stat, Jandel Scientific) using Student's *t*-test for independent samples at the $P \preceq 0.05$ level.

Results

Maintenance of the incubation parameters

During the experiments constant concentrations of sulfide and O_2 as well as a constant pH were achieved using a flow-through respirometer. During the incubation time of 7 h sulfide concentrations deviated by 6.7 ± 3.1% ($n = 49$) from the set values. The variation was independent of Po_2 or sulfide concentration used. The Po_2, adjusted at the beginning of the experiment, decreased during the incubation period which is probably due to a slight poisoning of the electrode. To determine the average Po_2 the animals were exposed to, initial and final Po_2 values were averaged. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide the deviation of Po_2 was 5.7 ± 4.1% ($n = 20$) which was lower than in the presence of 200 $\mu mol \cdot l^{-1}$ sulfide where Po_2 decreased by an average of 9.4 ± 4.6% ($n = 18$). The pH differed only slightly from the adjusted value of 8.00. The median pH during all sulfidic incubations was 7.99 ± 0.02.

Po_2- and sulfide-dependent O_2 consumption

In the absence as well as in the presence of sulfide *Arenicola marina* respired less O_2 with decreasing Po_2 (Fig. 1). In the absence of sulfide and between a Po_2 of 21.3 and 16.4 kPa, $\dot{M}O_2$ was reduced from 1.96 ± 0.13 to 1.71 ± 0.03 $\mu mol \cdot h^{-1} g^{-1} \cdot wwt$. Lowering the ambient Po_2 further the reduction in $\dot{M}O_2$ was more pronounced and decreased to 0.40 ± 0.06 $\mu mol \cdot h^{-1} \cdot g^{-1} wwt$ at 4.8 kPa. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide and normoxia $\dot{M}O_2$ was 2.01 ± 0.12 $\mu mol \cdot h^{-1} \cdot g^{-1} wwt$ and declined gradually to 0.48 ± 0.12 $\mu mol \cdot h^{-1} \cdot g^{-1} wwt$ at 4.9 kPa which is a 4.2-fold reduction. $\dot{M}O_2$ in the presence of 25 $\mu mol \cdot l^{-1}$ sulfide did not differ significantly from the corresponding value without sulfide at any Po_2. In contrast, at a concentration of 200 $\mu mol \cdot l^{-1}$ sulfide and normoxic conditions the specimens reduced their $\dot{M}O_2$ to 1.45 ± 0.10 $\mu mol \cdot h^{-1} \cdot g^{-1} wwt$ and decreased it further by a factor of 10 to 0.15 ± 0.03 $\mu mol \cdot h^{-1} \cdot g^{-1} wwt$ at 6.8 kPa. At anoxia no O_2 consumption could be detected at all.

Anaerobic metabolism

In the context of this investigation it is of interest to know at which Po_2 the energy metabolism switches from an aerobic to an anaerobic mode when different sulfide concentrations are present, i.e. is there a transition of the Pc_M from a lower (without sulfide) to a higher Po_2 region (in the presence of sulfide).

In lugworms incubated in the absence of sulfide steady-state levels of succinate at moderate hypoxia did not differ significantly from normoxic controls (0.20 ± 0.10 $\mu mol \cdot g^{-1} wwt$). With further decreasing Po_2 succinate increased linearly to 1.09 ± 0.26 $\mu mol \cdot g^{-1} wwt$ at 10.1 kPa and to 2.39 ± 0.39 $\mu mol \cdot g^{-1} wwt$ in anoxic conditions. In animals incubated with 25 $\mu mol \cdot l^{-1}$ sulfide succinate ranged from 0.93 to 1.12 $\mu mol \cdot g^{-1} wwt$ at a Po_2 of 20.9 and 9.8 kPa, respectively. With a further decrease in Po_2 the content of succinate increased further and amounted to 1.94 ± 0.72 $\mu mol \cdot g^{-1} wwt$ in the absence of O_2. At normoxia and 14.7 kPa significantly more succinate was produced than in control animals. When 200 $\mu mol \cdot l^{-1}$ of sulfide was added to the medium, the succinate contents at normoxia and 14.3 kPa were significantly increased when compared to controls and were also higher than those in the presence of 25 $\mu mol \cdot l^{-1}$ sulfide. Succinate ranged from 1.48 to 1.64 $\mu mol \cdot g^{-1} wwt$ between normoxia and 6.8 kPa and reached 2.64 $\mu mol \cdot g^{-1} wwt$ at anoxia (Fig. 2).

Without sulfide the tissue contents of strombine increased with decreasing Po_2 from 1.35 ± 0.66 $\mu mol \cdot g^{-1} wwt$ at normoxia to 7.32 ± 1.06 $\mu mol \cdot g^{-1} wwt$ at anoxia. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide strombine accumulated to 7.86 and 7.57 $\mu mol \cdot g^{-1} wwt$ at normoxia and anoxia, respectively. With 200 $\mu mol \cdot l^{-1}$ sulfide added to the incubation medium strombine contents ranged from 6.24 to 8.31 $\mu mol \cdot g^{-1} wwt$ (Fig. 3). In the absence of sulfide alanopine increased with decreasing Po_2 from 0.36 ± 0.24 $\mu mol \cdot g^{-1} wwt$ at normoxia to 2.78 ± 0.78 $\mu mol \cdot g^{-1} wwt$ at anoxia. At 25 $\mu mol \cdot l^{-1}$ sulfide the alanopine content ranged from 0.50 to 0.85 $\mu mol \cdot g^{-1} wwt$. In the presence of 200 $\mu mol \cdot l^{-1}$ sulfide the lugworm produced significantly more alanopine (2.15 ± 0.6 $\mu mol \cdot g^{-1} wwt$) than in the absence of sulfide only at normoxia (Fig. 4).

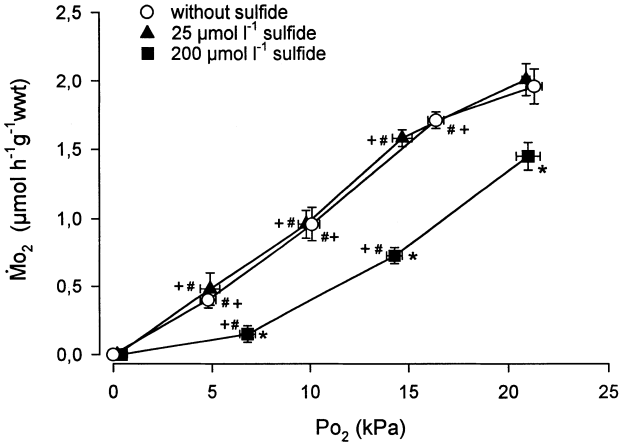

Fig. 1 Oxygen consumption ($\mu mol \cdot h^{-1} \cdot g^{-1} wwt$) of *Arenicola marina* during 7 h of incubation in a flow-through respirometer without and with 25 and 200 $\mu mol \cdot l^{-1}$ sulfide and various Po_2 (means ± SD, $n = 6$, with 25 $\mu mol \cdot l^{-1}$ sulfide $n = 5$, *= significantly different from the value without sulfide, $^{\#}$= significantly different from the following value, higher Po_2; $^{+}$ = significantly different from the corresponding value at normoxia)

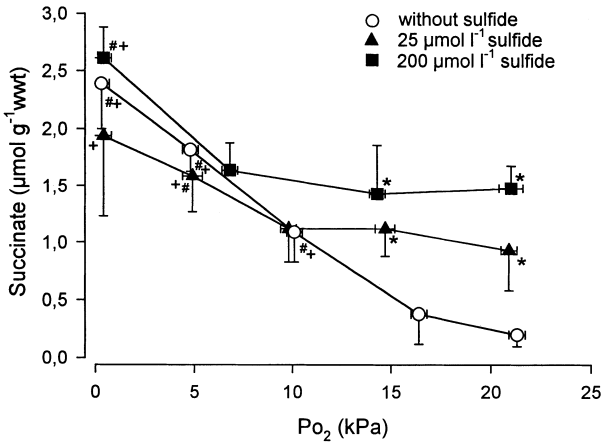

Fig. 2 Succinate ($\mu mol \cdot g^{-1} wwt$) in the body wall of *Arenicola marina* after 7 h of incubation in a flow-through respirometer without and with 25 and 200 $\mu mol \cdot l^{-1}$ sulfide and various Po_2 (means ± SD, $n = 5$, without sulfide $n = 6$, *= significantly different from the value without sulfide, $^{\#}$= significantly different from the following value, higher Po_2; $^{+}$ = significantly different from the corresponding value at normoxia)

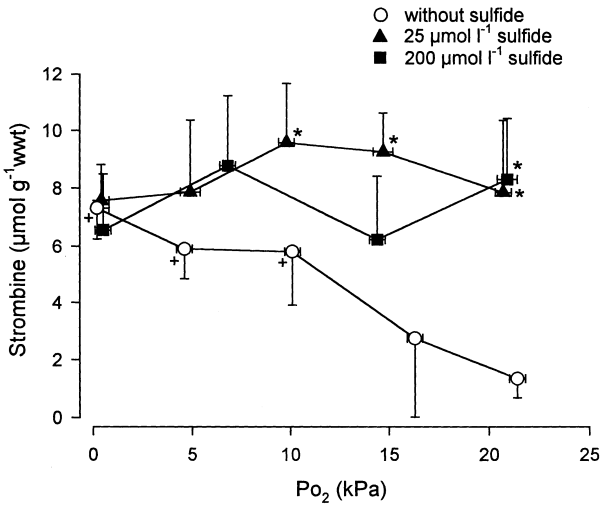

Fig. 3 Strombine ($\mu mol \cdot g^{-1}$wwt) in the body wall of *Arenicola marina* after 7 h of incubation in a flow-through respirometer without and with 25 and 200 $\mu mol \cdot l^{-1}$ external sulfide and various Po_2 (means ± SD, $n = 5$, without sulfide $n = 6$, * = significantly different from the value without sulfide, $^+$ = significantly different from the corresponding value at normoxia)

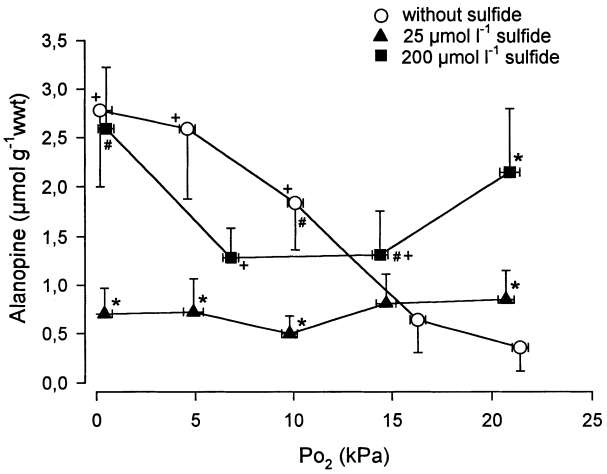

Fig. 4 Alanopine ($\mu mol \cdot g^{-1}$wwt) in the body wall of *Arenicola marina* after 7 h of incubation in a flow-through respirometer without and with 25 and 200 $\mu mol \cdot l^{-1}$ ambient sulfide and various Po_2 (means ± SD, $n = 5$, without sulfide $n = 6$, * = significantly different from the value without sulfide, # = significantly different from the following value, higher Po_2, $^+$ = significantly different from the corresponding value at normoxia)

The accumulation of sulfide in various body compartments

The concentrations of sulfide[2] were estimated in various body compartments of *Arenicola marina* in order to examine a possible permeation and accumulation of this sulfur compound present at 25 and 200 $\mu mol \cdot l^{-1}$ in the ambient medium under various Po_2. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide and under normoxic conditions the sulfide concentration in the body wall tissue was 39.0 ± 8.9 $\mu mol \cdot l^{-1}$ and increased to 66.6 ± 7.4 $\mu mol \cdot l^{-1}$ in the absence of O_2. In the blood and coelomic fluid sulfide concentrations amounted to 3.4 ± 1.2 and 0.8 ± 0.8 $\mu mol \cdot l^{-1}$ during normoxia and 10.5 ± 2.5 and 2.8 ± 0.5 $\mu mol \cdot l^{-1}$, respectively, during anoxia (Fig. 5a). At an ambient concentration of 200 $\mu mol \cdot l^{-1}$ sulfide, the concentration of this compound in the coelomic fluid increased from 0.3 ± 0.7 $\mu mol \cdot l^{-1}$ at normoxia to 20.1 ± 5.2 $\mu mol \cdot l^{-1}$ during anoxia. In the blood the corresponding concentrations were 7.8 ± 2.0 and 14.4 ± 4.3 $\mu mol \cdot l^{-1}$ sulfide. The sulfide concentration in the blood was significantly higher than in the coelomic fluid except during anoxic conditions. In the body wall tissue sulfide ranged from 150 to 158 $\mu mol \cdot l^{-1}$ which was significantly higher than in the body fluids (Fig. 6a).

[2] The thiol concentration in the body wall refers to the cell water content. This allows a comparison of the concentration in the body wall with the sulfide concentration in the medium as well as with the concentration of thiols in other compartments of the lugworm. Because the ratio of tissue wet weight to cell water is 0.8 a concentration of 100 $\mu mol \cdot l^{-1}$ cell water corresponds to 0.08$\mu mol \cdot g^{-1}$wwt

The detoxification of sulfide

The detoxification of sulfide to sulfur compounds of lower toxicity is a widespread adaptation to sulfide (reviews: Vetter et al. 1991; Vismann 1991a; Bagarinao 1992; Völkel and Grieshaber 1995). This process requires O_2 and it is therefore of interest to assess oxidation rates of sulfide at various sulfide levels and decreasing ambient Po_2.

Thiosulfate was the main oxidation product and it accumulated in all body compartments when ambient sulfide was present, but it was absent in control animals. Under normoxic conditions and 25 $\mu mol \cdot l^{-1}$ ambient sulfide the concentration of thiosulfate was 55.2 ± 13.4 $\mu mol \cdot l^{-1}$ in the body wall tissue and decreased to 29.1 ± 7.9 $\mu mol \cdot l^{-1}$ in the absence of O_2. In the blood and the coelomic fluid thiosulfate concentrations were the same (153 $\mu mol \cdot l^{-1}$) and decreased gradually and to the same extent to 38 ± 8 $\mu mol \cdot l^{-1}$ (blood) and 40 ± 16 $\mu mol \cdot l^{-1}$ (coelomic fluid) (Fig. 5b). In the presence of 200 $\mu mol \cdot l^{-1}$ sulfide and at a normoxic Po_2 thiosulfate accumulated up to 1780 ± 170 $\mu mol \cdot l^{-1}$ in the body wall tissue, 3370 ± 510 $\mu mol \cdot l^{-1}$ in the coelomic fluid, and 2530 ± 1120 $\mu mol \cdot l^{-1}$ in the blood. At a Po_2 of 14.3 kPa and the same ambient sulfide concentration thiosulfate accumulation was significantly reduced to 590 ± 40, 1050 ± 290 and 830 ± 240 $\mu mol \cdot l^{-1}$, respectively, in the body wall tissue, the coelomic fluid and in the blood. During anoxia only 80 $\mu mol \cdot l^{-1}$ thiosulfate accumulated in all three compartments (Fig. 6b).

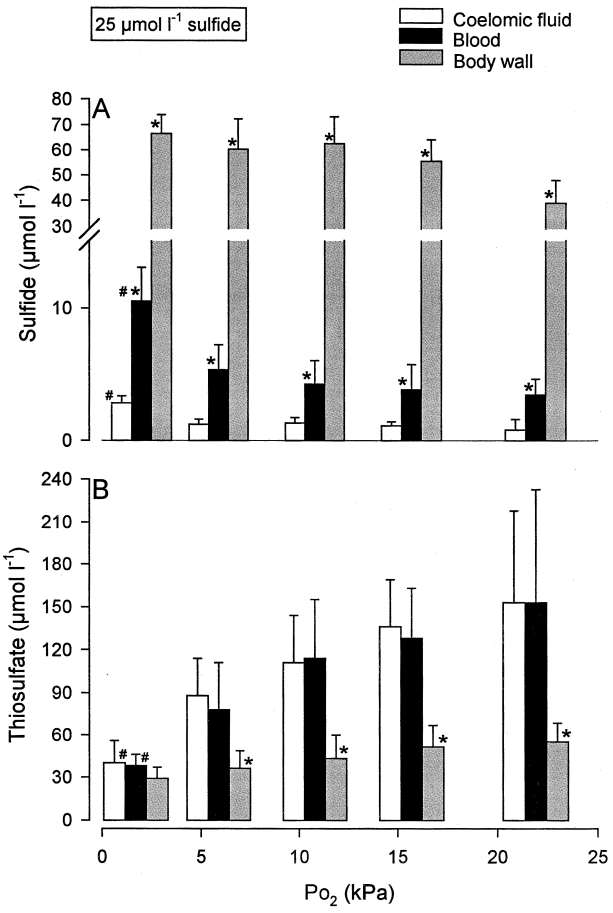

Fig. 5A, B Concentrations of (**A**) sulfide ($\mu mol \cdot l^{-1}$) and (**B**) thiosulfate ($\mu mol \cdot l^{-1}$) in the coelomic fluid, the blood, and the body wall of *Arenicola marina* after 7 h of incubation in a flow through respirometer in the presence of 25 $\mu mol \cdot l^{-1}$ sulfide and various Po_2 (means ± SD, $n = 5$, * = significantly different from the value of the coelomic fluid, in the body wall significantly different from the coelomic fluid and the blood, # = significantly different from the following value, higher Po_2)

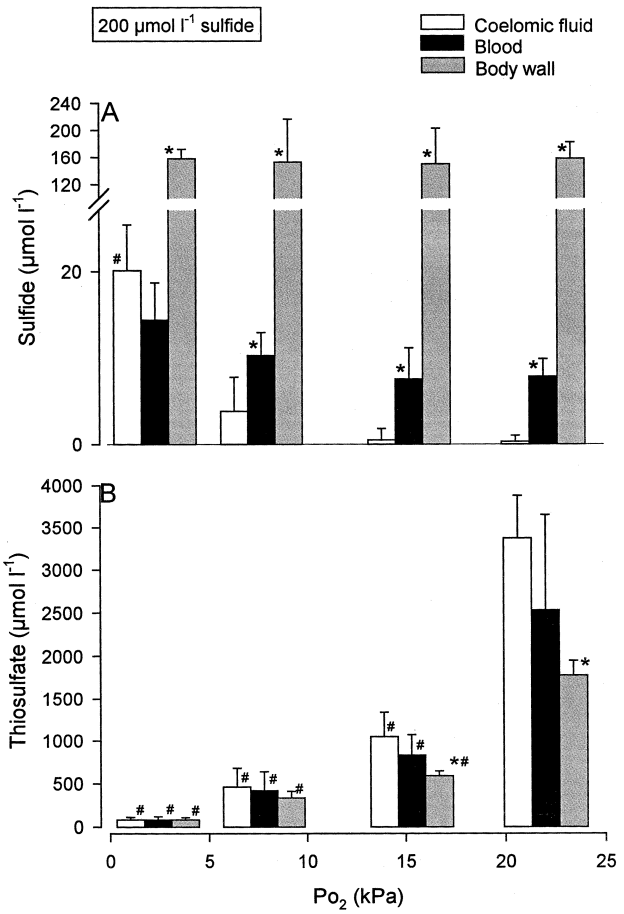

Fig. 6A, B Concentrations of (**A**) sulfide ($\mu mol \cdot l^{-1}$) and (**B**) thiosulfate ($\mu mol \cdot l^{-1}$) in the coelomic fluid, the blood, and the body wall of *Arenicola marina* after 7 h of incubation in a flow through respirometer in the presence of 200 $\mu mol \cdot l^{-1}$ sulfide at various Po_2 (means ± SD, $n = 6$, in the body wall $n = 3$, * = significantly different from the value in the coelomic fluid, in the body wall significantly different from the coelomic fluid and the blood, # = significantly different from the following value, higher Po_2)

Discussion

Po_2- and sulfide-dependent O_2 consumption

Various animal species show different patterns of O_2 consumption. Some keep their O_2 consumption more or less constant over a wide range of ambient Po_2 and are called oxyregulators, whereas others reduce $\dot{M}O_2$ with decreasing O_2 tensions and are termed oxyconformers (Dejours 1975). The transition from one respiratory mode to another has been termed the critical Po_2 or P_c if O_2 consumption alone is considered. If the mode of energy metabolism is also taken into account, one can distinguish a P_c at which respiration declines (Pc_R) and a second one at which anaerobiosis starts (Pc_M) (Pörtner and Grieshaber 1993).

When exposed to declining O_2 tensions *Arenicola marina* reacted as an oxyconformer. $\dot{M}O_2$ decreased slightly between a Po_2 of 21.3 and 16.4 kPa. The decline became more pronounced with a further reduction in ambient O_2 tension. Although this change in $\dot{M}O_2$ is significant, the latter Po_2 probably reflects the Pc_R. Normoxic $\dot{M}O_2$ of 1.96 ± 0.13 $\mu mol \cdot h^{-1} \cdot g^{-1}$wwt was relatively high due to the small size of animals. $\dot{M}O_2$ declines with increasing body weight (Toulmond 1975) and was found to be 1 $\mu mol \cdot h^{-1} \cdot g^{-1}$wwt in specimens weighing 6 g (Schöttler 1989) and 0.27 $\mu mol \cdot h^{-1} \cdot g^{-1}$ wwt in specimens of 33 g (Hauschild, unpublished results). Toulmond and Tchernigovtzeff (1984) reported in *Arenicola marina* (15–20 g fresh weight) a normoxic $\dot{M}O_2$ of 1.47 $\mu mol \cdot h^{-1} \cdot g^{-1}$wwt which remained constant down to a Po_2 of 16 kPa (Pc_R) and then declined gradually until anoxia. The slight decrease of $\dot{M}O_2$ in the higher Po_2 range observed in our study can be explained by the different experimental set up used. In the experiments carried out by Toulmond and Tchernigovtzeff (1984) the animals were placed in straight glass tubes and had to ventilate themselves in order to obtain oxygenated seawater. In our case a flow-through system

continuously pumped water over the animals in order to keep ambient conditions with regard to P_{O_2} and sulfide concentration constant. In relation to the water current, 431 $ml \cdot h^{-1}$ at 16.4 kPa, the volume of seawater ventilated by *Arenicola marina* is low: 10.4 $ml \cdot h^{-1} \cdot g^{-1}$wwt at 16 kPa (Toulmond and Tchernigovtzeff 1984). Therefore, in our set up the lugworms could hardly compensate for a decrease of P_{O_2} by enhanced ventilatory movements.

Sulfide can influence respiration in two ways: it could either decrease $\dot{M}O_2$ due to the inhibition of the electron transport chain by binding to cytochrome *c* oxidase or it could increase $\dot{M}O_2$ because the oxidation of sulfide to thiosulfate requires additional O_2. The presence of 25 $\mu mol \cdot l^{-1}$ sulfide had no effect on the $\dot{M}O_2$ of *Arenicola marina* throughout the whole range of ambient P_{O_2}. At an ambient concentration of 200 $\mu mol \cdot l^{-1}$ sulfide, however, $\dot{M}O_2$ decreased significantly at all three P_{O_2} values measured. Eaton and Arp (1993) who investigated the sulfide-dependent $\dot{M}O_2$ of *Urechis caupo* obtained similar results. Up to the external concentration of 30 $\mu mol \cdot l^{-1}$ sulfide, this species was able to maintain or even to increase its $\dot{M}O_2$ slightly, whereas at higher sulfide concentrations a decrease was observed. The sulfide-dependent reduction of $\dot{M}O_2$ coincided with the concentrations of sulfide found in the burrows of *Urechis caupo* during low tide (Arp et al. 1992). In the burrows of the lugworm Völkel et al. (1995) detected a sulfide concentration up to 30 $\mu mol \cdot l^{-1}$ in summer and up to 20 $\mu mol \cdot l^{-1}$ in autumn. It seems that *Arenicola marina* is adapted at least with regard to O_2 consumption to concentrations of sulfide occurring in its habitat.

Sulfide-dependent changes in O_2 consumption are also found in the calocaridid mud-shrimp *Calocaris mecandreae* when exposed to sulfide. This species was able to maintain or even to increase its O_2 consumption in the presence of up to approximately 130 $\mu mol \cdot l^{-1}$ sulfide under normoxic and also under hypoxic conditions (Johns et al. 1997), whereas at higher sulfide levels $\dot{M}O_2$ became lower than in control animals. Obviously O_2 can serve for sulfide oxidation and an aerobic energy metabolism leading to an increase in $\dot{M}O_2$, but it can also remain constant or decrease at certain sulfide levels. This competition for O_2 could limit O_2 provision for an aerobic ATP synthesis and, hence, lead to anaerobiosis.

Anaerobic metabolism

The anaerobic energy metabolism of many invertebrate species has been elucidated in detail (Grieshaber et al. 1994). In the lugworm anaerobiosis can be monitored by estimating the tissue contents of the opines, alanopine and strombine, which are the end products of anaerobic glycolysis (Siegmund et al. 1985). Succinate can serve as a particular sensitive indicator of an anaerobic metabolism in the mitochondria, because its steady-state levels increase about five- to tenfold as soon as O_2 becomes limiting in these organelles (Grieshaber et al. 1988).

Succinate contents remained low in the body wall tissue of lugworms during moderate hypoxia and in the absence of sulfide, but increased approximately fivefold at an ambient P_{O_2} of 10.1 kPa and were elevated by about tenfold during anoxia. Thus, the P_{c_M} is reached in *Arenicola marina* under the experimental conditions given at around 10 kPa. At a normoxic ambient P_{O_2} and sulfide concentrations similar to those occurring in the field, the tissue content of succinate was already significantly higher (4.7-fold) than in controls and was even more pronounced (7.4-fold) at high ambient sulfide. The tissue levels remained nearly the same over a wide range of ambient hypoxic P_{O_2} and increased further towards severe anoxic conditions. Although $\dot{M}O_2$ was not influenced by the presence of low sulfide concentrations, the P_{c_M} was shifted from 10.1 kPa to normoxia which means that the energy metabolism was at least partially changed from an aerobic to an anaerobic mode. Sulfide must, therefore, interfere with the mitochondria of *Arenicola marina* by either diminishing the O_2 supply to cytochrome *c* oxidase and/or partially inhibiting this part of the electron transport system. Thus, the effect of sulfide induced anaerobiosis is identical to an environmental anaerobiosis (Grieshaber et al. 1992).

Environmental anaerobiosis is also indicated by strombine which is mainly accumulated during ambient lack of O_2 (Kreutzer et al. 1989). Its tissue content was significantly increased at a P_{O_2} of 10.1 kPa when compared to normoxic controls. At both sulfide concentrations the strombine content was about six times higher at normoxia than without sulfide. Again the P_{c_M} was shifted to normoxia. In contrast no clear effect of sulfide on the alanopine production was observed according to the preferred production of alanopine as a typical end product of functional anaerobiosis in the lugworm (Kreutzer et al. 1989).

A dependency between O_2 consumption and anaerobiosis in the presence of various concentrations of ambient sulfide has also been shown in other species. Anderson et al. (1990) found in *Solemya reidi* an increase in respiration up to 100 $\mu mol \cdot l^{-1}$ sulfide and a decrease of $\dot{M}O_2$ with the concomitant onset of succinate accumulation in the presence of 250 $\mu mol \cdot l^{-1}$ sulfide. The oligochaete *Tubificoides benedii* can maintain a completely aerobic metabolism at sulfide concentrations up to 175 $\mu mol \cdot l^{-1}$ as indicated by a lack of succinate accumulation. At 300 $\mu mol \cdot l^{-1}$ sulfide, anaerobic pathways are utilized only to a small extent and even at sulfide concentrations as high as 450 and 600 $\mu mol \cdot l^{-1}$ a partially aerobic metabolism is sustained. However, if ambient O_2 concentrations are decreased, much lower sulfide concentrations cause the animal to utilize anaerobic pathways. At O_2 partial pressures as low as 1.5 kPa, where the worms can maintain a fully aerobic metabolism in the absence of sulfide, minimal concentrations of sulfide are sufficient to give rise to an anaerobic metabolism (Dubilier et al. 1994). From these comparative analyses it is obvious that different species can defend their aerobic metabolism even in the presence

of high ambient sulfide concentrations, whereas others succumb to anaerobiosis even at low ambient sulfide. The degree of sulfide oxidation varies within different species and this detoxification mechanism cannot always prevent the presence of sulfide at concentrations high enough to inhibit cytochrome *c* oxidase.

The detoxification of sulfide

Arenicola marina oxidizes sulfide in the mitochondria of the body wall tissue to thiosulfate (Völkel and Grieshaber 1994). Between 25 and 200 $\mu mol \cdot l^{-1}$ ambient sulfide the thiosulfate production increased with the Po_2 as well as with the sulfide concentration. Thiosulfate accumulated in the coelomic fluid, the blood, and the body wall tissue of the lugworm.

In the body wall the thiosulfate content was about 60% lower than in the coelomic fluid and in the blood, the concentrations of which did not differ significantly. In the body wall of *Halicryptus spinulosus* less thiosulfate was also determined than in the hemolymph (Oeschger and Vetter 1992). An accumulation of thiosulfate in the body fluids was observed in *Bythograea thermydron*, *Solemya reidi*, and in different fish species as well (Vetter et al. 1987, 1989; Bagarinao and Vetter 1989). In the lugworm thiosulfate is probably transported from the tissue cells of the body wall into the extracellular milieu where the charged ions are accumulated due to their low permeability in biological membranes (Holmes and Donaldson 1969). Hitherto nothing is known about the postulated thiosulfate transport from the muscle cells. The concentration difference between the compartments as well as the low permeability point to an efficient transport or exchange process. The removal of intracellular thiosulfate could prevent an inhibition of the sulfide oxidation via its product and other damaging effects of high intracellular thiosulfate concentrations. The low toxicological impact of thiosulfate on vertebrates is thought to result from the poor permeability of the ion, the low rate of metabolisation, and its excretion via the kidney (Cardozo and Edelmann 1952; Foulks et al. 1952; Sörbo 1972).

In *Arenicola marina* mitochondrial sulfide oxidation is linked to the respiratory electron transport chain (Völkel and Grieshaber 1996) and during sulfide exposure the lugworm needs O_2 to oxidize sulfide as well as to maintain an aerobic metabolism. The data presented here prove that both processes can proceed simultaneously and that, in addition, an anaerobic component can also be present. Hence, we wanted to know how much O_2 was used for thiosulfate production. The amount of O_2 *Arenicola marina* consumed for detoxification and an aerobic metabolism was estimated assuming a relative amount of coelomic fluid and body wall tissue of 45% (Siegmund 1982), and of blood of 5% (Toulmond 1971) for a lugworm. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide and normoxic Po_2 in two specimens of *Arenicola marina* weighing 5.6 g a total amount of 0.54 μmol thiosulfate was found after 7 h of incubation. Since the ratio of sulfide to O_2 and thiosulfate is 1:1:0.5 in *Arenicola marina* (Völkel and Grieshaber 1994), only about 1.4% of the consumed O_2 was needed to produce the amount of thiosulfate accumulated at normoxia. The percentage increased to 3.4% at a Po_2 of 4.9 kPa. Accordingly the amount of O_2 available to maintain aerobic metabolism was hardly affected by the detoxification. Yet sulfide induced at least a partial shift to anaerobiosis. Obviously the capacity of the sulfide oxidation was too low to prevent the inhibition of the cytochrome *c* oxidase which is confirmed by the accumulation of sulfide in the body wall tissue. Nevertheless the $\dot{M}O_2$ of the uninhibited electron transport chain remained at a level as high as during normoxia. This can only mean that under the sulfidic stress the animals increase their aerobic energy turn over using the still aerobically working mitochondria to a greater extent. Perhaps less O_2 than assumed was at the lugworm's disposal due to an underestimation of the amount of O_2 used to detoxify sulfide. The amount of thiosulfate excreted into the medium during the incubation period of 7 h could not be considered, since thiosulfate was highly diluted due to the flow-through conditions. In the medium thiosulfate produced via chemical sulfide oxidation was present as well, in concentrations from 2.2 to 7.2 $\mu mol \cdot l^{-1}$ at anoxia and normoxia, respectively.

In the presence of 200 $\mu mol \cdot l^{-1}$ sulfide the $\dot{M}O_2$ of *Arenicola marina* was already significantly lowered when compared with the controls without sulfide. About 50% of the consumed O_2 was used for thiosulfate production. Accordingly the amount of O_2 available to maintain aerobiosis was remarkably reduced. An onset of anaerobic metabolism occurred even at normoxia, but the lugworm could resort to a residual aerobic metabolism at the normoxic and hypoxic Po_2. This was confirmed by the determination of succinate which increased significantly at severe anoxia. Sulfide concentrations in the body compartments should corroborate the assumption of the simultaneous occurrence of sulfide oxidation, aerobic energy supply and anaerobiosis.

The accumulation of sulfide

Biological membranes are highly permeable to sulfide (Beerman 1924) with a higher permeability to undissociated H_2S than to HS^- (Jacques 1936; Julian and Arp 1992). The pH gradient between the seawater and the lugworm's body fluids causes lower internal sulfide concentrations (Groenendaal 1981; Völkel and Grieshaber 1992).

At each Po_2 less sulfide was detected in the coelomic fluid than in the blood which might be attributed to the higher pH in the latter leading to a lower amount of H_2S and consequently to a higher concentration of total sulfide. Moreover, the higher sulfide concentration in the blood could result from the detection of protein-bound sulfide as well as of endogenous mercapto-groups re-

leased during the preparation of the samples. In the body wall tissue the sulfide concentrations were clearly higher than in the body fluids. In the tissue of control animals sulfide was detected as well, yet not in the body fluids. An average concentration of 24.2 $\mu mol \cdot l^{-1}$ sulfide was found, independent of the Po_2. Probably instead of free sulfide mercapto-groups of body wall proteins were detected as sulfide with the used bromobimane method. This hypothesis was confirmed by the determination of a sulfide concentration below 1 $\mu mol \cdot l^{-1}$ in nearly protein-free pig fat-tissue (Hauschild, unpublished results).

Sulfide was detected as well in control animals of *Saduria entomon* and *Halicryptus spinulosus* (Vismann 1991b; Oeschger and Vetter 1992), but unfortunately the authors did not discuss their results. By subtraction of the constant value of 24.2 $\mu mol \cdot l^{-1}$ from the sulfide concentrations determined in animals incubated under sulfidic conditions, one should be able to obtain the amounts of sulfide resulting from the influx of the ambient medium. Nevertheless, in the presence of 25 $\mu mol \cdot l^{-1}$ sulfide the sulfide concentration in the body wall was 11 or 7 times higher than in the coelomic fluid and in the blood at anoxia. Moreover at each Po_2 the sulfide concentration in the body wall tissue was higher than the calculated value of about 15% assuming a tissue pH of 7.00 at anoxia (Kamp and Juretschke 1989). These data argue against the presence of total sulfide in a free diffusible form and suggest an immobilization of sulfide in the body wall tissue of the lugworm.

For meiofauna species, Powell et al. (1979) postulated an accumulation of sulfide in the body wall and its later excretion into the sulfide-free medium. In *Halicryptus spinulosus* and *Tubificoides benedii* sulfide was precipitated as iron sulfide in the cuticle (Oeschger and Janssen 1991; Dubilier et al. 1995). In contrast the separate preparation of the cuticle and the muscle tissue of the lugworm's body wall did not demonstrate an accumulation of sulfide in its cuticle. Hence, sulfide penetrated into the internal compartments of *Arenicola marina* without an external mechanism of defence. In the lugworm's body wall tissue sulfide could not be bound completely, since anaerobic metabolites were produced. Consequently, the respiratory chain was inhibited by sulfide. The cytochrome *c* oxidase in a mitochondrial preparation of the body wall was totally inhibited in the presence of 12 $\mu mol \cdot l^{-1}$ sulfide (Völkel and Grieshaber 1997). Even at normoxia in the presence of the low ambient sulfide concentration 14.8 $\mu mol \cdot l^{-1}$ sulfide was detected in the body wall tissue (the constant value of sulfide taken into account). This concentration is high enough to inhibit respiration. Nonetheless, according to the content of anaerobic metabolites the aerobic energy provision was not completely inhibited confirming the assumption of an immobilisation of sulfide in the body wall tissue. Thus, aerobic and anaerobic metabolism as well as sulfide oxidation took place simultaneously in the lugworm's body wall.

According to the reported data the sulfide adaptation of *Arenicola marina* results from both its ability to oxidize sulfide to thiosulfate and to shift to an anaerobic energy production. In the presence of 25 $\mu mol \cdot l^{-1}$ sulfide, a concentration occurring in the stagnant water of lugworm burrows during low tide (Völkel et al. 1995), its oxidative sulfide detoxification mechanism is limited even at normoxia when sufficient O_2 is supplied. Thus, the ability to maintain a completely aerobic metabolism under sulfidic conditions is not pronounced in the lugworm. Nonetheless, because of its highly developed anaerobic capabilities *Arenicola marina* can survive an exposure to sulfide even when the defence system is overwhelmed by sulfide diffusing into its body.

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