

# Thiosulfate Elimination and Permeability in a Sulfide-Adapted Marine Invertebrate

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## ABSTRACT

Oxidation of hydrogen sulfide to thiosulfate is one of the best-characterized mechanisms by which animals adapted to sulfide minimize its toxicity, but the mechanism of thiosulfate elimination in these animals has remained unclear. In this study, we examined the accumulation and elimination of thiosulfate in the sulfide-adapted marine worm *Urechis caupo*. The coelomic fluid of *U. caupo* exposed to 50–100  $\mu\text{mol L}^{-1}$  sulfide in hypoxic seawater ( $\text{Po}_2$  ca. 10 kPa) accumulated (mean  $\pm$  SD)  $132 \pm 41 \mu\text{mol L}^{-1}$  thiosulfate after 2 h, reaching  $227 \pm 113 \mu\text{mol L}^{-1}$  after an additional 4 h in aerated, sulfide-free seawater. In whole-animal thiosulfate clearance studies, the rate of thiosulfate elimination from the coelomic fluid followed a single exponential time course with a half-life of 6 h. The thiosulfate permeability coefficient of isolated preparations mounted in diffusion chambers was  $7.6 \times 10^{-5} \pm 7.7 \times 10^{-5} \text{ cm s}^{-1}$  for the hindgut and  $5.5 \times 10^{-7} \pm 2.7 \times 10^{-7} \text{ cm s}^{-1}$  for the body wall. These rates were independent of the direction of net efflux (mucosal-to-serosal or serosal-to-mucosal). Using a simple mathematical model of *U. caupo* that incorporates the thiosulfate permeability coefficients, the thiosulfate half-life was calculated to be 23 h without hindgut ventilation but less than 1 h with normal hindgut ventilation. Based on this information, we propose that passive thiosulfate diffusion across the hindgut is adequate to explain the observed rates of thiosulfate elimination.

## Introduction

Hydrogen sulfide is a metabolic poison that can be fatal to animals relying solely on aerobic respiration. Sulfide (the term sulfide refers here to  $\text{H}_2\text{S}$ ,  $\text{HS}^-$ , and  $\text{S}^{2-}$ ) occurs in a number of aquatic environments, including hydrothermal vents, cold seeps, mudflats, marshes, and at sites where human activities produce large deposits of reduced carbon compounds (Fenchel and Riedl 1970; Vismann 1991). In each of these environments, animals from diverse taxa are found that have numerous mechanisms to minimize sulfide's toxic effects (Somero et al. 1989; Vismann 1991; Grieshaber and Völkel 1998). Two important mechanisms for protecting sensitive tissues from sulfide toxicity are sulfide detoxification and sulfide binding by specialized molecules. The most universal means of sulfide detoxification appears to be the enzymatic or nonenzymatic oxidation of sulfide to nontoxic or less toxic compounds, especially thiosulfate ( $\text{S}_2\text{O}_3^{2-}$ ). In animals both with and without bacterial symbionts, thiosulfate concentrations in blood and hemolymph can reach 1–2  $\text{mmol L}^{-1}$  during sulfide exposure (see Grieshaber and Völkel 1998 for review).

The principle limitation of sulfide oxidation as a detoxification mechanism, other than the availability of oxygen, is likely to be accumulation of thiosulfate and other oxidation end-products. However, the mechanism by which thiosulfate is eliminated from an animal's tissues after sulfide exposure has been little studied. In this study, we examined the accumulation and elimination of thiosulfate in the marine worm *Urechis caupo* to clarify the mechanism by which thiosulfate is eliminated in sulfide-adapted marine organisms.

*Urechis caupo* is a large, burrowing echiuroid worm that inhabits U-shaped burrows that can accumulate sulfide in concentrations up to 50  $\mu\text{mol L}^{-1}$  during low tides (Fisher and MacGinitie 1928; Arp et al. 1992). *Urechis caupo* coelomic fluid in vitro catalyzes the oxidation of sulfide to thiosulfate, primarily because of the presence of heme in the coelomocytes (Powell and Arp 1989), but the extent to which thiosulfate is produced in *U. caupo* in vivo during sulfide exposure, and how this thiosulfate would then be eliminated from the tissues, has not been demonstrated.

In theory, a decline in blood or coelomic fluid thiosulfate could result from three mechanisms: sequestration of thiosulfate within tissues, further oxidation of thiosulfate to other sulfur-containing compounds, and the release of thiosulfate to the seawater. No thiosulfate storage sites have been identified in marine invertebrates, making thiosulfate sequestration un-

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likely. Thiosulfate oxidation has been studied in organisms with symbiotic bacteria capable of oxidizing thiosulfate to sulfate, but, to date, such bacterial symbionts have been identified only in sulfide-adapted mussels (Belkin et al. 1986; Anderson et al. 1987; Nelson et al. 1995). Finally, to our knowledge, catalyzed nonbacterial thiosulfate oxidation has never been described in sulfide-adapted animals.

This suggests that the release of thiosulfate to the seawater is the most likely mechanism for thiosulfate elimination. This mechanism could involve excretory organs, active transport across epithelia, or simple net outward diffusion across epithelia. It is not known whether *U. caupo*'s putative excretory organs, known as the anal vesicles or anal sacs, could contribute to thiosulfate elimination. While their physiology has not yet been described, in related echinurans, similar organs appear to act as excretory filters, allowing such test substances as India ink particles and inulin to pass into the vesicle lumen, which empties into the cloaca (Baltzer 1931; Harris and Jaccarini 1981). In contrast, outward flux of thiosulfate in *U. caupo*, whether active or passive, could occur across both the thin-walled hindgut and the body wall. The hindgut, which functions as a water lung, is tidally ventilated with up to twice the worm's body weight in seawater. During normal inflation for a 50-g worm, the gross surface areas of the hindgut and body wall are estimated to reach 200 cm<sup>2</sup> and 240 cm<sup>2</sup>, respectively, producing approximately 9 cm<sup>2</sup> g<sup>-1</sup> body weight in contact with seawater (Julian and Arp 1992). *Urechis caupo* has no vascular system, but the coelomic fluid circulates freely within the coelomic cavity, bathing all of the organs and the serosal surfaces of both the hindgut and the body wall.

## Material and Methods

### Collection and Maintenance of Animals

*Urechis caupo*, wet weight between 20 and 60 g, were collected from Pillar Point Harbor in San Mateo County, California, during low tides using a suction device (or "slurp gun"). Collected animals were maintained in the laboratory in aerated, recirculating, nutrient-free seawater at 18°–20°C. All animals in these studies were used within 2 wk of collection, but we have been able to maintain *U. caupo* under these conditions for several months.

### Thiosulfate Accumulation during Sulfide Exposure

To confirm that *U. caupo* produce thiosulfate during sulfide exposure, animals of similar size were each placed in a flow-through chamber and exposed to between 70 and 100 μmol L<sup>-1</sup> sulfide for 2 h, after which, coelomic fluid sulfide and thiosulfate concentrations were measured. To control the sulfide concentration in the chamber, a sulfide stock solution (2.5 mmol L<sup>-1</sup> Na<sub>2</sub>S · 7H<sub>2</sub>O in vacuum-degassed seawater) was admixed to the inflowing seawater, using a peristaltic pump.

During the exposure, the seawater Po<sub>2</sub> in the chamber was kept either hypoxic (≈10 kPa) or anoxic (<0.5 kPa) by bubbling the inflowing seawater with, respectively, a 1 : 1 mixture of air and compressed N<sub>2</sub>, or pure compressed N<sub>2</sub>. Three animals were tested at each Po<sub>2</sub> condition. Inflowing and outflowing Po<sub>2</sub> were continuously monitored using sulfide-insensitive O<sub>2</sub> electrodes (Diamond General, Ann Arbor, Mich.). At the end of the 2-h sulfide exposure, each animal was placed in flowing, sulfide-free, air-saturated seawater for a 4-h recovery period. Samples of coelomic fluid were withdrawn by hypodermic needle and syringe immediately before and after the 2-h sulfide exposure and at 2 and 4 h into the recovery period. All samples were then assayed for sulfide and thiosulfate by reverse phase high-performance liquid chromatography (Fahey and Newton 1987; Vetter et al. 1989). Sulfide exposure experiments were run at room temperature (24°C).

### Whole-Animal Thiosulfate Elimination Studies

*Urechis caupo* were injected intracoelomically with thiosulfate, and the rate of thiosulfate appearance in the external medium was measured in order to determine the rate at which thiosulfate is eliminated in vivo. A fresh thiosulfate solution consisting of 0.2 μm filter-sterilized artificial seawater (ASW; Instant Ocean, Aquarium Systems) with 50 mmol L<sup>-1</sup> Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, adjusted to 1,000 mosmol kg<sup>-1</sup> and pH 7.4, was used for all injections. The body wall was pierced using a 22-gauge needle and syringe, and 1.0 mL of thiosulfate solution was injected into the coelomic fluid. Hindgut ventilatory activity was not quantified in this study, but the animals appeared to continue normal hindgut ventilation after thiosulfate injection. The worms were then placed in an acrylic tube (2.5-cm diameter), which simulated a burrow, and this tube was submerged in an aerated bath of 350 mL ASW (0.2 μm filter-sterilized, 1,000 mosmol kg<sup>-1</sup>, pH 8.0) at room temperature (20°–22°C). Thiosulfate elimination was then measured over 3 h (three animals) or 16 h (four animals). Samples of the bathing solution were withdrawn for thiosulfate analysis every 30 min (3-h experiments) or at 1, 2, 4, 8, and 16 h (16-h experiments). Experiments lasting 3 h used an open bathing tray, while in the 16-h experiments, the bathing chamber was closed and aeration of the bathing solution was with humidified air to minimize evaporation. In all cases, the bathing solution sample was stored at -20°C immediately after collection, and the volume removed from the bath was replaced by an equivalent volume of filtered ASW.

Within 3 d of collection, all samples were diluted fivefold with ultradistilled water, and the thiosulfate concentration was measured by ion chromatography. A sample volume of 50 μL was injected into a Dionex model 4500i ion chromatograph equipped with an anion self-regenerating suppressor, IonPac AG4A guard column and IonPac AS4A separator column, and a conductivity detector (5 μmol L<sup>-1</sup> detection limit). The eluent

was a sodium bicarbonate solution ( $1.8 \text{ mmol L}^{-1} \text{ Na}_2\text{CO}_3$  and  $1.7 \text{ mmol L}^{-1} \text{ NaHCO}_3$ ) at  $2 \text{ mL min}^{-1}$ .

The average thiosulfate permeability coefficient  $P$  ( $\text{cm s}^{-1}$ ) was calculated from

$$\ln\left(1 - \frac{C_o}{C_\infty}\right) = AP\left(\frac{v_i + v_o}{v_i v_o}\right)t, \quad (1)$$

where  $C_o$  is the “outer” (bath) thiosulfate concentration ( $\text{mol cm}^{-3}$ ) at time  $t$ ,  $C_\infty$  is the theoretical thiosulfate equilibrium concentration,  $A$  is the surface area for diffusion ( $\text{cm}^2$ ), and  $v_i$  and  $v_o$  are the inner (worm) and outer (bath) volumes ( $\text{cm}^3$ ), respectively (from Sten-Knudsen 1978, eq. [47]). Estimates of the surface area for diffusion were from Julian and Arp (1992).

#### Determination of Thiosulfate Permeability Coefficient

Thiosulfate permeability coefficients were determined for hindgut and body wall tissue preparations *in vitro* using an “Ussing chamber”-style diffusion apparatus. Diffusion chamber design and tissue-mounting techniques have been described previously (Julian and Arp 1992). Buffered artificial seawater (BASW), consisting of (in  $\text{mmol L}^{-1}$ ): NaCl 375;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  37.7;  $\text{Na}_2\text{SO}_4$  18.0; KCl 9.35;  $\text{CaCl}_2$  8.0;  $(\text{NH}_4)_2\text{SO}_4$  7.57;  $\text{NaHCO}_3$  2.7; EGTA 0.085; HEPES 20.0 (Anderson et al. 1987), was used as the chamber-filling solution. All animals were anesthetized with  $0.5 \text{ mol L}^{-1} \text{ MgCl}_2$  ( $0.05 \text{ mL g}^{-1}$  body weight) before dissection.

The thiosulfate permeability coefficients for hindgut and body wall were determined for both net mucosal-to-serosal and net serosal-to-mucosal thiosulfate flux. Tissue preparations (five replicates at each orientation for hindgut and four replicates at each orientation for body wall) were mounted vertically between two diffusion half-chambers, with a cross-sectional diffusion area of  $1.3 \text{ cm}^2$  or  $2.0 \text{ cm}^2$  for body wall and  $0.81 \text{ cm}^2$  for hindgut. Each half-chamber was connected with vinyl tubing to a circulation reservoir and had a total volume of  $4 \text{ mL}$ , including both the half-chamber and the associated reservoir. The chambers and reservoirs were then filled with either BASW or BASW with  $50 \text{ mmol L}^{-1}$  thiosulfate. Both solutions were adjusted to pH 8.0 and  $1,000 \text{ mosmol kg}^{-1}$ . Circulation and aeration of the solution on each side of the tissue preparation was achieved with an air-bubble lift between the chamber half and its circulation reservoir. Beginning immediately after the addition of the thiosulfate solution,  $100\text{-}\mu\text{L}$  samples were withdrawn from each half-chamber every 5 min for a period of 2 h. The solution volume of each reservoir was maintained constant after each sample by replacement with an equal volume of the original BASW solution (i.e., either BASW or BASW with thiosulfate). Thiosulfate content of these samples was analyzed spectrophotometrically (Westly 1987). To correct for the periodic dilution during sampling, the Fick equation

was modified to include a dilution constant,  $F$ , which represents the fraction of the chamber volume replaced per unit time:

$$J = \frac{C_t F}{1 - e^{-Ft}}, \quad (2)$$

where  $J$  is the rate of thiosulfate flux ( $\text{mol s}^{-1}$ ), and  $C_t$  is the concentration at time  $t$ . Thiosulfate concentration time series data for each tissue sample were fit to equation (2) using a least-squares curve-fitting program (N-Fit, Island Products, Galveston, Tex.). Data sets were included in the results only if the calculated correlation coefficient of the curve-fit was at least 0.95.

Using a slightly different protocol, the permeabilities of hindgut and body wall tissue preparations were measured at both pH 7.4 and 8.0 to determine whether the measured thiosulfate permeability coefficient was dependent on the pH of the incubation medium. Tissue samples of hindgut (six replicates at pH 7.4, 14 replicates at pH 8.0) or body wall (six replicates at each pH) were positioned horizontally, serosal side up in a vertical diffusion chamber (Julian and Arp 1992). In all cases, the tissue was mounted such that the direction of net thiosulfate flux was outward (serosal-to-mucosal). The upper chamber was connected to a peristaltic pump for uninterrupted circulation of  $5 \text{ mmol L}^{-1}$  thiosulfate in BASW (for hindgut studies) or  $50 \text{ mmol L}^{-1}$  thiosulfate in BASW (for body wall studies) from a  $10\text{-mL}$  reservoir. Solutions in both chambers were adjusted to pH 7.4 or pH 8.0, and the osmolarity was adjusted to  $1,000 \text{ mosmol kg}^{-1}$ . The solution in the lower chamber was continuously stirred with a magnetic microstir bar and had a volume of  $0.7 \text{ mL}$  for hindgut or  $1.0 \text{ mL}$  for body wall. After mounting the tissue in the chambers, the two chamber halves were clamped together gently, sealed with vacuum grease, and then filled simultaneously. After 30 min, the contents of the lower chamber were withdrawn by syringe and stored at  $-20^\circ\text{C}$ . Analysis of these samples for thiosulfate was then performed within 3 d by ion chromatography, as described in “Whole-Animal Thiosulfate Elimination Studies.”

Since the upper chamber thiosulfate concentration is relatively constant (at  $5 \text{ mmol L}^{-1}$  or  $50 \text{ mmol L}^{-1}$ ), and since the mucosal concentration is initially zero,

$$P = -v \frac{\ln\left(1 - [C_i/C_o]\right)}{At}, \quad (3)$$

where  $C_i$  and  $C_o$  are the inside (lower chamber) and outside (upper chamber) thiosulfate concentrations, respectively, and  $v$  is the volume of inner (lower) chamber (Sten-Knudsen 1978).

### Coelomic Fluid Electrolyte Concentrations

For analysis of coelomic fluid electrolyte concentrations, *U. caupo* were collected by slurp gun from burrows in the subtidal zone. A 1.5-mL sample of coelomic fluid was immediately collected from each animal, after which the animals were returned to their burrows. For comparison of electrolyte concentration, seawater samples were taken from the water overlying the burrow sites. Immediately after collection, both the coelomic fluid and seawater samples were placed on ice for no longer than 20 min, after which they were centrifuged for 10 min at 5,000 g, followed by storage of the supernatants at  $-20^{\circ}\text{C}$ . Analyses of supernatants for the electrolytes  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , and  $\text{HCO}_3^-$  were then performed by a commercial laboratory (Meris Laboratories, San Jose, Calif.), using an automated clinical analyzer (Roche Diagnostics 747-200). The number of replicates was six for all samples, except for seawater  $\text{Ca}^{2+}$ , for which there were only two replicates.

### Statistical and Mathematical Analyses

Values are presented as means  $\pm$  one standard deviation (SD). Significant differences between two means ( $p < 0.05$ ) were determined by a two-tailed *t*-test. Theoretical rates for thiosulfate elimination were calculated iteratively using a modified form of the Fick equation:

$$J = C(P_H A_H + P_B A_B), \quad (4)$$

where  $C$  is the coelomic fluid thiosulfate concentration (the seawater thiosulfate concentration was assumed to remain at zero) and  $A$  is the tissue surface area, with the subscripts H and B representing the hindgut and body wall, respectively. Coelomic fluid volume was assumed to be 30% of total body weight (Arp et al. 1992). Hindgut and body wall surface area estimates were from Julian and Arp (1992).

## Results

### Thiosulfate Accumulation during Sulfide Exposure

The coelomic fluid of *Urechis caupo* exposed to  $75 \pm 20 \mu\text{mol L}^{-1}$  sulfide in hypoxic seawater ( $\text{Po}_2$  ca. 10 kPa) accumulated  $130 \pm 41 \mu\text{mol L}^{-1}$  thiosulfate after 2 h (Fig. 1). The coelomic fluid thiosulfate concentration continued to rise during the 4-h recovery period, during which the animals were exposed to aerated, sulfide-free seawater, reaching  $230 \pm 113 \mu\text{mol L}^{-1}$ . The coelomic fluid sulfide concentration reached a maximum of  $9.4 \pm 13 \mu\text{mol L}^{-1}$  at the end of the exposure and returned to undetectable levels during the recovery period (data not shown). When the exposure was carried out under anoxic conditions, the result was quite different, with the coelomic fluid thiosulfate concentration reaching only  $11 \pm 2 \mu\text{mol L}^{-1}$ , and the coelomic fluid sulfide concentration reaching  $15 \pm 4 \mu\text{mol}$

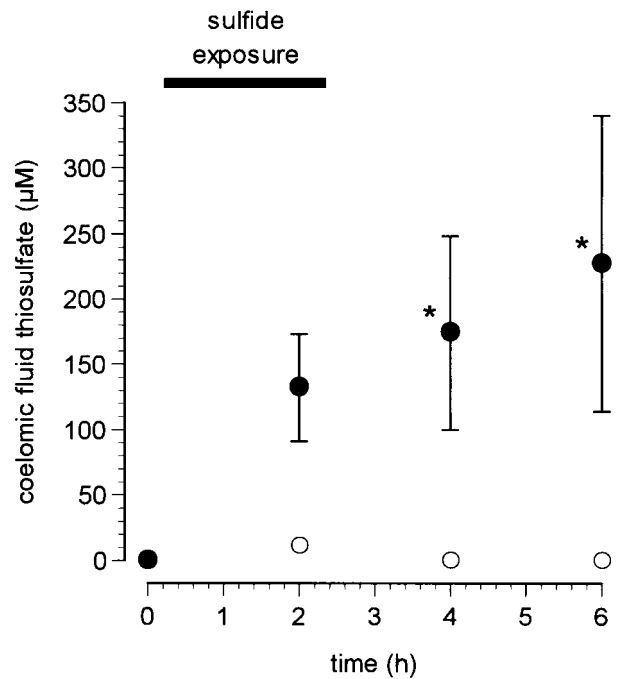


Figure 1. Accumulation of thiosulfate in the coelomic fluid of *Urechis caupo* exposed to sulfide. Animals were exposed to  $50\text{--}100 \mu\text{mol L}^{-1}$  sulfide for 2 h (indicated by horizontal bar) in either hypoxic seawater (closed symbols;  $\text{Po}_2$  ca. 10 kPa) or anoxic seawater (open symbols;  $\text{Po}_2 < 0.5$  kPa). Three animals were tested at each condition. After the exposure, the animals were placed in air-saturated, sulfide-free seawater for an additional 4 h. Coelomic fluid sulfide and thiosulfate concentrations were obtained by syringe and assayed for sulfide and thiosulfate by high-performance liquid chromatography. Asterisks indicate concentrations significantly different from zero (by one-sample *t*-test).

$\text{L}^{-1}$ . During the recovery period, both sulfide and thiosulfate were undetectable. Although ventilatory behavior was not quantified in these experiments, each *U. caupo* exposed to anoxic conditions ceased body wall peristaltic contractions and hindgut ventilation soon after sulfide was added (data not shown). This did not occur in animals exposed to anoxic seawater alone or to sulfide in hypoxic seawater.

### Thiosulfate Elimination in Whole Animals

After injection of thiosulfate into the coelomic fluid, thiosulfate began accumulating rapidly in the bath and was eliminated from the coelomic fluid with a half-life of approximately 6 h (Fig. 2). If the mechanism of thiosulfate elimination were simple diffusion, then the rate should follow a single exponential decay. The experimental data were variable but did in fact fit a single exponential with good accuracy (raw  $r^2 = 0.98$ ). This is consistent with elimination via passive diffusion but does not, under specific conditions, rule out active or facilitated

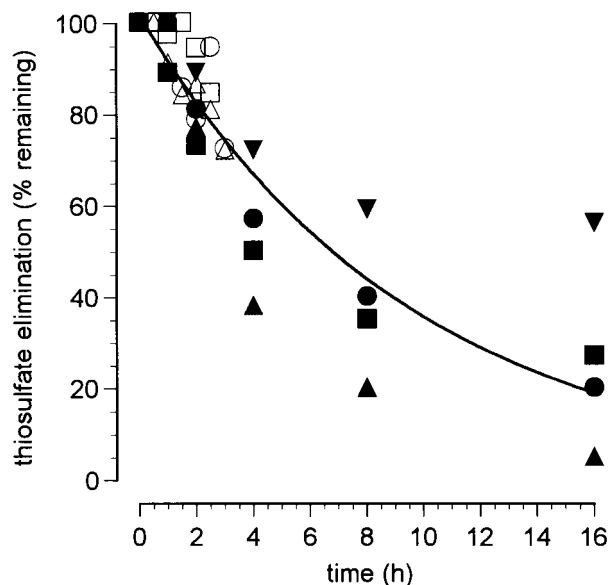


Figure 2. Elimination of injected thiosulfate in *Urechis caupo*. Animals were injected with 50  $\mu\text{mol}$  of sodium thiosulfate into the coelomic fluid. Each worm was then placed in an artificial burrow in a seawater bath, and thiosulfate appearance in the bath was monitored for either 3 h (three animals) or 16 h (four animals). Different symbols are used for each animal tested. Bath thiosulfate concentrations were used to calculate the percentage of injected thiosulfate remaining in the coelomic fluid, based on a theoretical equilibrium state in which thiosulfate is equally distributed throughout the system (with an assumption of no thiosulfate loss).

transport. The time constant of the decay indicates that over 90% of the thiosulfate would be eliminated within 24 h. In this analysis, the amount of thiosulfate remaining in the animal was calculated based on the theoretical equilibrium state that would exist if the injected thiosulfate were equally distributed throughout the worms' tissues and throughout the bath. If the thiosulfate were actually completely excluded from the intracellular space, or if it were completely excluded from all tissues (i.e., the thiosulfate was distributed just in the bath), the error in calculation would be less than 15%. This is because each animal's mass was at most 15% of the total mass (including the bath). Attempts at performing the thiosulfate elimination experiments for 32 h or more were not successful because the total thiosulfate concentration began to decrease markedly. Although this decrease was almost certainly caused by thiosulfate oxidation, it is not certain to what extent this might have been spontaneous, bacterial, or from an endogenous catalyst.

To estimate the permeability coefficient of *U. caupo* epithelia, equation (1) was fit to the elimination data ( $r^2 = 0.81$ ,  $p < 0.001$ ). To limit any effect of thiosulfate oxidation on this estimate, only the first 8 h of data were used in the analysis. To account for the effect of hindgut inflation on surface area, the

total epithelial surface area was estimated to be 95  $\text{cm}^2$  (hindgut uninflated) or 440  $\text{cm}^2$  (maximal hindgut inflation; Julian and Arp 1992). With these parameters, the calculated permeability coefficients were  $6.3 \times 10^{-6} \text{ cm s}^{-1}$  (hindgut uninflated) and  $1.4 \times 10^{-6}$  (hindgut inflated). It is important to note that these values represent total epithelial surface area and, thus, do not distinguish between the individual contributions of the hindgut and body wall. Furthermore, this estimate of the permeability coefficient assumes that efflux is due solely to passive diffusion, which may not be true.

#### Hindgut and Body Wall Permeability

To determine the contribution of the hindgut and body wall to thiosulfate efflux, these tissues were isolated and placed in standard Ussing diffusion chambers. Orientation had no significant effect on the thiosulfate permeability coefficient for both the hindgut ( $P = 1.8 \times 10^{-5} \pm 2.3 \times 10^{-5} \text{ cm s}^{-1}$  serosal-to-mucosal vs.  $1.3 \times 10^{-5} \pm 1.9 \times 10^{-5} \text{ cm s}^{-1}$  mucosal-to-serosal;  $n = 5$ ,  $p = 0.72$ ) and the body wall ( $P = 1.0 \times 10^{-7} \pm 0.58 \times 10^{-7} \text{ cm s}^{-1}$  serosal-to-mucosal vs.  $1.8 \times 10^{-7} \pm 0.86 \times 10^{-7} \text{ cm s}^{-1}$  mucosal-to-serosal;  $n = 4$ ,  $p = 0.20$ ). Similarly, for both hindgut and body wall, the permeability coefficients at pH 7.4 and 8.0 were not significantly different from each other (Table 1). However, in all experiments, the hindgut was significantly more permeable to thiosulfate than was the body wall under the same experimental conditions ( $p < 0.001$  in every case).

In general, the permeability coefficients measured in the orientation studies were less than those measured for the same tissue in the pH studies. Although the method of thiosulfate analysis differed for these two studies (spectrophotometry for the orientation studies and ion chromatography for the pH studies), the difference in apparent permeability is more likely caused by increased thiosulfate oxidation in the orientation studies. In the orientation experiments, all solutions were continuously aerated, and the duration of exposure was always longer. Both of these factors would increase thiosulfate oxidation, thereby decreasing the apparent permeability coefficient. For this reason, the thiosulfate permeability coefficients obtained from the pH studies were likely to be more accurate, so these data were used for the remaining calculations and mathematical modeling. Furthermore, because the thiosulfate permeability coefficients for pH 7.4 and 8.0 were not significantly different, these were pooled for each tissue. The pooled thiosulfate permeability coefficient was  $7.6 \times 10^{-5} \pm 7.7 \times 10^{-5} \text{ cm s}^{-1}$  ( $n = 20$ ) for the hindgut and  $5.5 \times 10^{-7} \pm 2.7 \times 10^{-7} \text{ cm s}^{-1}$  ( $n = 12$ ) for the body wall. For this pooled data, the thiosulfate permeability coefficient of the hindgut is almost 100-fold greater than that of the body wall ( $p < 0.001$ ).

For a worm of approximately 50-g body mass with a hindgut volume of 1.0 mL seawater  $\text{g}^{-1}$  body mass, the hindgut serosal-to-mucosal thickness is approximately 46  $\mu\text{m}$  (Menon and Arp

Table 1: Effect of pH on *Urechis caupo* hindgut and body wall thiosulfate permeability in vitro

	$P$ (cm s <sup>-1</sup> )		$p$
	pH 7.4	pH 8.0	
Hindgut .....	$8.3 \times 10^{-5} \pm 9.4 \times 10^{-5}$	$7.2 \times 10^{-5} \pm 5.6 \times 10^{-5}$	.83
Body wall .....	$6.7 \times 10^{-7} \pm 4.3 \times 10^{-7}$	$3.3 \times 10^{-7} \pm 1.3 \times 10^{-7}$	.093

Note. Thiosulfate permeability coefficient ( $P$ , in cm s<sup>-1</sup>) for isolated *Urechis caupo* hindgut and body wall tissue preparations. Tissues were tested at pH 7.4 or pH 8.0 ( $n = 6$  for each, except hindgut at pH 8.0, for which  $n = 14$ ).  $p$  indicates statistical significance between tissues at pH 7.4 and 8.0.

1992). Multiplying this by the average thiosulfate permeability coefficient, the calculated thiosulfate diffusion coefficient is  $3.5 \times 10^{-7}$  cm<sup>2</sup> s<sup>-1</sup>. The body wall is much thicker, with an average serosal-to-mucosal thickness of approximately 300  $\mu$ m (Menon and Arp 1993), which yields an average thiosulfate diffusion coefficient,  $D$ , of  $1.6 \times 10^{-8}$  cm<sup>2</sup> s<sup>-1</sup>. By correcting for tissue thickness (and with the assumption of homogeneity), the permeability of the hindgut and body wall are seen to be more similar (20-fold difference) than is indicated by the permeability coefficients (140-fold difference). However, the results still suggest that the hindgut is intrinsically more permeable to thiosulfate than is the body wall.

#### Coelomic Fluid Electrolyte Concentrations

*Urechis caupo* had no significant differences between its coelomic fluid electrolyte concentrations and the electrolyte concentrations in surrounding seawater for any of the electrolytes tested (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Cl<sup>-</sup>, and HCO<sub>3</sub><sup>-</sup>; Table 2). This is consistent with the presence of "leaky" epithelia, which are unable to maintain significant transepithelial concentration gradients.

#### Predicted Thiosulfate Elimination Rates

If efflux across the hindgut and body wall epithelial surfaces is sufficient to account for the elimination of thiosulfate in vivo, then the half-life calculated from the in vitro thiosulfate per-

meability experiments should be no greater than that observed in whole animals. Since the surface area of *U. caupo* is dependent on hindgut inflation volume, the total permeability of the animal will vary with hindgut distention. Using the measured hindgut and body wall thiosulfate permeability coefficients, along with estimates of hindgut and body wall surface area, equation (4) was used to calculate the half-life of thiosulfate for a range of hindgut distention volumes (Fig. 3). If the hindgut is completely empty, then it is assumed that all thiosulfate efflux must cross the body wall. Under this condition, the calculated thiosulfate half-life is 23 h, which is much longer than that observed in the whole-animal studies (6 h). However, since the hindgut is over 100 times more permeable to thiosulfate than the body wall, regular ventilation of the hindgut with even small volumes of seawater should greatly increase the rate of thiosulfate efflux. For example, at an average hindgut volume of 0.35 mL per g body weight, the thiosulfate half-life drops to less than 1 h. This hindgut volume is characteristic of normally ventilating *U. caupo* (Julian and Arp 1992), suggesting that for a normally ventilating worm, efflux of thiosulfate across the epithelia is sufficient to explain the rate of thiosulfate elimination seen in the whole-animal experiments. The greatly decreased half-life that results from hindgut ventilation suggests that thiosulfate efflux across the hindgut is much more important than that across the body wall.

## Discussion

### Sulfide Oxidation

Sulfide is very permeable across both the hindgut and the body wall, and *Urechis caupo* has no means of preventing sulfide from entering its tissues (Julian and Arp 1992). Furthermore, *U. caupo* mitochondria in tissue homogenates are more than 90% inhibited by 5  $\mu$ mol L<sup>-1</sup> sulfide (Powell and Arp 1989). Despite this, *U. caupo* can maintain aerobic metabolism in the presence of 50  $\mu$ mol L<sup>-1</sup> sulfide, provided that sufficient oxygen is available (Eaton and Arp 1993). This suggests that *U. caupo* is capable of detoxifying sulfide as it diffuses in from the environment.

During a long tidal exposure (2 h), *U. caupo* can be exposed to sulfide up to 65  $\mu$ mol L<sup>-1</sup> with a Po<sub>2</sub> of about 10 kPa (Arp

Table 2: Electrolyte concentrations in *Urechis caupo* coelomic fluid plasma and seawater

	Seawater	Plasma	$p$
Sodium .....	458 $\pm$ 30	468 $\pm$ 11	.48
Potassium .....	9.8 $\pm$ .7	10.4 $\pm$ .3	.072
Calcium .....	8.9, 11.9	10.6 $\pm$ 1.1	.86
Chloride .....	630 $\pm$ 66	574 $\pm$ 33	.092
Bicarbonate .....	2.2 $\pm$ .5	3.0 $\pm$ 2.5	.44

Note. Concentration (in mmol L<sup>-1</sup>) of electrolytes in seawater and coelomic fluid plasma from freshly collected *U. caupo*, presented as mean  $\pm$  SD ( $n = 6$ , except for seawater Ca<sup>2+</sup>, for which  $n = 2$ ).  $p$  indicates statistical significance between seawater and plasma concentrations.

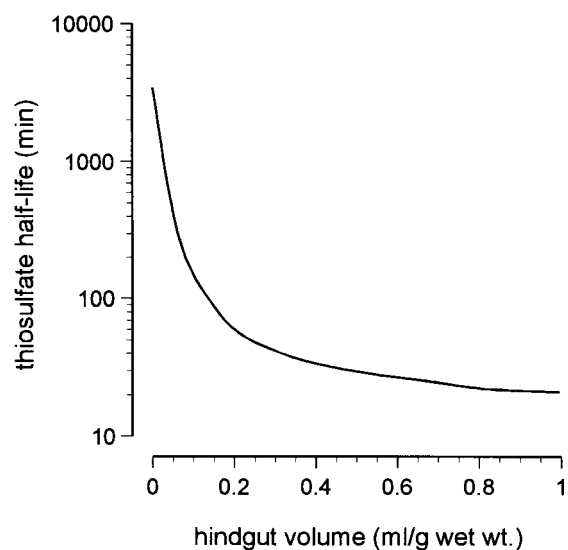


Figure 3. Effect of hindgut ventilation ( $\text{mL} \times \text{g wet weight}^{-1}$ ) on predicted rate of thiosulfate elimination in *Urechis caupo*. The half-life of thiosulfate in the coelomic fluid was estimated based on the measured hindgut and body wall thiosulfate permeability coefficients, along with estimates of hindgut and body wall surface area. Estimates of hindgut and body wall surface area at various degrees of hindgut inflation are from data in Julian and Arp (1992). Average hindgut volume for an animal of this size would be  $0.4 \text{ mL} \times \text{g wet weight}^{-1}$ .

et al. 1992). After 2 h under similar conditions in the laboratory, the coelomic fluid of *U. caupo* accumulated less than  $15 \mu\text{mol L}^{-1}$  sulfide, but more than  $200 \mu\text{mol L}^{-1}$  thiosulfate. When exposed to the same sulfide concentration, but under anoxic conditions, comparatively little thiosulfate was produced. These results demonstrate that sulfide is oxidized to thiosulfate in *U. caupo* and that this process is limited by the availability of  $\text{O}_2$  in the ambient seawater. Regardless of ambient  $\text{Po}_2$ , the coelomic fluid sulfide concentration was on average  $<20\%$  of that in the surrounding seawater, indicating that a mechanism of sulfide detoxification other than sulfide oxidation plays a role in limiting sulfide toxicity. Furthermore, the coelomic fluid thiosulfate concentration of *U. caupo* exposed to sulfide under anoxic conditions continued to increase following the animals' transfer to aerated, sulfide-free seawater. These two findings suggest that at least some of the sulfide that diffused into the animal during sulfide exposure was temporarily bound within the tissues or coelomic fluid and then slowly released and oxidized during the recovery phase.

Continued ventilation of the hindgut during sulfide exposure is likely to greatly increase sulfide influx by increasing both epithelial surface area and sulfide permeability. However, the increased  $\text{O}_2$  uptake that can also result from hindgut ventilation could, by enhancing oxidation of sulfide, provide a net benefit (Julian and Arp 1992; Julian et al. 1996). This is now

supported by the observation that hindgut activity continued when *U. caupo* were exposed to sulfide under oxygenated conditions but ceased when the sulfide exposure was under anoxic conditions.

#### Thiosulfate Clearance

In sulfide-exposed rats, thiosulfate is excreted in the urine (Curtis et al. 1972), as would be expected from kidney ultrafiltration in the absence of a thiosulfate reabsorption mechanism. In *U. caupo*, the anal vesicles are probably the excretory organs, as opposed to the misnamed nephridia, which actually serve as collection and storage sites for mature gametes (Pilger 1993). Although the physiology of the anal vesicles is unknown, their anatomical structure suggests that they might be able to filter the coelomic fluid (Seto 1997). To isolate the contribution of any excretory organs from that of efflux across the epithelia, we determined the thiosulfate permeability of isolated hindgut and body wall tissue preparations. These values were then used to estimate the rate of thiosulfate elimination in vivo that would be caused solely by efflux across these epithelial surfaces. Based on these data, we determined that efflux across the hindgut alone is sufficient to eliminate thiosulfate at least as rapidly as that seen in the whole-animal experiments, suggesting that excretory organs are not required for thiosulfate elimination.

During the whole-animal thiosulfate elimination experiments, the total thiosulfate in the experimental system was diminished at the end of the 16-h experiments. Because the half-life of thiosulfate in seawater is about 1 wk in the absence of bacteria (F. Millero, personal communication), the decrease in thiosulfate seen in these experiments was probably not due primarily to spontaneous oxidation. While bacteria have been identified in the body wall epithelium of *U. caupo*, their density is low (Menon and Arp 1993; Arp et al. 1995) and there is no evidence that they are capable of thiosulfate oxidation. Another possibility is that iron or other components of *U. caupo* coelomic fluid or tissues acted as a catalyst for thiosulfate oxidation. However, whether through catalysis or bacterial metabolism, the presence of rapid thiosulfate oxidation would not be necessary in order to achieve the rate of thiosulfate elimination seen in the whole-animal experiments.

#### Mechanism of Thiosulfate Efflux

Because thiosulfate is a charged, relatively large molecule, cell membranes are impermeable to thiosulfate without the presence of a transport molecule. Although transmembrane thiosulfate transporters have been identified in bacterial cells (Sirko et al. 1995), no such transporters have been identified in animals. In fact, because of the inability of thiosulfate to cross cell membranes, it has been used as a marker of extracellular fluid volume (Holmes and Donaldson 1969). However, in a recent study of the hydrothermal vent crab *Bythograea ther-*

*mydron*, Gorodesky and Childress (1994) found that two crabs appeared to be able to eliminate thiosulfate from their hemolymph against a concentration gradient of almost 1 mmol L<sup>-1</sup>. This implies an active component to thiosulfate elimination in *B. thermydron*. In contrast, Vetter et al. (1987), studying the same organism, did not find evidence for active thiosulfate elimination. In *U. caupo*, we found that the thiosulfate permeability of both hindgut and body wall were not dependent on the direction of net flux (serosal-to-mucosal or mucosal-to-serosal). This suggests, but does not prove, the absence of a significant unidirectional facilitated transport or active transport mechanism.

In the absence of specialized transport molecules, passive diffusion across epithelia would be via paracellular diffusion, in which thiosulfate would follow a path between cells (rather than across cell membranes). In a "leaky" epithelium, this pathway has a relatively low resistance, and the epithelium is unable to maintain large concentration gradients. We found that all measured electrolytes in *U. caupo* coelomic fluid were not significantly different in concentration from those of the surrounding seawater, which is consistent with the presence of leaky epithelia. In further agreement with this, previous measurements of *U. caupo* hindgut and body wall have shown that the electrical resistance of these tissues is very low (<100 Ω cm<sup>2</sup>), and that the transepithelial electrical potential is less than 1 mV (Julian and Arp 1992). In addition, ultrastructural studies of intercellular junctions in *U. caupo* epithelia have demonstrated the presence of septate junctions and the absence of tight junctions (Menon and Arp 1993). Septate junctions are characteristic of invertebrate integument and appear to offer a low resistance to diffusion (Lillywhite and Maderson 1988). Thus, *U. caupo* epithelium is likely permeable to small molecules, and transepithelial active transport of thiosulfate would be unlikely to contribute significantly to thiosulfate efflux. Since the thiosulfate concentration in marine sediment pore water is generally very low, primarily because thiosulfate can be reduced by most free-living sulfate-reducing bacteria (Jørgensen 1990), the gradient for thiosulfate should be outward, and any buildup of thiosulfate in the burrow water would be reduced by normal, continuous burrow irrigation by peristaltic contractions of the body wall (Lawry 1966).

#### Permeability Coefficients

In this study, we found that thiosulfate was approximately 100-fold more permeable through the hindgut ( $P = 7.6 \times 10^{-5} \pm 7.7 \times 10^{-5} \text{ cm s}^{-1}$ ) than through the body wall ( $P = 5.5 \times 10^{-7} \pm 2.7 \times 10^{-7} \text{ cm s}^{-1}$ ). The difference in permeability between hindgut and body wall is at least partially caused by the greater thickness of the body wall, as was evident from the decreased difference between the two tissues in the diffusion coefficient,  $D$ , compared with the permeability coefficient,  $P$ . As noted above, it is important to recognize that determination

of the permeability coefficient assumes that thiosulfate efflux across the epithelia is by passive diffusion.

The variability of the permeability coefficient was consistently higher for hindgut than it was for body wall. Because of the very high compliance of the hindgut tissue, we were unable to mount replicate tissue samples with the same degree of stretch, despite concerted effort. In a previous study, the permeability of *U. caupo* hindgut to total sulfide (H<sub>2</sub>S, HS<sup>-</sup>, and S<sup>2-</sup>) was two to six times higher in stretched hindgut than in unstretched hindgut, depending on pH (Julian and Arp 1992). It is likely that effects of stretch on thiosulfate permeability were similar. The thiosulfate permeability coefficients for hindgut and body wall were each much lower than the permeability coefficients for H<sub>2</sub>S and HS<sup>-</sup> measured in the same tissues at similar degrees of stretch (hindgut:  $P_{\text{H}_2\text{S}} = 30 \times 10^{-3} \text{ cm s}^{-1}$ ,  $P_{\text{HS}^-} = 2.7 \times 10^{-3} \text{ cm s}^{-1}$ ; body wall:  $P_{\text{H}_2\text{S}} = 2.8 \times 10^{-3} \text{ cm s}^{-1}$ ,  $P_{\text{HS}^-} = 1.0 \times 10^{-3} \text{ cm s}^{-1}$ ; Julian and Arp 1992), as expected from the smaller diameters of the H<sub>2</sub>S and HS<sup>-</sup> molecules.

An important question is how, in the absence of a transmembrane transport mechanism, thiosulfate exits cells and reaches the coelomic fluid, hemolymph, or blood of a sulfide-adapted animal. Since all proposed nonbacterial sulfide oxidation mechanisms in invertebrates occur within cells, the charged thiosulfate molecule must first cross the cell membrane before it can passively diffuse out of the animal paracellularly. This mechanism remains unknown. Importantly, such a transport mechanism need not be active, since the concentration gradient should always be outward.

#### Conclusion

*Urechis caupo* exposed to sulfide under conditions simulating that of a long tidal immersion, that is, 2 h duration, 50–100 μmol L<sup>-1</sup> sulfide, 10 kPa O<sub>2</sub> (Arp et al. 1992), were able to maintain coelomic sulfide concentrations at less than 20% of that in the external environment and accumulated more than 200 μmol L<sup>-1</sup> thiosulfate. When thiosulfate was injected into the coelomic fluid to a concentration above that which would be expected to result from such a sulfide exposure, *U. caupo* were able to eliminate the thiosulfate with a half-life of 6 h, which would be sufficient under natural conditions to eliminate almost all accumulated thiosulfate before the next tidal exposure. In this study, we determined that efflux across the hindgut is likely the primary mechanism of thiosulfate elimination under normal conditions. Furthermore, we hypothesize that this efflux is primarily via passive diffusion. While there may be other mechanisms for thiosulfate elimination in *U. caupo*, such as thiosulfate excretion via anal vesicles, active transepithelial thiosulfate transport, or thiosulfate oxidation, we conclude that the contribution of these mechanisms is probably minor.

*Note Added in Proof*

After this article went to press, Hauschild et al. (1999) published a report of thiosulfate elimination in the mudflat lugworm *Arenicola marina*. Hauschild et al. (1999) determined a thiosulfate permeability coefficient for *A. marina* body wall of  $3.64 \times 10^{-7} \text{ cm s}^{-1}$ , which is very similar to the coefficient of  $5.5 \times 10^{-7} \text{ cm s}^{-1}$  that we determined for *U. caupo* body wall. (*A. marina* does not have a hindgut.) Based on their whole-animal thiosulfate uptake and elimination experiments and in vitro measurements of permeability and short-circuit current, Hauschild et al. (1999) concluded that thiosulfate elimination in *A. marina* is primarily via passive diffusion across the body wall.

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