

Physiology of sulfide detoxification in the isopod *Saduria (Mesidotea) entomon*

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ABSTRACT: The physiology of sulfide detoxification in the isopod *Saduria (Mesidotea) entomon* (L.) was studied in vitro as well as in vivo using a sulfide oxidation assay and high-performance liquid chromatography. *S. entomon* is unable to prevent hydrogen sulfide from entering its body. Hydrogen sulfide is transported by the blood to the hepatopancreas, where it is detoxified in the fluid portion by an O₂-dependent oxidation to S₂O₃²⁻ and SO₃²⁻ (S⁰ or SO₄²⁻ formation could not be demonstrated). An O₂-independent binding of sulfide, probably by iron, also occurs in the hepatopancreas. In addition, H₂S is oxidized to S₂O₃²⁻ in the muscle. This oxidation is suggested to be localized in the mitochondrial fraction of the muscle. Glutathione does not play an important role in the detoxification. This study supports the hypothesis that hydrogen sulfide is an important factor in the distribution of marine invertebrates in soft sediments.

INTRODUCTION

Hydrogen sulfide is known to be toxic to aerobic organisms at nanomolar to micromolar concentrations (National Research Council 1979). This toxicity is caused by inhibition of metalloenzymes, and especially cytochrome oxidase and blood pigments (Evans 1967). Nevertheless, hydrogen sulfide is produced internally as a by-product in the degradation of sulfur-containing proteins, certain coenzymes and metabolites. Sulfide is also produced by microbial fermentation in the alimentary canal (Siegel 1975). Detoxification mechanisms are necessary in order to avoid the toxic effects of this internally produced sulfide. Sulfide oxidation has been demonstrated in the liver and kidney of rats (Baxter et al. 1958, Sörbo 1958). Exposed to sulfide, dogs and rats excrete sulfate in the urine (Dziewiatkowski 1945, Yokoyama et al. 1971, Curtis et al. 1972). Sulfide is also oxidized by the blood plasma of vertebrates (Haggard 1921, Evans 1967).

Marine sediments are typically anoxic a few mm or cm beneath the surface and contain up to millimolar concentrations of sulfide (Fenchel & Riedl 1970). Sulfide is produced principally by sulfate-respiring bacteria and to a lesser extent by the fermentative degradation of proteins (Fenchel 1969). Thus, sediment-dwelling invertebrates face the risk of exposure to hydrogen sulfide in the environment.

Some invertebrates cope with the sulfide by irriga-

tion with water from above the sediment so that their surroundings remain oxidized (e.g. Meyers et al. 1987, 1988). In sulfide-rich sediments this is not always effective, viz. at low tide or when sulfide is present in the bottom water or when the animals establish new tubes or move within the sediment. Invertebrates from sulfide-rich environments are therefore likely to show an increased tolerance to hydrogen sulfide. Differential tolerance to sulfide may influence the distribution of marine infaunal invertebrates.

Studies indicating that hydrogen sulfide is an important environmental factor in the distribution of animals are accumulating. Thus a high tolerance to hydrogen sulfide in fauna from sulfide-rich environments has been shown by e.g. Jacobowa & Malm (1931), Theede et al. (1969), Oertzen & Schlungbaum (1972), Degn & Kristensen (1981), Oeschger & Schmaljohann (1988), Bagarinao & Vetter (1989), Oeschger & Storey (1990), and Vismann (1990). Sulfide catalysis has been demonstrated in a number of sediment-dwelling species (Patel & Spencer 1963, Powell et al. 1979, Powell & Somero 1985, 1986, Anderson et al. 1987, Vetter et al. 1987, Powell & Arp 1989, Vismann 1990). The ecological significance of hydrogen sulfide is also demonstrated in comparative studies on meiofauna, polychaetes, crustaceans and fishes (Vargo & Sastry 1978, Powell et al. 1979, Vetter et al. 1987, Bagarinao & Vetter 1989, 1990, Vismann 1990).

Tolerance to hydrogen sulfide (excluding species

with symbiotic sulfide-oxidizing bacteria) may be achieved by the following mechanisms: (1) exclusion of hydrogen sulfide; (2) possession of a cytochrome oxidase and an oxygen-transporting blood pigment which are insensitive to hydrogen sulfide; (3) dependence on anaerobic energy metabolism; (4) detoxification of hydrogen sulfide.

Detoxification can be effected by (1) enzymatic oxidation of hydrogen sulfide to non-toxic sulfur compounds; (2) non-enzymatic oxidation by metallic ions or metallo-protein complexes; (3) immobilization of hydrogen sulfide by binding to compounds such as metallic ions or proteins. Especially ferrous iron (e.g. Buchanan et al. 1980, De Ridder et al. 1985) and the tripeptide glutathione (e.g. Smith & Abbanat 1966, Siegel 1975) have been suggested as taking part in the detoxification of hydrogen sulfide.

Crustaceans are generally not tolerant to low oxygen tensions, but the few species which are tolerant to hypoxia may also be tolerant to hydrogen sulfide. Detoxification of sulfide has been demonstrated in some crustaceans from sulfide-rich environments (Vargo & Sastry 1978, Vetter et al. 1987). In the species studied by Vetter et al. (1987), sulfide detoxification takes place in the hepatopancreas. In crustaceans, this organ consists of one to several pairs of bilaterally arranged mesenteric glandular appendages extending from the midgut. The hepatopancreas is an exocrine gland excreting numerous enzymes into its extracellular lumen, where many metabolic activities are known to occur, including digestion and detoxification of poisonous substances (see e.g. Gibson & Barker 1979).

The isopod *Saduria (Mesidotea) entomon* (L.) is an example of an invertebrate living in an environment with high risk of exposure to external sulfide. *S. entomon* is a scavenger and burrows in sandy or muddy sediments, but it does not construct tubes. In the Baltic Sea it is an important faunal component, both as a consumer and as a food item for cod (Haathela 1962). As shown by Hagerman & Szaniawska (1988, 1990) *S. entomon* is very tolerant to hypoxia/anoxia.

The purpose of the present work was to elucidate physiological mechanisms involved in sulfide detoxification in *Saduria entomon*. The study included *in vitro* as well as *in vivo* experiments. The questions I attempted to answer were the following: (1) In which tissues and at what rates is hydrogen sulfide detoxified? (2) What is the chemical nature of the detoxification? Using intact isopods it was further investigated whether hydrogen sulfide can be prevented from entering the organism, and how the concentrations of hydrogen sulfide, glutathione, and detoxification products vary in different tissues during exposure to hydrogen sulfide.

MATERIALS AND METHODS

Sampling. *Saduria entomon* was collected at depths exceeding 15 m in the Gulf of Gdańsk, Poland (salinity 7 to 8‰, temperature 8°C) and in the area between Scania and Bornholm, Denmark (7 to 8‰ S, 8°C). The specimens were transported to the laboratory in Helsingør, Denmark, and kept at constant temperature (6°C) in large tanks with natural sediment and aerated water (7‰ S) until experiments started. The isopods were fed once weekly with bivalve meat. The crustacean *Carcinus maenas*, which lives in sulfide-free habitats, was used as a control in the sulfide oxidation activity analysis. *C. maenas* was collected in the Kattegat, Denmark.

The specimens used in analysis were dried and blood samples taken by carefully inserting a 100 µl hypodermic syringe (Terumo) into the heart from a postero-dorsal direction. The carapace was removed and tissue samples were taken. The hepatopancreas of *Saduria entomon* consists of 3 pairs of glandular appendages. Each appendage was removed with its contents using a pair of forceps to lift the organ at the area where it opens into the midgut. The fluid component of the gland was obtained by puncturing the gland, allowing the fluid to drain out. The cellular component of the gland was carefully washed before analysis. During the experiments it was noted that the hepatopancreas of the specimens used showed differences in color. The hepatopancreas of the control groups and the hypoxic groups were in general white to yellow in color, whereas the hepatopancreas of the sulfide-exposed individuals all had turned black.

Sulfide oxidation activity. Tissue samples of *Saduria entomon* and *Carcinus maenas* were homogenized on ice in a buffer (pH 8.0, 50 mM Tris/HCl and 0.1 % of the laboratory detergent 'Prox'). The samples were centrifuged for 10 min at 10 000 × *g*. Sulfide oxidation of the supernatant was measured colorimetrically using benzyl viologen (1.1'-dibenzyl-4,4'-bipyridinium). The reaction mixture contained 40 mM glycine/NaOH buffer (pH 9.0), 2 mM benzyl viologen, 0 to 5 mM Na₂S, and 200 µl sample in a total volume of 2 ml. The method is basically that of Powell & Somero (1985) as modified by Vismann (1990).

Elemental sulfur. Elemental sulfur was analyzed by high-performance liquid chromatography (HPLC) using 1 pump (Jasco 880 PU), a manual injector (Rheodyne 7105) fitted with a 20 µl loop, and a UV/VIS detector (Jasco 875-UV) set at 254 nm. The column was a Hamilton PRP-1 reversed phase column (15 cm × 4.1 mm i.d.). Treatment of the column prior to analysis, extraction of samples in chloroform followed by uptake in methanol, and the HPLC protocol were performed according to Lauren & Watkinson (1985).

Thiols. Monobromobimane (mBBr) and primary thiols form fluorescent adducts (mBSR), which can be separated by HPLC (e.g. Fahey & Newton 1987, Vetter et al. 1989). Tissue samples were rapidly homogenized on ice in a deoxygenated buffer [200 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid) and 5 mM EDTA; pH 8.0] with a sonicator (Mullard 7685/2). The homogenate (100 μ l) was added to 10 μ l mBBr (70 mM). The mixture was allowed to derivatize in darkness for 15 min. Proteins were precipitated by addition of 100 μ l acetonitrile followed by heating (60°C) for 10 min. Finally, 200 μ l of the mixture was combined with 300 μ l methane sulfonic acid (25 mM; pH 3.9) to dilute the acetonitrile and to stabilize the sample. Samples which were not analyzed immediately were stored at -20°C until analysis (max. 30 d). No significant effect on sample fluorescence has been shown for storage up to 20 mo at +4, -20 or -70°C (Fahey & Newton 1987). The samples were centrifuged at 15 000 $\times g$ for 10 min before the HPLC analysis.

Thiols were analyzed by HPLC via 2 pumps (Jasco 880 PU), a manual injector (Rheodyne 7105) fitted with a 20 μ l loop, a solvent mixing module (Jasco 880-31) and a fluorescence detector (Perkin-Elmer LC 1000, excitation = 395 nm, emission = 480 nm). The fluorescence detector output (mV) was logged on a computer with 2 s intervals. HPLC separation was carried out on a Spherisorb column (S5 ODS1; 25 cm \times 4.6 mm i.d.). Flow rate was 1.2 ml min⁻¹. The buffer system consisted of 2 buffers: Buffer A, 10% methanol and 0.25% acetic acid; Buffer B, 90% methanol and 0.25% acetic acid. Both buffers were prepared in glass-distilled water. The buffers were adjusted to pH 3.9, filtered and stripped of air bubbles. The elution profile was as follows: 0 to 10 min: 8% Buffer B (isocratic); 10 to 20 min: 8 to 40% Buffer B (linear gradient); 20 to 25 min: 40% Buffer B (isocratic); 25 to 30 min: 40 to 90% Buffer B (linear gradient). Elution was followed by column regeneration: 30 to 32 min: 90 to 100% Buffer B (linear gradient); 32 to 35 min: 100% Buffer B (isocratic); 35 to 45 min: 8% Buffer B (isocratic).

Peaks of hydrogen sulfide (H₂S), thiosulfate (S₂O₃²⁻), sulfite (SO₃²⁻) and reduced glutathione (GSH) were integrated on the digitalized chromatograms and converted to concentration (per gram tissue) by a computer. Identification and conversion for each protocol were based on chromatograms of standard solutions (0 to 1 mM) and controls (Vetter et al. 1989).

In vivo exposure to H₂S. *Saduria entomon* were exposed to H₂S in an aquarium (30 l) with natural sediment. A 10 mM sulfide stock solution (covered with paraffin oil to prevent gas diffusion and made fresh every day) was continuously pumped into the aquarium by a peristaltic pump (Gilson 312). The aquarium was aerated with nitrogen and atmospheric air. The water in

the aquarium was covered with small plastic spheres. With this experimental set-up it was possible to control both oxygen tension and H₂S concentration. Oxygen was monitored using a polarographic oxygen sensor (Radiometer, E 5047) connected to a Radiometer PHM 73 acid-base analyzer. The electrode was only used for short intervals to avoid H₂S contamination of the electrode. H₂S concentration was monitored continuously with an Ag-AgS electrode connected to a potentiometer (Radiometer, Ion85) with a calomel electrode as reference. In addition, sulfide concentration was analyzed with HPLC during the experiments.

RESULTS

In vitro experiments

Sulfide oxidation activity

Sulfide oxidation activity in different tissues of *Saduria entomon* incubated at 5 mM sulfide is shown in Table 1. The highest oxidation activity was found in the hepatopancreas, while oxidation activity in homogenates of muscle and blood was not significantly higher than the biological basal level (ca 0.20 to 0.25 μ mol min⁻¹ g⁻¹; see Powell & Somero 1985 and Vismann 1990). The activity of gill homogenate was just above the basal level. Sulfide oxidation in the cellular and fluid components of the hepatopancreas showed that most of the oxidation was confined to the fluid component (Table 1). For comparison, sulfide oxidation in the hepatopancreas of *Carcinus maenas* from a sulfide-free habitat is also shown. Sulfide oxidation activity was tested for saturation kinetics in the hepatopancreas components in *S. entomon* (data not shown); sulfide oxidation activity in the cellular component was very low and regression analysis did not indicate saturation kinetics. Thus, the sulfide oxidation activity of the cellular component represented a non-specific oxidation. In the fluid components, however, the relation between substrate concentration and activity is well described by the Monod function ($R^2 = 0.99$, $p < 0.001$, $n = 10$), indicating presence of a catalyst with a half-saturation constant (K_m) and a maximal catalytic rate (V_{max}) of 0.52 mM and 128.35 μ mol min⁻¹ g⁻¹, respectively. However, there was a very large discrepancy between the results with benzyl viologen and those obtained with HPLC (see below and 'Discussion').

Sulfide removal and oxidation products

The change in reduced sulfur in homogenates of the fluid component of the hepatopancreas incubated with

Table 1 *Saduria entomon* and *Carcinus maenas*. Sulfide oxidation activity at 5 mM H₂S in homogenates of different tissues. Activities are expressed in $\mu\text{mol min}^{-1} \text{g}^{-1} \pm \text{SEM}$. Number of samples in parentheses

Species	Tissue	Mean sulfide oxidation
<i>S. entomon</i>	Gill (11)	0.36 ± 0.06
	Muscle (8)	0.31 ± 0.06
	Blood (12)	0.19 ± 0.04
	Midtgut fluid (2)	7.60 ± 0.11
	Hepatopancreas (8)	33.38 ± 8.11
	Cellular component of hepatopancreas (14)	2.27 ± 0.26
	Fluid component of hepatopancreas (7)	104.88 ± 27.63
<i>C. maenas</i>	Hepatopancreas (8)	0.78 ± 0.12
	Cellular component of hepatopancreas (3)	0.43 ± 0.10
	Fluid component of hepatopancreas (3)	0.15 ± 0.05

1 mM H₂S (under an oxygen tension $p_w\text{O}_2 = 145$ torr) in relation to time is seen in Fig. 1. Elemental sulfur was not measurable in any in vitro or in vivo experiments [detection limit with 20 μl injection: 1 to 3 ng (Lauren & Watkinson 1985)]. Thus, this compound has been omitted from all tables and figures. In Fig. 1, the slope of regression lines gives the rate of H₂S removal and of SO₃²⁻ and S₂O₃²⁻ accumulation (Table 2). When corrected for the corresponding control, H₂S was removed at a rate of $4.44 \mu\text{mol min}^{-1} \text{g}^{-1}$, and the accumulation rate of SO₃²⁻ and S₂O₃²⁻ was 1.18 and $1.42 \mu\text{mol min}^{-1} \text{g}^{-1}$, respectively. About 60% of the H₂S removed was oxidized to SO₃²⁻ and S₂O₃²⁻. The fraction of H₂S removed and not recovered as SO₃²⁻ or S₂O₃²⁻ was $1.84 \mu\text{mol min}^{-1} \text{g}^{-1}$. The possibility that this fraction was oxidized to SO₄²⁻ was tested in an additional incubation, in which O₂-dependent oxidation was inhibited by a low oxygen tension ($p_w\text{O}_2 < 1$ to 2 torr) in the medium. Rates of H₂S removal and of SO₃²⁻ and S₂O₃²⁻ production in this hypoxic incubation are given in Table 2. Inhibition of SO₃²⁻ and S₂O₃²⁻ production was clearly seen. However, the H₂S removed and not recovered as SO₃²⁻ or S₂O₃²⁻ in the hypoxic incubation amounted to $1.49 \mu\text{mol min}^{-1} \text{g}^{-1}$, which is not significantly different from the $1.84 \mu\text{mol min}^{-1} \text{g}^{-1}$ in the normoxic incubation. Thus, it is most likely that the H₂S removed and not oxidized to SO₃²⁻ and S₂O₃²⁻ is turned into something other than SO₄²⁻. In a methodological test, either methane sulfonic acid (pH 3.9) or HEPES buffer (pH 8.0) were added to subsamples of homogenate of H₂S-exposed hepatopancreas. The subsamples were immediately analyzed by HPLC. It was observed that the acidic samples showed $40.9 \pm 14.6\%$ higher H₂S content compared to the alkaline samples (data not shown), i.e. ca 40% of the H₂S measured was acid labile H₂S. Thus, in addition to the $4.44 \mu\text{mol min}^{-1} \text{g}^{-1}$ of H₂S removed, some H₂S is immobilized as acid labile H₂S.

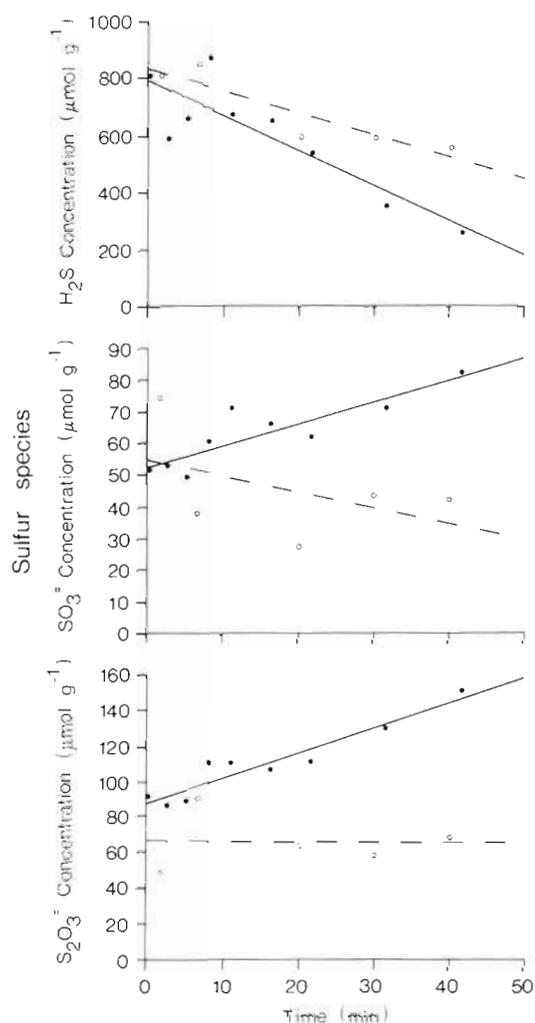


Fig. 1. *Saduria entomon*. Concentration of H₂S, SO₃²⁻, and S₂O₃²⁻ in homogenates of the fluid component of hepatopancreas in isopods incubated with 1 mM H₂S, as a function of time. (●) Hepatopancreas, (○) control. Solid and broken lines are calculated best-fit straight lines of hepatopancreas and control, respectively

Heat stability of the sulfide oxidation

The occurrence of enzymatic catalysis of sulfide oxidation in the hepatopancreas fluid was tested in a number of heat stability experiments. The rate of H₂S removal and of SO₃²⁻ and S₂O₃²⁻ accumulation in a sample [fractionated into a heated (30 min at 100 °C) and a non-heated sample] exposed to 1 mM sulfide is shown in Table 2. Accumulation of SO₃²⁻ and S₂O₃²⁻ under normoxia was seen to decrease by ca 30 and 38%, respectively, in the heated sample. In the heated and unheated hypoxic incubations the same trend was observed regarding S₂O₃²⁻ accumulation. The decrease in SO₃²⁻ and S₂O₃²⁻ accumulation in the heated samples indicates the occurrence of enzymatic H₂S oxidation. Removal of H₂S in the heated samples was significantly increased in both the normoxic and the hypoxic incubations. Thus, denatured proteins and metallic ions, which may be more available in the heated samples, seem to allow an increased non-specific binding of H₂S. This, together with the pool of acid labile H₂S and the color change in the hepatopancreas of sulfide-exposed individuals, suggests the presence of iron in the fluid component of the hepatopancreas.

In vivo experiments

Exclusion of sulfide from the organism

H₂S concentration in the blood of isopods adapted to severe hypoxia (3 ± 2 torr) for 10 d and then exposed to a slowly increasing sulfide concentration is shown in Fig. 2. Regression lines of H₂S concentration in the blood and water, respectively, as a function of time are given by: intercept = 1.73 ± 0.85 μmol g⁻¹, slope = 2.18 ± 0.44 μmol g⁻¹ h⁻¹ (R² = 0.86, p < 0.01); and

Table 2. *Saduria entomon*. Rate of removal/production of H₂S, SO₃²⁻, and S₂O₃²⁻ of homogenate of the fluid component of hepatopancreas in isopods incubated with 1 mM H₂S at normoxia (p_wO₂ = 145 torr) and hypoxia (p_wO₂ < 1 to 2 torr) before and after heating (100 °C for 30 min). Rates are expressed as μmol min⁻¹ g⁻¹ ± SEM

Treatment	SO ₃ ²⁻	S ₂ O ₃ ²⁻	H ₂ S
Normoxia			
No heat	0.68 ± 0.14	1.39 ± 0.18	-12.27 ± 2.59
Heat	-0.15 ± 0.31	0.51 ± 0.29	-19.85 ± 5.51
Control	-0.50 ± 0.56	-0.03 ± 0.56	-7.83 ± 2.05
Hypoxia			
No heat	0.01 ± 0.03	0.69 ± 0.13	-2.18 ± 1.26
Heat	0.09 ± 0.06	0.42 ± 0.10	-4.19 ± 0.80
Control	-0.03 ± 0.05	0.43 ± 0.10	-0.39 ± 0.77

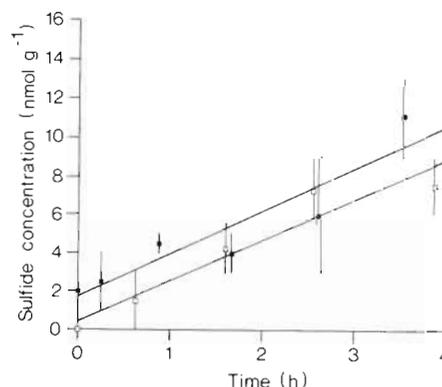


Fig. 2. *Saduria entomon*. Concentration of H₂S in blood as a function of increasing external H₂S concentration. (■) Blood, (□) water. Best-fit straight lines calculated using a linear regression program. Data points represent triplicate measurements ± SE

intercept = 0.48 ± 0.78 μmol g⁻¹, slope = 2.08 ± 0.35 μmol g⁻¹ h⁻¹ (R² = 0.92, p < 0.01). The sulfide concentration in the blood reflected the environmental concentration, so it was not possible for *Saduria entomon* to prevent sulfide from entering its body.

Natural levels of H₂S, SO₃²⁻, S₂O₃²⁻, and GSH

Natural levels of reduced sulfur compounds in *Saduria entomon* kept for 10 d at normoxia (145 ± 12 torr) and hypoxia (3 ± 2 torr) are shown in Figs. 3 & 4. No significant difference (p > 0.05) in the levels of H₂S, SO₃²⁻, and S₂O₃²⁻ was seen between normoxia and hypoxia in any of the tissues (Fig. 3). In the blood, all levels of H₂S, SO₃²⁻, and S₂O₃²⁻ were on average < 2 nmol g⁻¹, except that S₂O₃²⁻ was ca 8 nmol g⁻¹ at hypoxia. In the muscle the average levels of H₂S, SO₃²⁻, and S₂O₃²⁻ were ca 13, 30, and 150 nmol g⁻¹ respectively. In the hepatopancreas average levels were ca 50, 100, and 35 nmol g⁻¹ respectively. Levels of GSH between normoxia and hypoxia in blood and hepatopancreas were not significantly different (p > 0.05). In the muscle, however, a significant difference (p < 0.05) was observed between normoxia and hypoxia. The average level of GSH in blood and hepatopancreas was ca 20 and 2400 nmol g⁻¹ respectively. In the muscle, GSH level under normoxia (ca 360 nmol g⁻¹) increased to ca 1200 nmol g⁻¹ under hypoxia.

Reduced sulfur compounds in relation to hypoxia and H₂S exposure

Levels of reduced sulfur compounds in isopods kept for 10 d under hypoxia followed by 1 d of exposure to H₂S (80 μM) are shown in Figs. 3 & 4. When comparing

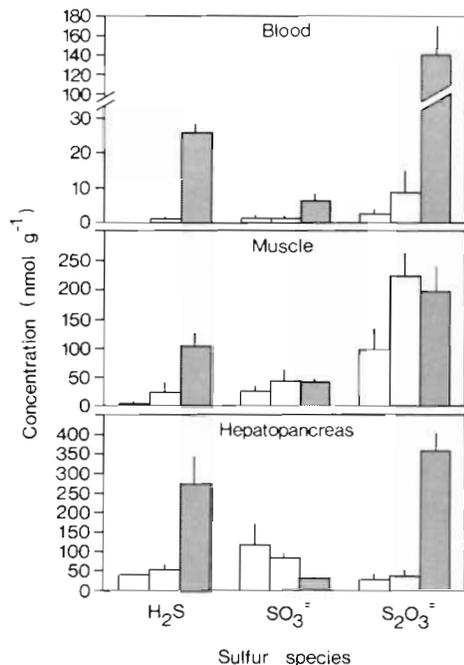


Fig. 3. *Saduria entomon*. Concentration of H₂S, SO₃²⁻, and S₂O₃²⁻ in different tissues of isopods (n = 5) as a function of p_wO₂ with/without H₂S. Specimens were adapted to experimental p_wO₂ conditions for 10 d prior to analysis. Open bars: 145 torr; light gray bars: 3 torr; dark gray bars: 3 torr + 80 μM H₂S. Standard error bars also shown.

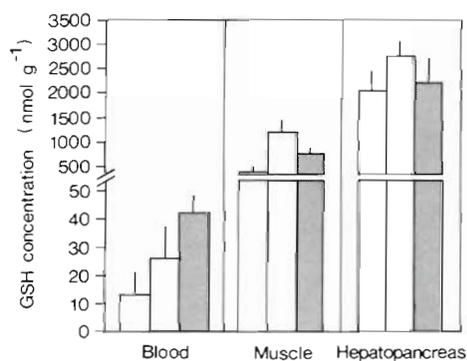


Fig. 4. *Saduria entomon*. Concentration of GSH in different tissues of isopods (n = 5) as a function of p_wO₂ with/without H₂S. Specimens were adapted to the experimental p_wO₂ for 10 d prior to analysis. Bars as in Fig. 3.

specimens under hypoxia with and without H₂S, all tissues showed higher H₂S levels when exposed to H₂S (blood, $p < 0.000001$; muscle, $p < 0.01$; hepatopancreas, $p < 0.05$). No significant differences were found in the levels of SO₃²⁻. However, S₂O₃²⁻ accumulated in blood ($p < 0.01$) and hepatopancreas ($p < 0.001$) during H₂S exposure, and the concentration of H₂S in blood, muscle, and hepatopancreas increased from natural levels to ca 25, 100, and 275 nmol g⁻¹ respectively. In

fact, the hepatopancreas concentrates H₂S. No significant differences in GSH levels of any tissue were observed between specimens under hypoxia with and without H₂S.

Reduced sulfur compounds in relation to duration of H₂S exposure

Concentrations of H₂S, SO₃²⁻, S₂O₃²⁻, and GSH in tissues exposed to H₂S and hypoxia (3 ± 2 torr) are shown in Figs. 5 & 6. Mean concentrations of H₂S, SO₃²⁻, S₂O₃²⁻, and GSH in the water were 1.57 ± 0.20 mM, 0.03 ± 0.01 mM, 0.11 ± 0.03 mM, and not detectable, respectively. Each data point in these 2 figures represents the mean of 3 different individuals, as animals were killed when analyzed. Hence, the standard error bars are relatively high, and the results are only considered as trends. H₂S, SO₃²⁻ and S₂O₃²⁻ in the blood accumulated slowly to ca 0.5, 0.10 and 0.25 μmol g⁻¹, respectively, at the end of the experiment. In the muscle, H₂S increased during the experiment to reach a concentration of ca 0.75 μmol g⁻¹. SO₃²⁻ and S₂O₃²⁻ concentrations in the muscle increased to a steady level (0.25 and 1 μmol g⁻¹ respectively) within 1 h. As in the muscle, the trend in SO₃²⁻ concentration in the hepatopancreas reached a steady level of 0.25 μmol g⁻¹ within 1 h. In the hepatopancreas, H₂S and S₂O₃²⁻ increased during the first 400 min of H₂S exposure, but tended to decrease during the last 300 min of the experiment. GSH (Fig. 6) in the blood increased slowly during the first 500 min of H₂S exposure and at a higher rate thereafter. After 100 min of H₂S exposure, GSH in the muscle increased rapidly to a steady level of ca 2.5 μmol g⁻¹. In the hepatopancreas, GSH concentration quickly increased to a level of ca 7 μmol g⁻¹, which was maintained until 300 min of H₂S exposure. During the last 400 min of the experiment, GSH concentration in the hepatopancreas decreased. This decrease coincided with the decrease in H₂S and S₂O₃²⁻.

DISCUSSION

Besides chemical transformation (detoxification) of H₂S, the only strategies in marine invertebrates for avoiding poisoning are the selective exclusion of H₂S, reliance upon anaerobic respiration or possession of H₂S-insensitive enzymes. The crustacean gill is known to regulate ionic exchange (e.g. Lucu 1990). Selective exclusion of H₂S should, up to a critical concentration, result in lower H₂S concentration in the blood than in the external environment. To my knowledge, exclusion of H₂S has not been demonstrated in any invertebrate

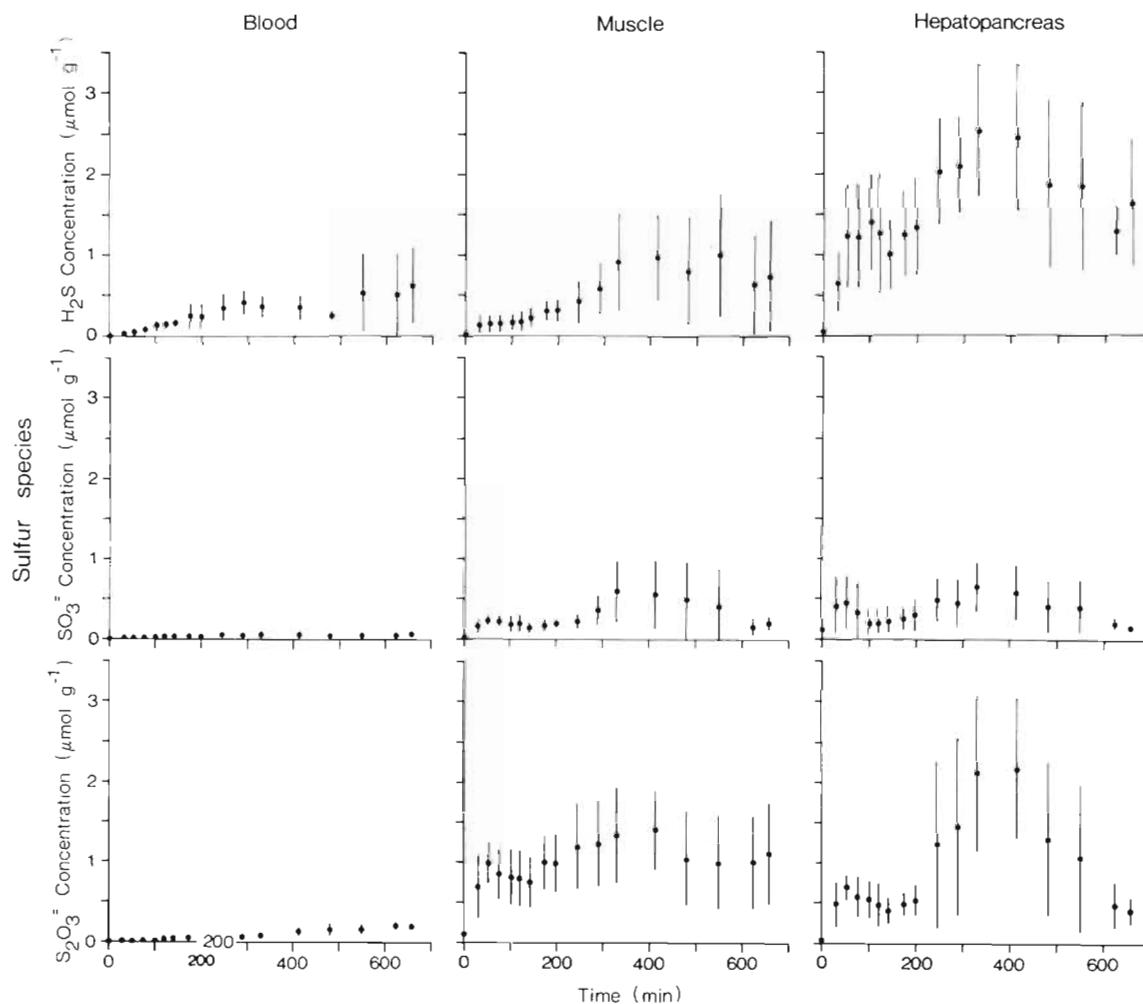


Fig. 5. *Saduria entomon*. Concentrations of H_2S , SO_3^{2-} , and $\text{S}_2\text{O}_3^{2-}$ in different tissues of isopods exposed to $p_w\text{O}_2 = 3$ torr and $1.57 \text{ mM H}_2\text{S}$, as a function of time. Specimens were adapted to normoxia ($p_w\text{O}_2 = 145$ torr) for 10 d prior to the experiment. Each data point represents measurements for 3 individuals \pm SE

(e.g. Powell et al. 1979, Vetter et al. 1987). In the present study, a linear increase in external H_2S concentration from ca $2 \mu\text{M}$ to $10 \mu\text{M}$ (Fig. 2) was instantaneously paralleled in the blood. If exclusion of H_2S is possible in *Saduria entomon* the H_2S concentration in the blood should be lower than that in the external environment, as only very low sulfide concentrations were used. *S. entomon* is therefore not able to prevent H_2S from entering its body.

Very few species are known to survive low oxygen tensions in the presence of H_2S on the basis of anaerobic respiration. In a study of the energy metabolism in vent fauna by Hand & Somero (1983), animals (the clam *Calyptogena magnifica* excepted) were found to possess enzymes characteristic of aerobic respiration in amounts similar to those in animals from sulfide-free environments. During anoxia *Saduria entomon* accumulates lactate when forced to be active, but

if left undisturbed it is quiescent and accumulates alanine instead (Hagerman & Szaniawska 1990). *S. entomon* is one of the very few crustaceans which are adapted to long-term anaerobiosis. Hagerman & Szaniawska (1990) found *S. entomon* to survive up to 300 h of anoxia. On the other hand, *S. entomon* has been shown to maintain stable aerobic respiration at decreasing $p_w\text{O}_2$ down to < 5 to 10 torr (Hagerman & Szaniawska 1988). In practice, *S. entomon* must be considered able to extract all O_2 from the water. Respiratory overshoot (indicating anaerobiosis during hypoxia) in *S. entomon* recovering from several hours of severe hypoxia was only seen occasionally by Hagerman & Szaniawska (1988). In accordance with Hagerman & Szaniawska (1988) it is assumed in the present study that *S. entomon* respire aerobically. Aerobic/anaerobic respiration and concentrations of metabolites with increasing hypoxia and/or presence of

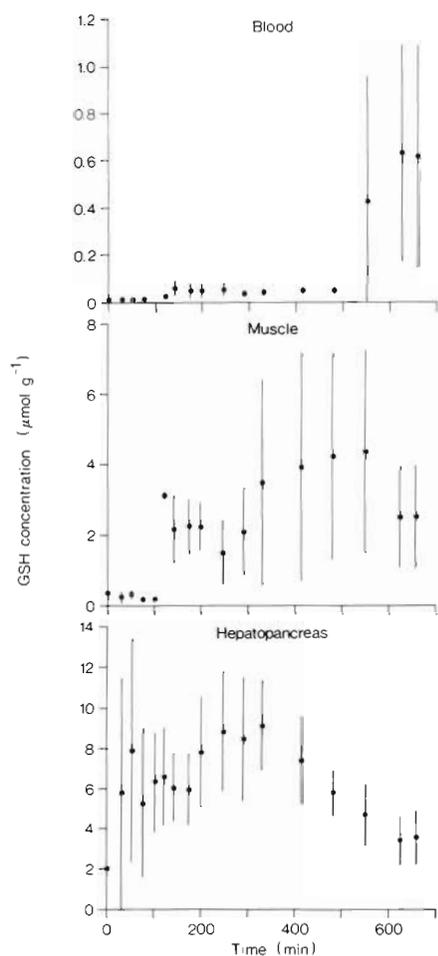


Fig. 6. *Saduria entomon*. Concentrations of GSH in different tissues of isopods exposed to $p_wO_2 = 3$ torr and 1.57 mM H_2S , as a function of time. Specimens were adapted to normoxia ($p_wO_2 = 145$ torr) for 10 d prior to the experiment. Each data point represents measurements for 3 individuals \pm SE

H_2S are presently being studied by the author and L. Hagerman.

Saduria entomon exposed to a mixture of O_2 and H_2S in the environment survives by detoxifying H_2S in the hepatopancreas. This has also been found for other sulfide-tolerant crustaceans (Vetter et al. 1987). The cellular component of the crustacean hepatopancreas is known to be involved in synthesis, storage, and secretion of enzymes, absorption of nutrients and excretion of waste products (Gibson & Barker 1979). Although an intracellular phase of digestion has been demonstrated (Barker & Gibson 1977), the activity of digestive enzymes is normally confined to the fluid component. It is thus physiologically plausible that enzymatic detoxification of H_2S is also confined to the fluid component of the gland.

Detoxification can be quantified as the rate of H_2S removal. The rate of sulfide removal measured with the

benzyl viologen method is the highest ever measured (see e.g. Powell & Somero 1985, 1986, Vetter et al. 1987, Bagarinao & Vetter 1989, Vismann 1990). From data in Moriarty & Nicholas (1969) sulfide oxidation in *Thiobacillus concretivorus* can be calculated to be ca $50 \mu\text{mol min}^{-1} \text{g}^{-1}$. In the bivalve *Solemya reidi*, the gills contain a high number of sulfide-oxidizing bacteria, and the gill sulfide oxidation activity has been found to be ca $6 \mu\text{mol min}^{-1} \text{g}^{-1}$ (Powell & Somero 1985). Therefore, the sulfide oxidation rate as estimated with the benzyl viologen method seems unrealistically high. In addition, the benzyl viologen method has a number of shortcomings: (1) the method is performed with an anoxic reaction medium, making production of SO_3^{2-} , $S_2O_3^{2-}$, or SO_4^{2-} impossible; (2) it is an indirect method, in which nothing is known about the electron-producing reactions leading to color development; (3) the H_2S concentration normally used (5 mM) exceeds realistic in vivo concentrations. In agreement with the conclusion reached by Bagarinao & Vetter (1990), I regard the method as having no in vivo physiological significance. Although the H_2S removal rate estimated with HPLC is lower than that estimated with the benzyl viologen method by a factor of ca 10, the hepatopancreas of *Saduria entomon* still removes H_2S at a very high rate. The benzyl viologen assay showed no sulfide oxidation activity in the muscle (Table 1). However, $S_2O_3^{2-}$ was seen to accumulate in vivo in the muscles of isopods exposed to 1.5 mM H_2S (Fig. 5). According to Bagarinao & Vetter (1990) any mitochondrial sulfide detoxification occurring in the muscle would be inhibited at the H_2S concentration used in the benzyl viologen assay. Thus, the in vivo experiment indicates that mitochondrial sulfide detoxification occurs in the muscle of *S. entomon*. This process appears to operate only above a threshold of external sulfide concentration, as no $S_2O_3^{2-}$ accumulated in the muscle of individuals exposed in vivo to 80 μM H_2S (Fig. 3).

In the in vitro experiments, most of the H_2S removed by the hepatopancreas was oxidized to $S_2O_3^{2-}$ and SO_3^{2-} in an O_2 -dependent reaction. Specific catalysis of H_2S oxidation to $S_2O_3^{2-}$ has also been found in rat liver and kidney (Baxter & VanReen 1958a, Baxter et al. 1958, Sörbo 1958, 1960). However, the oxidation rates observed in these mammal studies are not higher than could be obtained by any low-molecular-weight iron chelate or iron-protein complex, e.g. ferritin or hemoglobin (Baxter & VanReen 1958b, Sörbo 1958, Siegel 1975). The removal of H_2S and production of $S_2O_3^{2-}$ observed in vitro by Baxter et al. (1958) was only ca 1% and 6%, respectively, of the rates found in *Saduria entomon*.

Vertebrates are known to oxidize H_2S to SO_4^{2-} , which is excreted with the urine (e.g. Dziewiatkowski

1945, Yokoyama et al. 1971, Curtis et al. 1972), and $S_2O_3^{2-}$ is produced as an intermediate compound (see e.g. Baxter & VanReen 1958a, Baxter et al. 1958, Sörbo 1958). Accumulation of $S_2O_3^{2-}$, instead of SO_4^{2-} excretion, has been demonstrated in several marine animals (e.g. Vetter et al. 1987, Bagarinao & Vetter 1989, O'Brien & Vetter 1990). A significant level of SO_4^{2-} production is probably also absent in *Saduria entomon*. Compared to production of SO_3^{2-} or SO_4^{2-} , that of $S_2O_3^{2-}$ has the highest efficiency in terms of H_2S removed per O_2 used. In the marine environment, the correlation between H_2S and low O_2 availability makes $S_2O_3^{2-}$ production the most likely O_2 -dependent mechanism of sulfide detoxification. The importance of O_2 availability for oxidation products is demonstrated in comparative studies by Powell et al. (1979, 1980). When exposed to a mixture of H_2S and O_2 , the O_2 -dependent strategy of *S. entomon* is to produce $S_2O_3^{2-}$, which accumulates in the muscle and the blood since it has a low ability to cross membranes (Holmes & Donaldson 1969). In the muscle and blood the accumulated $S_2O_3^{2-}$ is likely to be slowly excreted or oxidized to SO_4^{2-} when H_2S is no longer present. The fluid component of the hepatopancreas is a part of the external environment, and the $S_2O_3^{2-}$ produced in the gland is likely to leave via the digestive system.

Approximately 40% of the H_2S removed by the hepatopancreas of *Saduria entomon* is removed by an O_2 -independent mechanism. The basis for this may be H_2S binding to metallic ions, metal-protein complexes, formation of disulfide bonds, or oxidation to linear polysulfides. The present results strongly suggest that sulfide is bound to iron. In several crustaceans, the hepatopancreas is known to accumulate iron (Gibson & Barker 1979). In the priapulid worm *Halicryptus spinulosus*, iron present in the cuticular mucus coat is suggested to be a rechargeable buffer protecting against sulfide toxicity (Oeschger & Janssen 1991). Although this buffer cannot account for the long-term protection against sulfide seen in *H. spinulosus*, it allows the animal to gain time for metabolic adaptations (Oeschger & Janssen 1991). Iron has also been suggested to take part in binding of H_2S in echinoderms (Buchanan et al. 1980, De Ridder et al. 1985). I therefore suggest that a buffer is present in the hepatopancreas of *S. entomon*. This buffer represents an additional mechanism for eliminating H_2S toxicity.

In mice, H_2S has been shown to react with glutathione disulfide (GSSG) forming GSSH and GSH, which protect against acute H_2S toxicity (Smith & Abbanat 1966). Reduced glutathione stimulates the oxidation of $S_2O_3^{2-}$ to SO_3^{2-} , which in turn is oxidized to SO_4^{2-} (Sörbo 1964, Koj & Frenedo 1967, Koj 1968). Glutathione is abundant in animal tissues and involved in numerous reactions (Meister 1975, 1983).

In cells in a normal state, glutathione predominantly occurs in the reduced form. Oxidized glutathione (GSSG) is rapidly reduced to GSH by enzymatic catalysis. These oxidation-reduction processes are important in avoiding disruption of the thiol status of cell membranes by oxidative stress (Kosower & Kosower 1983). If the reaction between GSSG and H_2S occurs in vivo it would be observed as an increase in GSH concentration. The physiological significance of the process is uncertain, as only a limited amount of GSSG is present, and hypoxia itself has been shown to decrease GSSG formation (Siegel 1975, Jaeschke 1990). Although only significant in muscle tissue, the effect of hypoxia on GSSG formation was observed as an increase in GSH in all tissues of *Saduria entomon* (Fig. 4). Exposure to 80 μM H_2S of isopods adapted to hypoxia for 1 d did not change the GSH concentrations in any tissues, and they even tended to decrease. The effect of hypoxia on GSH makes it impossible to evaluate whether the increase in GSH seen in the other in vivo experiment (Fig. 6) was caused by hypoxia or reactions between H_2S and GSSG. The decrease in GSH and $S_2O_3^{2-}$ in the hepatopancreas observed after 400 min of H_2S exposure (Figs. 5 & 6) was not a GSH-stimulated oxidation of $S_2O_3^{2-}$ to SO_3^{2-} , as no simultaneous increase in SO_3^{2-} was seen. Since a decrease in GSH in the hepatopancreas was accompanied by an increase in blood GSH, the observed trends after 400 min of H_2S exposure might simply reflect tissue damage and onset of death. Although no mortality was observed during the exposure period it was noted that 5 specimens left in the set-up were all dead after 780 min.

In order for H_2S entering *Saduria entomon* via the gills to be detoxified, it must be transported to the hepatopancreas. Since H_2S enters directly into the blood, which is in circulatory connection with the hepatopancreas (Gibson & Barker 1979), the blood seems to mediate this transport. In *Bythograea thermydron*, Childress et al. (1987) found H_2S to be transported in a free form in the blood (i.e. no interaction with hemocyanin or special transport proteins). The blood of *B. thermydron* does not exhibit sulfide oxidation activity (Vetter et al. 1987), as is also the case for *S. entomon*. The lack of sulfide oxidation activity in crustacean blood supports the hypothesis that H_2S is transported in a free form. In other words, hemocyanin seems different from, e.g. hemoglobin, methemoglobin, or hemein, which have all been shown to react with H_2S (Baxter & VanReen 1958b, Sörbo 1958, Patel & Spencer 1963, Smith & Gosselin 1966, Powell & Arp 1989). The O_2 -transport by hemocyanin can therefore proceed in the presence of H_2S . This is further supported by the O_2 -dependent production of SO_3^{2-} and $S_2O_3^{2-}$ in the hepatopancreas of *S. entomon*.

The final step in the transport of H₂S by blood is diffusion of H₂S from the blood into the hepatopancreas. A passive net transport out of the animal into the fluid component of the hepatopancreas only occurs when the H₂S concentration is higher in the blood. However, the in vivo experiments all showed H₂S concentration to be higher in the hepatopancreas of *Saduria entomon*. Although not recognized, this enigma can also be seen in other studies (e.g. Childress et al. 1984, Bagarinao & Vetter 1989). However, the acid labile bound H₂S in the hepatopancreas of *S. entomon* might be an explanation. Since *S. entomon* cannot exclude H₂S, the fact that H₂S concentration in blood was lower than that in water shows that H₂S is transported via blood to hepatopancreas. In *Riftia pachyptila*, Childress et al. (1984) ascribed the apparent concentration of H₂S in the trophosome to be an overestimate, due to the presence of acid labile H₂S characteristic of bacterial ferredoxins.

In conclusion, the present study adds new evidence for the hypothesis that physiological adaptations to H₂S are not unique to animals from the hydrothermal vents. Adaptations to H₂S allow numerous species to extend their ecological niche; this makes H₂S an important factor in the distribution of marine invertebrates in soft sediments. In *Saduria entomon*, adaptations to low p_wO₂ and H₂S can explain the species' present distribution in the Baltic Sea.

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