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EXCRETION OF THIOSULPHATE, THE MAIN DETOXIFICATION PRODUCT OF SULPHIDE, BY THE LUGWORM *ARENICOLA MARINA* L.

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Summary

Thiosulphate, the main sulphide detoxification product, is accumulated in the body fluids of the lugworm *Arenicola marina*. The aim of this study was to elucidate the fate of thiosulphate. Electrophysiological measurements revealed that the transepithelial resistance of body wall sections was 76±34 Ω cm^2 (mean ± S.D., *N*=14), indicating that the body wall of the lugworm is a leaky tissue in which mainly paracellular transport along cell junctions takes place. The body wall was equally permeable from both sides to thiosulphate, the permeability coefficient of which was $1.31\times10^{-3}\pm0.37\times10^{-3}$ cm h^{-1} (mean ± S.D., *N*=30). No evidence was found for a significant contribution of the gills or the nephridia to thiosulphate permeation. Thiosulphate flux followed the concentration gradient, showing a linear correlation (*r*=0.997) between permeated and supplied (10–100 mmol l^{-1}) thiosulphate. The permeability of thiosulphate was not sensitive to the presence of various metabolic inhibitors, implicating a permeation process independent of membrane proteins and showing that the lugworm does not need to use energy to dispose of the sulphide detoxification product. The present data suggest a passive permeation of thiosulphate across the body wall of *A. marina*. In live lugworms, thiosulphate levels in the coelomic fluid and body wall tissue decreased slowly and at similar rates during recovery from sulphide exposure. The decline in thiosulphate levels followed a decreasing double-exponential function. Thiosulphate was not further oxidized to sulphite or sulphate but was excreted into the sea water.

Key words: thiosulphate, permeability coefficient, excretion, lugworm, *Arenicola marina.*

Introduction

The sulphide-tolerance of many intertidal invertebrates depends to a large extent on their ability to oxidize toxic sulphide to less toxic sulphur compounds (Grieshaber and Völkel, 1998). In marine sediments, the exposure of animals to sulphide often occurs in the presence of limiting oxygen concentrations, so thiosulphate is the main oxidation product, probably because of the favourable ratio of sulphide detoxification to oxygen consumption (Vismann, 1991). Moreover, thiosulphate is non-acidic, soluble and still contains a large amount of energy (Bagarinao, 1992). In contrast to the highly toxic sulphide (National Research Council, 1979), the toxicity of thiosulphate is low (Sörbo, 1972). Thiosulphate is not bound to protein (Cardozo and Edelmann, 1952), and cytochrome *c* oxidase is not inhibited by thiosulphate (Vetter et al., 1989). The charged thiosulphate ion does not easily permeate biological membranes (Holmes and Donaldson, 1969). Consequently, the diffusion of sulphide into an organism and its subsequent detoxification result in a net concentration of thiosulphate within the organism. Hitherto, little was known about the further fate of thiosulphate. It may be degraded, oxidized or excreted. No thiosulphate transport system has yet been described for invertebrates, and the involvement of excretory organs in the elimination of this compound remains to be clarified.

In most endosymbiont-containing species, energy-rich thiosulphate serves as a further substrate for the bacteria, resulting in the production of sulphate as the final end-product of symbiotic energy provision (Childress and Fisher, 1992). When symbiont-free species, in which this kind of exploitation is impossible, are exposed to sulphide, thiosulphate is accumulated in the body compartments. Until now, only a few studies have dealt with the metabolism and excretion of thiosulphate.

In the haemolymph of the symbiont-free hydrothermal vent crab *Bythograea thermydron*, the thiosulphate concentration remained constant during 4 h of recovery following sulphide exposure (Vetter et al., 1987). No active excretion of thiosulphate was observed (Vetter, 1991). However, Gorodezky and Childress (1994) reported a release of thiosulphate from the haemolymph into the sea water *via* the gills of *B. thermydron*. These authors suggested that the major part of this release is achieved by diffusion across the gills, but

an active transport system capable of excreting thiosulphate against a concentration gradient could also be involved. In the fat innkeeper worm *Urechis caupo*, measurements of thiosulphate permeability in isolated sections of body wall tissue demonstrated that thiosulphate is 120 times more permeable through hindgut tissue than it is through body wall tissue (Wieting et al., 1995). In this species, thiosulphate is probably eliminated mainly by diffusion across the highly permeable hindgut (Arp et al., 1995). Anderson (1995) compared sulphide oxidation in isolated gills of two bivalves, the symbiont-free species *Mytilus californianus* and the symbiont-containing species *Lucinoma aequizonata*. Both bivalves produced thiosulphate as the sulphide detoxification product. In *M. californianus*, thiosulphate was excreted from the gills into the sea water, whereas it was oxidized further by the symbionts of *L. aequizonata*.

In the present study, we investigated the metabolism and the excretion of thiosulphate by the lugworm *Arenicola marina*. In the presence of oxygen, the lugworm is able to detoxify some of the sulphide that enters the body by oxidation to thiosulphate in the mitochondria of its body wall tissue (Völkel and Grieshaber, 1994). The detoxification product must be transported out of the mitochondria and the cells by mechanisms as yet unknown because it accumulates in low concentrations in the body wall tissue but in high concentrations in the coelomic fluid and in the blood (Hauschild and Grieshaber, 1997). We have studied the excretion of thiosulphate from these compartments. First, the permeation process of thiosulphate was characterized electrophysiologically using isolated sections of the body wall. The excretion of thiosulphate was further investigated in live animals and, to study the reverse effect, uptake of thiosulphate was also measured. Isolated sections of the body wall were used to elucidate the mechanisms of the thiosulphate permeation. From these experiments, we wanted to obtain information on (1) the excretion and further oxidation of thiosulphate, (2) the thiosulphate permeability of the body wall of the lugworm, (3) the participation of the nephridia and gills in excretion, and (4) the involvement of passive and/or active mechanisms in the elimination of thiosulphate.

Materials and methods

Animal collection and maintenance

Specimens of *Arenicola marina* L. were collected from intertidal flats near St Pol de Léon (Brittany, France) in March and October between 1993 and 1995. The worms were kept in the laboratory in darkened tanks containing a bottom layer of sediment (10–20 cm) from their natural habitat. The tanks were circulated with aerated artificial sea water at 15±1 °C. Prior to the experiments, the worms were kept for at least 24 h in aerated sea water to allow them time to empty their intestines.

Solutions

The artificial sea water (salinity 35 ‰) contained 435 mmol l^{-1} NaCl, 28 mmol l^{-1} $MgSO_4$, 23 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl and 10 mmol l^{-1} $CaCl_2$, buffered with 10 mmol l^{-1} Hepes. The pH was adjusted to 8.00 using 5 mmol l^{-1} KOH. In the substitution experiments, differing amounts of NaCl were replaced with equimolar concentrations of $Na_2S_2O_3$, Na_2SO_3 or Na_2SO_4. The number of replicates was three.

Tissue preparation

Lugworms were anaesthetized hypothermically, the head and tail were cut off and the trunk was opened by a longitudinal incision. The body wall was detached from the intestines, rinsed with artificial sea water and fixed on its mucosal side on a dissecting board. A Lucite ring was then glued with tissue adhesive (Histoacryl Blau, Braun, Melsungen, Germany) to the serosal side of the body wall, and the surrounding tissue was cut away. The preparation was mounted in an Ussing chamber with a 0.5 cm aperture specially designed to minimize edge damage (De Wolf and van Driessche, 1986) and immediately perfused with artificial sea water. The Ussing chamber consisted of two half-chambers (volume 1.5 ml) which were separated by the vertically mounted tissue into the mucosal and the serosal side of the body wall preparation. A 3.4 mm thick space holder, which was mounted between the two half-chambers, kept the distance between the contact areas to 0.3 mm. Silicone grease was used to seal the edges on both sides, so that ion exchange between the two half-chambers could only occur through the tissue mounted between them.

Electrophysiological measurements

Throughout the experiment, the two sides of the body wall section were continuously perfused with artificial sea water at a flow rate of 7 ml min^{-1} in the mucosal compartment and of 3 ml min^{-1} in the serosal compartment. Voltage and current electrodes were Ag/AgCl wires in 1 mol l^{-1} KCl solution connected, using 1 mol l^{-1} KCl agar bridges, to the bathing compartments. For transepithelial measurements, the tissue was voltage-clamped to 0 mV using a low-noise voltage-clamp (van Driessche and Lindemann, 1978). Short-circuit current (I_{sc}) was continuously recorded on a stripchart recorder and a computer (Apple Mac IIcx) with a MacLab interface and a chart recorder program (Analog Digital Instruments, Castle Hill, Australia). Transepithelial resistance (R_t) was calculated from superimposed 10 mV pulses of 500 ms duration using Ohm's law. The resistance values were corrected for the solution resistance.

Determination of the permeability coefficient

To determine the permeability coefficient (P_t) of the body wall of the lugworm, a tissue preparation was mounted in an Ussing chamber. One side of the body wall was continuously perfused with artificial sea water containing various amounts of thiosulphate at a flow rate of 1.7 ml min^{-1}. The other side of the tissue was perfused by a peristaltic pump recirculating a small volume of artificial sea water (1.8 ml) at a flow rate of 0.3 ml min^{-1}. After 1.5 h, samples of sea water and body wall tissue were prepared to determine the concentration of

permeated thiosulphate and of thiosulphate taken up into the tissue. Since the concentration of supplied thiosulphate remained constant as a result of the flow-through conditions, and because the concentration was initially zero in the sea water into which thiosulphate permeated, the permeability coefficient was calculated using the following equation (Sten-Knudsen, 1978):

$$P_t = -v \frac{\log_e(1 - C_1 C_2^{-1})}{A t}, \quad (1)$$

where P_t is the permeability coefficient ($cm\,h^{-1}$), v is the volume into which thiosulphate permeated (cm^3), C_1 is the concentration of permeated thiosulphate ($mol\,cm^{-3}$), C_2 is the concentration of thiosulphate supplied ($mol\,cm^{-3}$), A is the cross-sectional area of the tissue (cm^2) and t is incubation time (h).

Characterization of the permeation process

To compare the permeability of thiosulphate from both sides of the body wall of the lugworm, a set of experiments was carried out in which sea water containing $10\,mmol\,l^{-1}$ thiosulphate was supplied either on the mucosal or on the serosal side. In the following experiments, thiosulphate was always supplied on the serosal side. To determine the concentration-dependence of the permeation process, artificial sea water containing thiosulphate at concentrations of $10–100\,mmol\,l^{-1}$ was supplied. The permeability coefficient of body wall sections containing gills or nephridia was also determined. Sections of body wall containing gills were dissected in the area of the fourth to the sixth segment, and sections of body wall containing nephridia were dissected between the tenth and the sixteenth segment. In addition, the permeability of thiosulphate was measured in the presence of various metabolic inhibitors such as cyanide, 2,4-dinitrophenol, iodoacetic acid and ouabain at concentrations of 2 and $5\,mmol\,l^{-1}$.

Desorption of thiosulphate in sections of body wall

Two sections of body wall tissue from one lugworm were glued on two Lucite rings and incubated for 1 h in artificial sea water containing $20\,mmol\,l^{-1}$ thiosulphate. One section was prepared for analysis of thiosulphate levels and the other was mounted in an Ussing chamber. Both sides of the tissue were perfused using a peristaltic pump to recirculate a small volume (1.8 ml) of sea water. After an incubation time of 1.5 h, samples of sea water on the mucosal and the serosal sides and of the body wall tissue were prepared for analysis of the concentration of thiosulphate. Assuming the section of body wall to be approximately cylindrical, its volume V was calculated according to the equation:

$$V = \pi r^2 h, \quad (2)$$

where r is radius and h is height. The total amounts of thiosulphate in the sea water and in the tissue before and after the incubation were calculated to balance the desorption.

Excretion of thiosulphate by live worms

Lugworms were incubated for 16 h in aerated artificial sea water with an initial sulphide concentration of $2.5\,mmol\,l^{-1}$, which was prepared by adding washed crystals of $Na_2S{\cdot}9H_2O$ to the sea water and adjusting the solution to pH 8.00 using $1\,mmol\,l^{-1}$ KOH. The worms were then incubated for 2 h in aerated sulphide-free sea water to remove all internal sulphide. The tissues of three specimens were prepared for analysis of thiosulphate, sulphite and sulphate levels. This time (t=0) was taken as the start of the recovery period. A specimen of each of the remaining worms was transferred into one of five glass vessels containing 10 ml of aerated sea water. After 1, 2, 4, 7, 12 and 24 h of recovery, the concentrations of the sulphur compounds were determined in the sea water and in the different body compartments of the worms. The experiment was replicated five times.

Influx of thiosulphate into live worms

To determine the influx of thiosulphate, lugworms were incubated in a flow-through chamber (volume 500 ml) in sea water containing $10\,mmol\,l^{-1}$ thiosulphate at a flow rate of $280\,ml\,h^{-1}$. After 0, 1, 2, 4 and 7 h, a worm was removed and prepared for analysis of thiosulphate levels in the coelomic fluid, the blood and the body wall tissue. The permeability coefficient was calculated from the equation of Sten-Knudsen (1978) with A being the surface area (cm^2) of the worm. The worms were considered to have an approximately cylindrical shape, and the following equation was used to determine their surface area:

$$A = 2\pi r(r + h), \quad (3)$$

where r is radius and h is height (length of the worm).

Determination of thiosulphate, sulphite and sulphate levels

Sea water, coelomic fluid, blood and body wall tissue were analysed for thiosulphate and sulphite levels using high-performance liquid chromatography (HPLC) after derivatization with monobromobimane (Fahey and Newton, 1987; Newton et al., 1981; Vetter et al., 1989). Details of this procedure have been published previously (Hauschild and Grieshaber, 1997).

Sulphate levels in the sea water and the coelomic fluid were determined using high-performance ion chromatography (HPIC) with a DX-100 ion-chromatograph (Dionex, Idstein, Germany), a Dionex AS 9 column and an AG 9 pre-column. The solvent, consisting of $1.8\,mmol\,l^{-1}$ Na_2CO_3 and $1.7\,mmol\,l^{-1}$ $NaHCO_3$, was pumped through the column at a flow rate of $1.3\,ml\,min^{-1}$. Detection was carried out using a bipolar pulsed conductivity cell. To obtain a low baseline conductivity, a Dionex micromembrane suppressor (AMMS 1) with $12.5\,mmol\,l^{-1}$ H_2SO_4 as solvent was used. Immediately after collection, 1 % formaldehyde was added to preserve the samples, which were then stored at −20 °C. Prior to measurement, samples of the coelomic fluid were mixed with acetonitrile (1:3) to denature the proteins and then centrifuged for 10 min at 14 550 ***g*** (Biofuge A, Heraeus Christ, Osterode, Germany).

The accuracy of these two procedures was ±5 %.

Treatment of the data

Results are presented as means ± standard deviations (S.D.). Differences between values were evaluated using a statistical software package (Sigma Stat, Jandel Scientific) using Student's *t*-test for independent samples and analysis of variance (ANOVA) to compare multiple groups. Both statistical tests were accepted as significant at $P \leqslant 0.05$.

Results

Electrophysiological measurements

Isolated sections of the body wall tissue of the lugworm were mounted in an Ussing chamber and characterized electrophysiologically. Within 30 min of incubation, I_{sc} and R_t became stable in the voltage-clamped tissue perfused with sea water on both sides. The initial transepithelial voltage V_t was –0.93±0.82 mV (N=14) with the serosal side as the reference. The initial I_{sc} levelled out at –25.7±19.1 μA cm^{-2} (N=14). R_t of the body wall was 76±34 Ω cm^2 (N=14).

Fig. 1 shows the time course of I_{sc} during a typical experiment in which various amounts of NaCl in the sea water (initial concentration 435 mmol l^{-1}) were replaced by equimolar concentrations of $Na_2S_2O_3$. The substitution of 50 % of the NaCl with $Na_2S_2O_3$ on the mucosal side induced a change in I_{sc} in the negative direction and an increase in R_t. When normal sea water was supplied again on the mucosal side, I_{sc} and R_t returned almost to their previous levels. In the case of the substitution of 100 % of the NaCl with $Na_2S_2O_3$, the changes in the electrical variables were more pronounced. When NaCl was substituted with $Na_2S_2O_3$ on the serosal side, I_{sc} became more positive and R_t increased. The effects were reversible with normal sea water. The substitution of only 3 % of the NaCl with $Na_2S_2O_3$ on the mucosal and on the serosal sides of the body wall section did not influence either I_{sc} or R_t.

Table 1. *Variations in the short-circuit current and the transepithelial resistance of sections of body wall from the lugworm* Arenicola marina *induced by the substitution of $Na_2S_2O_3$, Na_2SO_3 and Na_2SO_4 for 50 % NaCl in the sea water on the mucosal and the serosal side of the tissue*

	ΔI_{SC} (μA cm^{-2})		ΔR_t (Ω cm^2)	
Substitution	Mucosal	Serosal	Mucosal	Serosal
$Na_2S_2O_3$	–5.1±3.4	12.9±6.1	13.3±2.1	12.3±2.1
Na_2SO_3	–5.2±1.6	5.5±4.0	17.8±6.5	16.7±5.7
Na_2SO_4	–8.2±4.9	4.4±0.9	13.3±4.0	12.0±1.0

I_{SC}, short-circuit current; R_t, transepithelial resistance.
Values are means ± S.D., (N=3).

The magnitude of the changes in I_{sc} and R_t depended on the amount of NaCl replaced with $Na_2S_2O_3$ (Fig. 2). When 100 % of the NaCl was substituted with $Na_2S_2O_3$ on the mucosal side, I_{sc} changed by –11.5±2.8 μA cm^{-2}. When 25 % of the NaCl was substituted with $Na_2S_2O_3$, I_{sc} changed by –5.9±4.3 μA cm^{-2}. The corresponding values for the serosal side were 22.8±21.9 μA cm^{-2} and 2.4±1.0 μA cm^{-2}, respectively. When 25 % or 100 % of the NaCl was replaced with $Na_2S_2O_3$ on the mucosal side, R_t increased by 4.5±2.2 Ω cm^2 and 24.3±18.8 Ω cm^2, respectively. The replacement of 25 % or 100 % NaCl by $Na_2S_2O_3$ on the serosal side increased R_t by 5.3±2.3 Ω cm^2 and 18.3±14.5 Ω cm^2, respectively.

To evaluate the effects of thiosulphate on the electrical variables, NaCl was also substituted with the oxidized sulphur compounds Na_2SO_3 and Na_2SO_4 (Table 1). The changes in I_{sc} and R_t induced by the replacement of 50 % NaCl by Na_2SO_3 and Na_2SO_4 were not significantly different from the effects induced by the replacement of 50 % NaCl by $Na_2S_2O_3$.

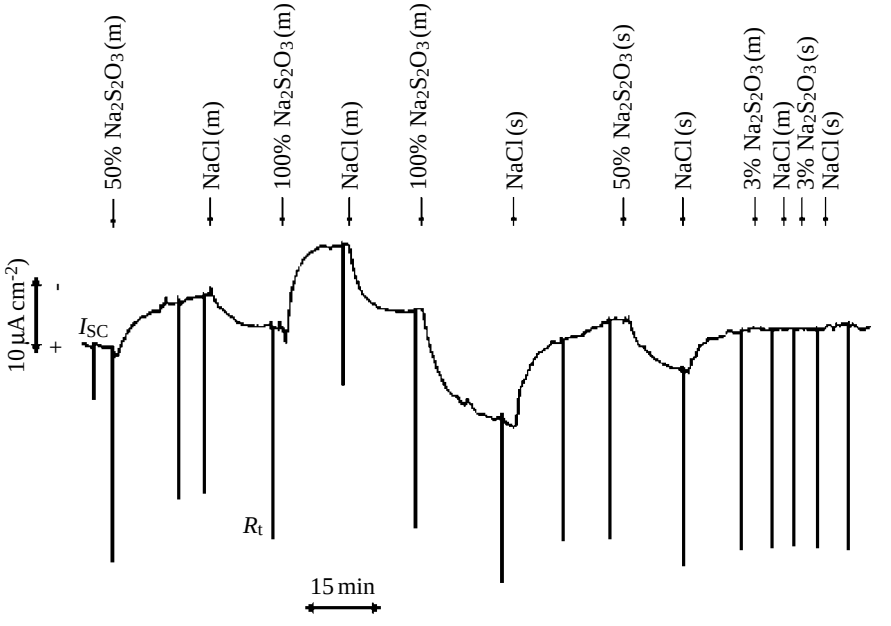

Fig. 1. The time course of a typical experiment showing the dependence of the short-circuit current I_{sc} and the transepithelial resistance R_t on the concentration-dependent substitution of $Na_2S_2O_3$ for NaCl in the sea water on the mucosal (m) and the serosal (s) sides of body wall sections of the lugworm *Arenicola marina*. I_{sc} was monitored continuously. The arrows indicate the application of sea water of differing composition. R_t, illustrated by the deflections, was determined after each change of composition.

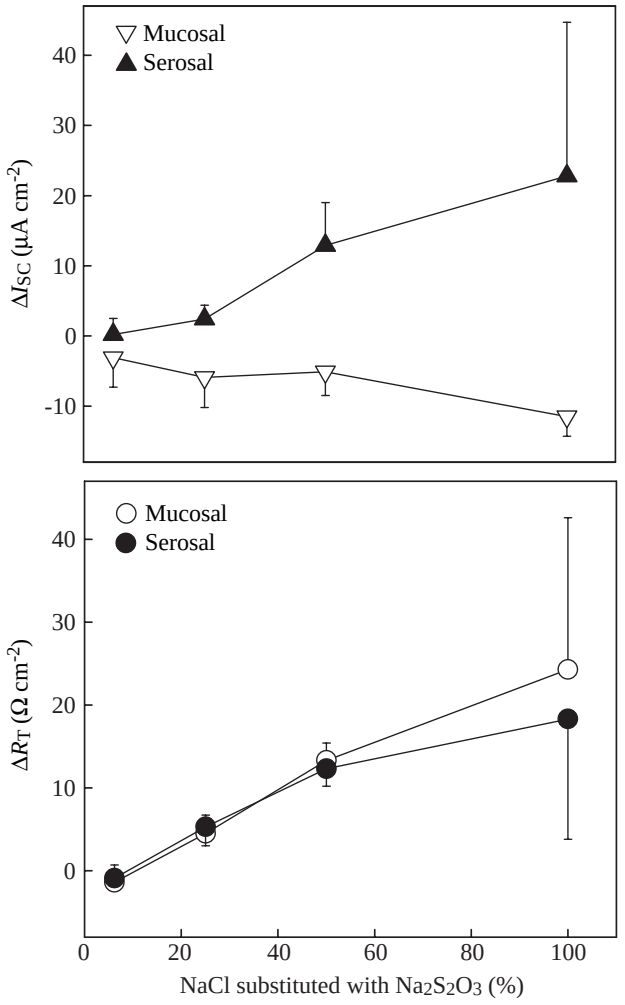

Fig. 2. Variations in the short-circuit current I_{sc} (μA cm^{-2}) and in the transepithelial resistance R_t (Ω cm^2) of body wall sections from the lugworm *Arenicola marina* resulting from the concentration-dependent substitution of NaCl with $Na_2S_2O_3$ in the sea water on the mucosal and the serosal sides of the tissue. Values are means ± S.D., *N*=3.

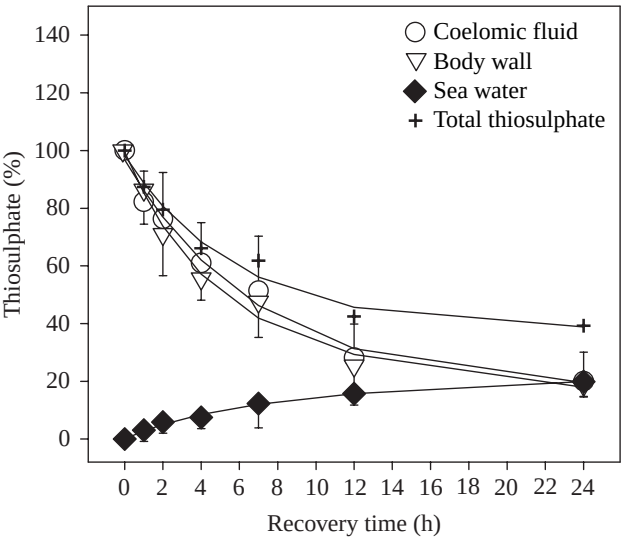

Fig. 3. Changes in the amount of thiosulphate (%) in the coelomic fluid and the body wall of the lugworm *Arenicola marina* and in the sea water, and the total thiosulphate in the system (worm and sea water), during recovery (h) from exposure to sulphide. Values are means ± S.D., *N*=5.

Excretion of thiosulphate by live worms

To stimulate the production of thiosulphate within the lugworm, specimens of *A. marina* were incubated in the presence of oxygen and sulphide. The lugworms were then transferred into small volumes of aerated sea water. During recovery, the concentrations of thiosulphate in the coelomic fluid and in the body wall tissue decreased slowly and at similar rates (Fig. 3). After 1 h, 82±8 % of the thiosulphate, the initial concentration of which was approximately 2.8 mmol l^{-1}, remained in the coelomic fluid. Thiosulphate levels in the coelomic fluid decreased to 51±19 % after 7 h of recovery. After 24 h, 20±10 % of the thiosulphate remained in the coelomic fluid. The corresponding values of thiosulphate in the body wall tissue, where the initial concentration was approximately 0.6 mmol l^{-1}, were 86±7 % after 1 h, 48±12 % after 7 h and 19±4 % after 24 h of recovery. The percentages in both these compartments did not differ significantly. The decline of thiosulphate levels in the coelomic fluid and in the body wall tissue followed a decreasing double-exponential function. Note that the thiosulphate concentration in the body wall tissue refers to the total cell water content, which comprises interstitial and intracellular fluid. This allows a comparison of the concentration in the body wall tissue with the concentration in the sea water and in other compartments of the lugworm. Because the ratio of cell water to tissue wet mass is 0.8, a concentration of 100 μmol l^{-1} cell water corresponds to 0.08 μmol g^{-1} wet mass.

Thiosulphate was excreted into the sea water and is expressed as percentage of total thiosulphate in the worm at the beginning of recovery. After 1 h of recovery, 3.1±2.6 % of the thiosulphate was found in the medium. The percentage increased to 12.3±8.4 % after 7 h. After 24 h of recovery, 19.9±5.3 % of the thiosulphate was present in the sea water.

To evaluate whether thiosulphate was oxidized further, the concentrations of sulphite were also determined. The internal concentration was approximately 38 μmol l^{-1} in the coelomic fluid and approximately 7 μmol l^{-1} in the body wall tissue at the beginning of the recovery period. During recovery, sulphite concentration decreased in a double-exponential manner in both body compartments, and there were no significant differences in the percentage of sulphite remaining between these two compartments throughout the recovery period (Fig. 4). After 1 h, 105±27 % of the sulphite remained in the coelomic fluid. The percentage decreased to 67±18 % after 7 h and to 11±5 % after 24 h of recovery. The corresponding values in the body wall tissue were 107±33 % after 1 h, 66±39 % after 7 h and 44±41 % after 24 h of recovery. The percentage of sulphite in the sea water was 2.5±5.6 % of total sulphite present in the worm prior to recovery after 1 h, and increased to

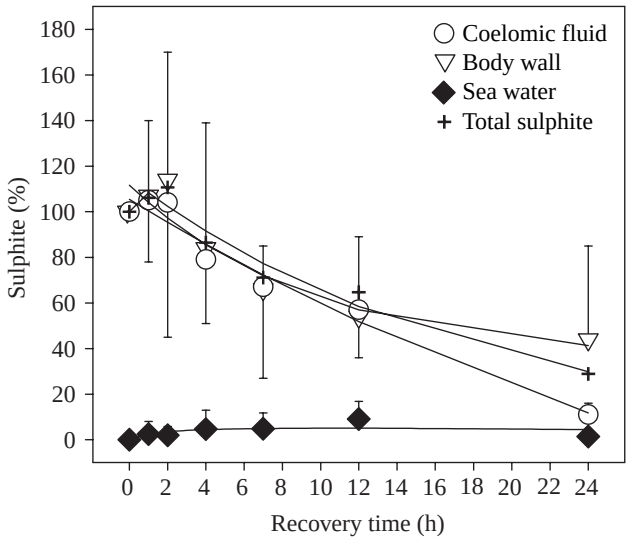

Fig. 4. Changes in the amount of sulphite (%) in the coelomic fluid and the body wall of the lugworm *Arenicola marina* and in the sea water, and the total sulphite in the system (worm and sea water), during recovery (h) from exposure to sulphide. Values are means ± S.D., *N*=5.

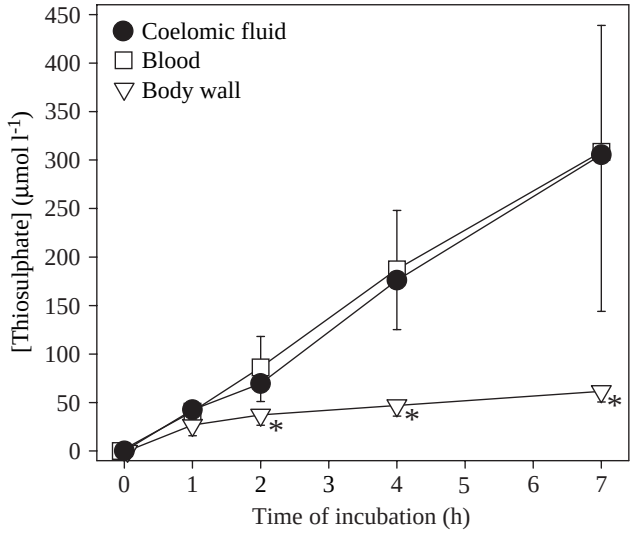

Fig. 5. Thiosulphate concentrations (µmol l^{-1}) in the coelomic fluid, the blood and the body wall of the lugworm *Arenicola marina* during incubation in sea water containing 10 mmol l^{-1} thiosulphate under flow-through conditions. Values are means ± S.D., *N*=5. *Significantly different from the concentration in the body fluids (P<0.05).

4.9±6.9 % after 7 h. After 24 h of recovery, 1.5±2.0 % of the sulphite was detected in the sea water.

Sulphate is another possible oxidation product of thiosulphate. The initial concentrations of sulphate were 25.0±1.0 mmol l^{-1} in the coelomic fluid and 24.6±0.4 mmol l^{-1} in the sea water. During recovery, the concentrations did not change significantly either in coelomic fluid or in sea water. The percentage ranged from 98 % to 102 % of the initial concentration of sulphate in the coelomic fluid and from 97 % to 101 % of the initial concentration of the sulphate in the sea water (data not shown).

Influx of thiosulphate into live worms

Lugworms were incubated for 7 h in sea water containing 10 mmol l^{-1} thiosulphate to determine whether and to what extent exogenously offered thiosulphate permeates into *A. marina*. Thiosulphate was detected in the coelomic fluid, the blood and the body wall tissue of the lugworm (Fig. 5). The concentrations in the body fluids did not differ significantly and amounted to 49±9 µmol l^{-1} thiosulphate in the coelomic fluid and 41±11 µmol l^{-1} in the blood after 1 h. In these compartments, the thiosulphate concentrations increased to 306±162 µmol l^{-1} and 309±130 µmol l^{-1}, respectively, after 7 h. In the body wall tissue, 30±10 µmol l^{-1} thiosulphate were detected after 1 h. After 7 h of incubation, the thiosulphate concentration increased to 62±9 µmol l^{-1}, which is significantly lower than the corresponding value in the body fluids. The permeability coefficient P_t of thiosulphate through the body wall tissue was calculated to be $0.84\times10^{-3}\pm0.43\times10^{-3}$ cm h^{-1} during the total incubation period of 7 h.

Permeation of thiosulphate across different sections of body wall

To quantify thiosulphate permeation across the body wall of *A. marina*, further experiments were performed using isolated tissue. The concentrations of permeated thiosulphate were determined to calculate the permeability coefficient. In the first set of experiments, 10 mmol l^{-1} thiosulphate was supplied either on the mucosal or on the serosal side of the body wall section. The permeability coefficients were $1.31\times10^{-3}\pm0.13\times10^{-3}$ cm h^{-1} for thiosulphate supplied on the mucosal side and $1.24\times10^{-3}\pm0.29\times10^{-3}$ cm h^{-1} for thiosulphate supplied on the serosal side (Fig. 6). There was no significant difference between these values, whereas the concentrations of thiosulphate that diffused into the body wall tissue from both sides did differ significantly. From the mucosal side, 165±21 µmol l^{-1} thiosulphate was taken up into the tissue, whereas 713±170 µmol l^{-1} thiosulphate was detected in the body wall tissue when it was supplied on the serosal side.

The dependence of the permeation on the concentration of thiosulphate supplied on the serosal side of the body wall tissue was investigated. Permeated thiosulphate concentration increased linearly from 3.64±1.28 to 36.9±14.21 µmol l^{-1} when 10–100 mmol l^{-1} thiosulphate were provided (r=0.997, P=0.05) (Fig. 7). Over the whole concentration range, a permeability coefficient of $1.31\times10^{-3}\pm0.37\times10^{-3}$ cm h^{-1} (*N*=30) was calculated. The concentration of thiosulphate in the body wall tissue increased linearly with the increase in the external concentration of thiosulphate (r=0.994, P=0.05). When 10 mmol l^{-1} thiosulphate was supplied, 710±170 µmol l^{-1} was detected in the body wall tissue. The concentration increased to 7350±1670 µmol l^{-1} when 100 mmol l^{-1} thiosulphate was supplied.

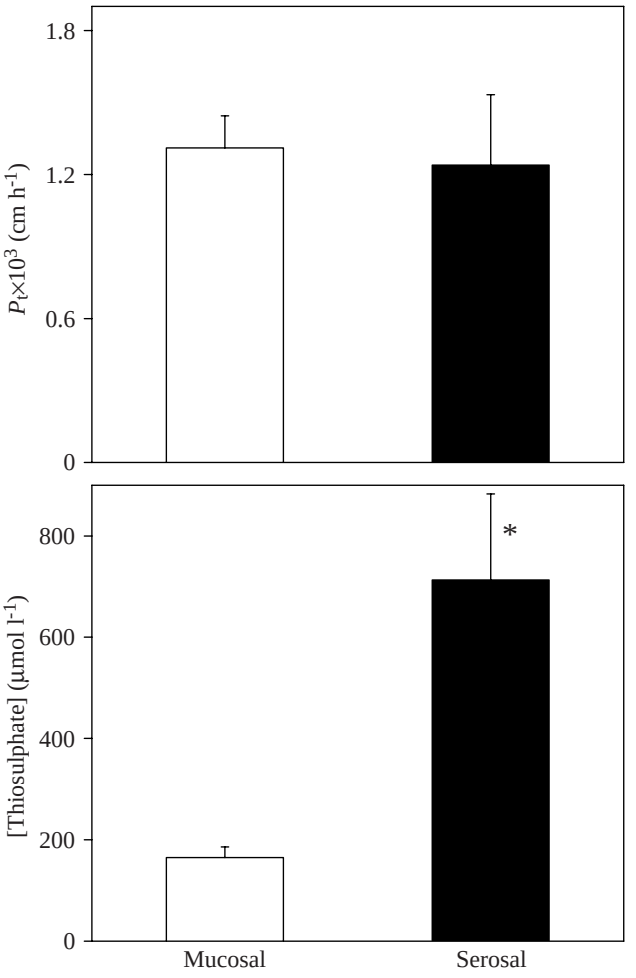

Fig. 6. Permeability coefficients P_t and thiosulphate concentrations in the tissue of body wall sections of the lugworm *Arenicola marina* exposed for 1 h to sea water containing 10 mmol l^{-1} thiosulphate on the mucosal or the serosal side. Values are means ± S.D., N=5. *Significantly different from the value for the mucosal side (P<0.05).

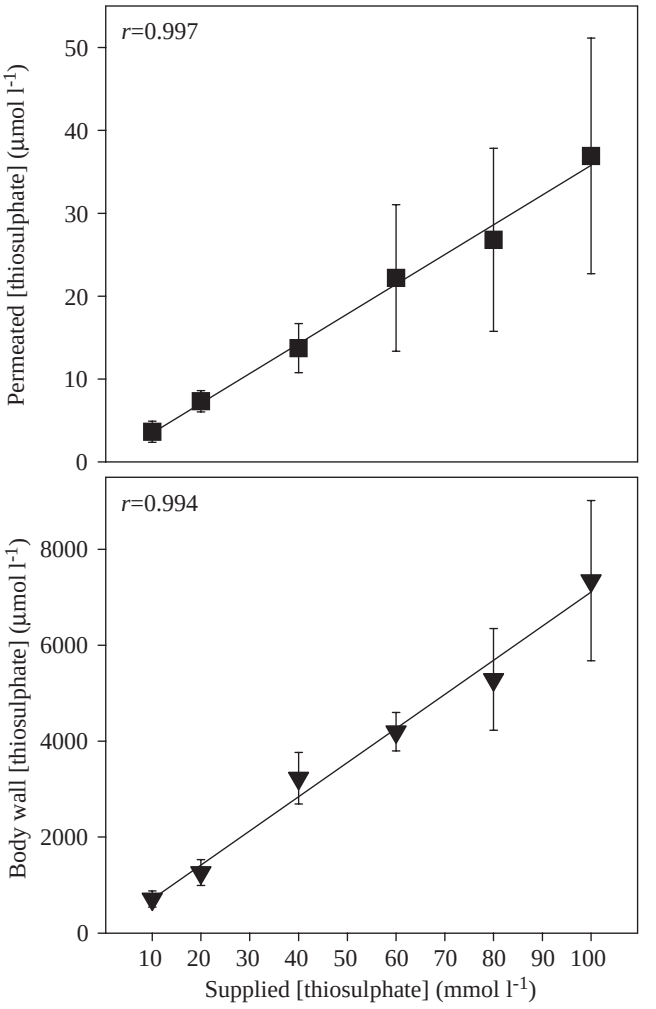

Fig. 7. Concentrations of permeated thiosulphate (μmol l^{-1}) and thiosulphate in the body wall tissue (μmol l^{-1}) of the lugworm *Arenicola marina* in relation to the concentration of thiosulphate supplied for 1 h on the serosal side of the tissue (mmol l^{-1}). Values are means ± S.D., N=5.

The permeation of 20 mmol l^{-1} thiosulphate was determined in the presence of various metabolic inhibitors. The process was not sensitive to 2 and 5 mmol l^{-1} cyanide, 2,4-dinitrophenol, iodoacetic acid and ouabain. Neither the permeability coefficients nor the concentrations of thiosulphate taken up into the tissue differed significantly from the values obtained in the absence of metabolic inhibitors.

In addition, the permeability coefficients of body wall sections containing gills or nephridia were determined (N=6). The permeability coefficient of body wall tissue with gills, $1.01\times10^{-3}\pm0.26\times10^{-3}$ cm h^{-1}, was almost identical to the value determined for body wall tissue alone, which was $1.00\times10^{-3}\pm0.23\times10^{-3}$ cm h^{-1}. Body wall sections containing nephridia exhibited a permeability coefficient of $1.22\times10^{-3}\pm0.27\times10^{-3}$ cm h^{-1}, which again did not differ significantly from the value for body wall tissue without gills.

Desorption of thiosulphate from sections of body wall

Sections of body wall loaded with thiosulphate were mounted in an Ussing chamber, and the concentration of thiosulphate desorbed into the sea water was determined in each half-chamber. To compare the values, the difference between the thiosulphate concentration in the body wall tissue before and after the experiment was used as the 100 % value. Of the desorbed thiosulphate, 114±20 % was detected in the sea water (Fig. 8), 39±12 % of which was on the mucosal side. Significantly more thiosulphate, 75±16 %, was desorbed into the sea water on the serosal side of the body wall section.

Discussion

The sulphide tolerance of *A. marina* results to some extent from the detoxification of sulphide within the mitochondria leading to the production of the less toxic sulphur compound thiosulphate (Völkel and Grieshaber, 1994, 1995, 1996). Thiosulphate accumulates in the body fluids and to a lesser

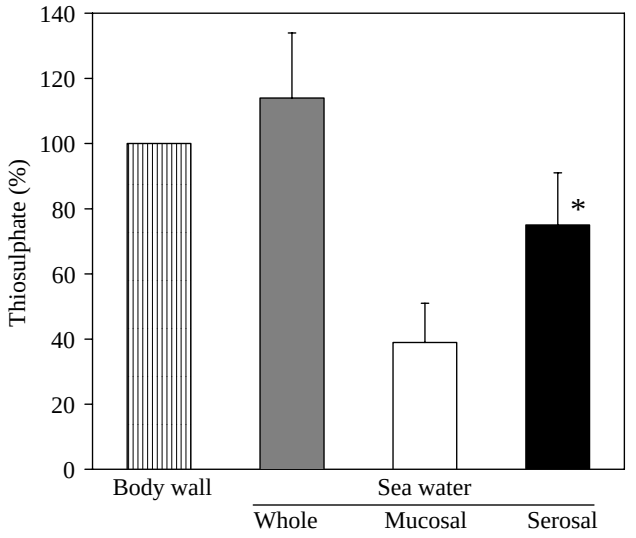

Fig. 8. Balance of the excretion of thiosulphate (%) into the sea water from the body wall on the mucosal and the serosal sides of sections of body wall from the lugworm *Arenicola marina* after 1 h of incubation in an Ussing chamber. Values are means ± S.D., N=8. *Significantly different from the value for the mucosal side (P<0.05).

extent in the body wall tissue of the lugworm (Hauschild and Grieshaber, 1997). The present study deals with the fate of this thiosulphate.

Electrophysiological characterization of the body wall

Ions can permeate epithelia in two ways, *via* paracellular and transcellular pathways (Erlij and Martínez-Palomo, 1978). Paracellular transport takes place along the junctions between the cells; it is bidirectional and follows passively the concentration gradient. Transcellular transport takes place through the cells and is mediated by membrane proteins. Depending on the permeability of the cell junctions, one can distinguish between leaky and tight epithelia (Koefoed-Johnson and Zerahn, 1982). In leaky epithelia, transepithelial ion transport is due mainly to passive paracellular diffusion. In tight tissues, where tight junctions seal off the tissue, the transcellular pathway is the main route of ion transport.

With a V_t close to zero and an R_t of 76 Ω cm^2, the lugworm's body wall belongs to the class of leaky epithelia characterized by low spontaneous transepithelial potential differences and small transepithelial resistances (Frömter, 1972). The low R_t of *A. marina* matches the value of 57 Ω cm^2 found in *Urechis caupo* (Julian and Arp, 1992), another sediment-dwelling invertebrate with known sulphide tolerance. Septate junctions have been detected in the body wall epithelium of this species (Menon and Arp, 1993). The comparatively leaky septate junctions substitute for tight junctions in most invertebrate phyla (Green, 1984). The epidermis of freshwater and terrestrial organisms typically has more septa than that of marine invertebrates, which experience reduced concentration gradients across the epithelial layer (Lillywhite and Maderson, 1988). In *A. marina*, septate junctions have been detected in the caudal epidermis and the gill epithelium (Jouin et al., 1985; Jouin and Toulmond, 1989), implying that they are also present in the body wall. At any rate, the values of these two electrical variables certainly qualify the lugworm's body wall as a leaky tissue in which mainly paracellular transport occurs.

Electrophysiological studies on the integument of marine invertebrates are rare, and to our knowledge have been limited to specialized epithelia such as gills. Therefore, it was not possible to compare the values of V_t and I_{sc} from the present study with those of other marine invertebrates. The measured I_{sc} of chloride and thiosulphate and of sulphite and sulphate were compared on the basis of charge and molecular mass.

To investigate the influence of thiosulphate on the electrical variables, varying amounts of NaCl in the sea water were replaced by equimolar concentrations of $Na_2S_2O_3$. The substitution of NaCl with $Na_2S_2O_3$ on the mucosal side of the body wall provoked a change of I_{sc} in a negative direction. This change was induced by an increased flow of chloride from the serosal to the mucosal side, demonstrating that thiosulphate permeates the body wall to a lesser extent than chloride. When NaCl was substituted with $Na_2S_2O_3$ on the serosal side, the reverse effect was observed. R_t increased in consequence of the substitution on both sides because of a decrease in the amount of ions which permeated the tissue.

According to these results, the lugworm's body wall is less permeable to thiosulphate than to chloride. But replacing 3 % of the NaCl with $Na_2S_2O_3$ did not influence either I_{sc} or R_t, which is indicative of a low thiosulphate permeation in live animals. There was no difference in the changes in I_{sc} and R_t when thiosulphate was supplied on the mucosal and the serosal side of the tissue, suggesting a passive permeation process. Substitution of NaCl with Na_2SO_3 and Na_2SO_4 had the same effects on the electrical variables as substitution with $Na_2S_2O_3$. Since the permeability of the lugworm's body wall did not differ for the different ions, no special transport system seems to exist for any of the oxidized sulphur compounds.

Efflux and influx of thiosulphate in live worms

The electrophysiological measurements suggest that the body wall of the lugworm has a low permeability to thiosulphate. Further experiments were carried out to determine the importance of the postulated thiosulphate permeation *via* the body wall in live animals. First, we investigated whether and to what extent the thiosulphate that accumulated in the body fluids was excreted into the sea water. To study the reverse effect, the uptake of the detoxification product from sea water containing thiosulphate was also examined.

When lugworms were allowed to recover from exposure to sulphide, thiosulphate levels decreased slowly and at similar rates in the coelomic fluid and in the body wall tissue. After 7 h, approximately 50 % of thiosulphate had disappeared, and after 24 h, approximately 20 % of the initial concentration remained in both body compartments. The reduction in the concentration of thiosulphate followed a decreasing double-exponential function. In the first hour, thiosulphate levels

decreased by approximately 16 %, and during 12 and 24 h of recovery thiosulphate levels decreased by only approximately 2.3 % h^{-1}. The lugworm excreted thiosulphate into the sea water, where the percentage increased from 3 % of the initial whole-body concentration at 1 h to 20 % after 24 h of recovery. The dependence of thiosulphate excretion on the concentration gradient and the slow decrease in the level of detoxification product in both body compartments indicate a passive excretion process in *A. marina*.

Excretion of thiosulphate into the sea water has also been observed in other species. Although Vetter et al. (1987) found no evidence that thiosulphate was cleared rapidly from the haemolymph of *Bythograea thermydron*, Gorodezky and Childress (1994) did report a loss of thiosulphate from the haemolymph in this species. The latter authors suggested that most of the accumulated thiosulphate is released by passive diffusion across the gills, possibly supplemented by an active transport process that is capable of transporting thiosulphate against a concentration gradient. A decrease in thiosulphate concentration was observed in the coelomic fluid of the peanut worm *Sipunculus nudus* after thiosulphate injection. Thiosulphate could be detected in the sea water. The apparent excretion rate increased linearly when the concentration of injected thiosulphate increased from 0.1 to 10 mmol l^{-1}, following first-order kinetics (M. K. Grieshaber and S. Hanisch, unpublished results), indicating a passive excretion process in *S. nudus*.

The thiosulphate concentrations in the coelomic fluid and in the body wall tissue of *A. marina* were different. The concentration of thiosulphate in the body wall tissue was only approximately 20 % of the concentration in the coelomic fluid, and the ratio of the concentrations between the compartments remained constant during recovery. However, the measurement of thiosulphate levels in the tissues using the bimane method did not indicate whether thiosulphate was localized intra- or extracellularly since the cells were destroyed during homogenization. Thiosulphate is produced in the mitochondria of *A. marina* (Völkel and Grieshaber, 1994, 1996). There is insufficient evidence available to determine whether sulphide oxidation takes place within or outside the mitochondrial inner membrane (Völkel and Grieshaber, 1997). Even in the latter case, thiosulphate must permeate the cell membrane before accumulating in the extracellular milieu. Because of the low permeability of the charged ion in biological membranes (Holmes and Donaldson, 1969), thiosulphate is probably actively transported out of the cells. Nothing is known about this transport system, but an examination of this process is necessary.

During the experiments, the total amount of thiosulphate found in the worms and in the sea water decreased from 87 % at 1 h to 39 % at 24 h of recovery (Fig. 3). This could be due to chemical or bacterial degradation or to oxidation of thiosulphate in the worms and in the sea water. The curve fits for thiosulphate in the coelomic fluid and in the body wall are double-exponential functions, indicating that there are probably two time-dependent processes occurring here, diffusion and oxidation of thiosulphate. Almgren and Hagström (1974) described the spontaneous decomposition of thiosulphate in sea water leading to the formation of sulphite and sulphur. The formation of polythionates (Cline and Richards, 1969) and a slow oxidation to sulphate (Millero, 1986) have also been observed.

Since sufficient oxygen was available during recovery, thiosulphate may have been further oxidized by the lugworm. We were not able to measure sulphur and polythionates levels during the present study because the appropriate techniques were not available in our laboratory. During the experiment, sulphite levels decreased in the coelomic fluid and in the body wall tissue and increased in the sea water, as was observed for thiosulphate. However, the sulphite concentrations were only approximately 2 % of the thiosulphate concentrations. This ratio remained constant over the whole recovery time. Hence, one can exclude the possibility of further oxidation of thiosulphate to sulphite.

Another possible oxidation product is sulphate, an end-product of sulphide oxidation in species living in symbiosis with sulphur bacteria (Childress and Fisher, 1992). Excretion of sulphate into the sea water has been observed in *Solemya reidi* (Felbeck, 1983) and *Phallodrilus leukodermatus* (Giere et al., 1988), but the mechanisms of the excretion have not yet been elucidated. During recovery, no significant change in the concentrations of sulphate were observed either in the coelomic fluid of *A. marina* or in the sea water. Because of the marine milieu, sulphate concentrations were high in the lugworms and in the medium (24–25 mmol l^{-1}). Unfortunately, *A. marina* does not survive incubations in sulphate-free sea water. Therefore, we could not measure a concentration difference for sulphate using our methods. Radioactively labelled ^{35}S must be used to solve this problem.

To study the uptake of thiosulphate by living animals, *A. marina* was incubated in sea water containing thiosulphate. Thiosulphate was detected in the coelomic fluid, in the blood and in the body wall tissue. Accordingly, an influx of thiosulphate and an efflux from the internal compartments into the ambient sea water occur, depending on the concentration gradient. In its natural habitat, the lugworm's ability to take up thiosulphate is of no significance since the pore water of marine sediments contains at most only low levels (10 μmol l^{-1}) of thiosulphate (Jørgensen, 1990).

In contrast to the exponential decrease in internal thiosulphate levels, a linear rate of accumulation by the body fluids was observed. The difference may result from the varying experimental conditions. Influx was determined using flow-through conditions resulting in the maintenance of a high concentration gradient, whereas excretion had to be measured in a small volume of sea water to facilitate the detection of excreted thiosulphate.

Mechanisms of thiosulphate permeation across the body wall

Isolated sections of body wall mounted in an Ussing chamber were used to elucidate the mechanisms of thiosulphate permeation *via* the body wall of *A. marina*. In the

first set of experiments, the permeability coefficient was determined to quantify the permeation of thiosulphate. There was no significant difference in the permeability coefficient when thiosulphate was supplied on the mucosal or the serosal side of the body wall: the body wall of *A. marina* is equally permeable to thiosulphate from both sides of the tissue.

The permeability coefficient of the body wall tissue of *A. marina* was $1.31\times10^{-3}\pm0.37\times10^{-3}$ (N=30), which is similar to the value of $2\times10^{-3}\pm10^{-3}$ cm h^{-1} (N=17) determined for the body wall of *U. caupo* (Wieting et al., 1995). In this species, however, the permeability coefficient of the hindgut, which is $240\times10^{-3}\pm180\times10^{-3}$ cm h^{-1} (N=22), is 120 times higher than the value for the body wall (Wieting et al., 1995). Thus, Arp et al. (1995) concluded that thiosulphate is primarily eliminated by diffusion across the highly permeable hindgut in *U. caupo*.

In *A. marina*, the gills or the nephridia may participate in the elimination of thiosulphate. The permeability coefficient of body wall sections containing gills was the same as the value for body wall tissue alone, excluding an involvement of the gills in the excretion of thiosulphate. The value for tissue containing nephridia was increased by approximately 22 %, which was also not significantly different from the value for body wall tissue alone. Nevertheless, in these experiments, isolated sections of body wall tissue containing nephridia were used. To obtain evidence for the role of the nephridia in thiosulphate excretion *via* the urine, further experiments should be performed to measure urinary thiosulphate levels in living animals.

In the lugworm, the measured permeability coefficient was approximately 30 % higher than the value calculated from the influx of thiosulphate in live animals, indicating that the influx is based on permeation across the body wall. The difference in the permeability coefficients may result from the experimental conditions. A planar exchange surface was present in the Ussing chamber, whereas the surface area and thickness of the body wall changed as a result of contractions of the lugworm's musculature during the incubation of whole animals.

Although the permeability coefficients for thiosulphate supplied on the serosal and on the mucosal side of the body wall tissue did not differ, the amount of thiosulphate taken up into the tissue differed significantly depending on which side the thiosulphate was supplied. The internal concentration was 713 ± 170 μmol l^{-1} when 10 mmol l^{-1} thiosulphate were supplied on the serosal side, but was only 165 ± 21 μmol l^{-1} when 10 mmol l^{-1} thiosulphate were supplied on the mucosal side. This can be explained by the morphology of the body wall tissue of *A. marina.* The body wall consists of the following layers: cuticle, epidermis, subepidermal connective tissue, circular muscle, a layer of intercircular connective tissue, longitudinal muscle and coelomic epithelium (Wells, 1950). The different concentrations demonstrate that the internal tissues restrict the permeation to a small extent, whereas the epithelial cells sealed by the cell junctions function as barrier.

Since thiosulphate permeates into the tissue more easily from the serosal side than from the mucosal side, one might expect that desorption of thiosulphate into the sea water would also proceed faster on the serosal than on the mucosal side. The measurement of desorption in isolated sections of body wall confirmed this assumption. Significantly more thiosulphate was desorbed into the half-chamber adjacent to the serosal side than into that adjacent to the mucosal side. The total amount of thiosulphate detected in the sea water was in accordance with the decrease in thiosulphate concentration in the body wall section, indicating that the thiosulphate desorbed is chemically unchanged.

How might the permeation of thiosulphate across the body wall epithelium of *A. marina* work? On the one hand, it is possible to conceive of a passive permeation through cell junctions or cells. Permeation through cells is unlikely because of the high negative charge of the thiosulphate ion and the electrophysiological values obtained during this study. On the other hand, one could imagine a specific transport system such as an anion exchanger. In the marine sponge *Microciona prolifera*, a band-3-like protein may be involved in facilitated sulphate transport (Kuhns et al., 1995). No protein capable of transporting thiosulphate has been described in marine invertebrates. A characteristic feature of mediated transport is limited transport capacity, resulting in saturation kinetics. This type of kinetics should also occur if an active mechanism, connected to a biochemical process that produces energy, is involved. In contrast, in the case of passive permeation, one would expect a linear correlation between the concentrations of supplied and permeated thiosulphate.

In *A. marina*, the concentration of thiosulphate permeated *via* the body wall tissue (3.64–36.9 μmol l^{-1}) and the concentration of thiosulphate in the body wall tissue (710–7350 μmol l^{-1}) increased linearly when solutions having a thiosulphate concentration of between 10 and 100 mmol l^{-1} were supplied on the serosal side. This concentration-dependence suggests the existence of a passive permeation process independent of membrane proteins. In *B. thermydron*, there was a linear correlation between the concentration of thiosulphate in the haemolymph and the rate of elimination (Gorodezky and Childress, 1994). Two of the four crabs investigated were able to excrete thiosulphate against the concentration gradient. Nevertheless, the authors concluded that, in addition to diffusion, an active process was also involved in the elimination of thiosulphate by *B. thermydron*.

To determine whether active or passive mechanisms are involved in the permeation of thiosulphate in *A. marina*, various metabolic inhibitors were added to the sea water. The permeation was insensitive to cyanide or dinitrophenol. Moreover, the presence of iodoacetic acid, an inhibitor of glycolysis, and ouabain, a specific inhibitor of Na^+/K^+-ATPase, did not influence permeation. These results confirm that the measured thiosulphate flux resulted from passive permeation.

In conclusion, the elimination of thiosulphate from the lugworm results from permeation across the body wall tissue. Neither the gills nor the nephridia contribute significantly to the excretion of thiosulphate. Because of the passive nature of

the permeation, *A. marina* does not need to use energy to dispose of the sulphide detoxification product. During recovery from sulphide exposure, thiosulphate is not further metabolized but is passively released from the internal compartments and is lost to the ambient sea water.

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