The Journal of Experimental Biology 211, 3816-3825
Published by The Company of Biologists 2008
doi:10.1242/jeb.021303

Hypotaurine and sulfhydryl-containing antioxidants reduce H_2S toxicity in erythrocytes from a marine invertebrate

J. A. Ortega, J. M. Ortega and D. Julian*
University of Florida, P.O. Box 118525, Department of Zoology, Gainesville, FL 32611, USA
*Author for correspondence (e-mail: djulian@ufl.edu)

Accepted 16 October 2008

SUMMARY

Hypotaurine (HT) has been proposed to reduce sulfide toxicity in some deep-sea invertebrates by scavenging free radicals produced from sulfide oxidation or by scavenging sulfide *via* the reaction of HT with sulfide, forming thiotaurine (ThT). We tested whether HT or several antioxidants could reduce the total dissolved sulfide concentration in buffered seawater exposed to H_2S, and whether HT, ThT or antioxidants could increase the viability of *Glycera dibranchiata* erythrocytes exposed to H_2S *in vitro*. We found that 5 and 50 mmol l^{-1} HT reduced the dissolved sulfide in cell-free buffer exposed to H_2S by up to 80% whereas the antioxidants glutathione ethyl ester (GEE), N-acetylcysteine (NAC), L-ascorbic acid (ASC), Tempol and Trolox had no consistent effect. Exposure of erythrocytes to 0.10%–3.2% H_2S (producing 0.18–4.8 mmol l^{-1} sulfide) decreased the fraction of viable cells, as evidenced by loss of plasma membrane integrity, with virtually no cells remaining viable at 1.0% or more H_2S. Addition of HT (0.5–50 mmol l^{-1}) significantly increased the fraction of viable cells (e.g. from 0.01 to 0.84 at 0.32% H_2S) whereas ThT (0.5 and 5 mmol l^{-1}) decreased cell viability. GEE (0.03–3 mmol l^{-1}) and NAC (0.001–1 mmol l^{-1}), which contain sulfhydryl groups, increased cell viability during H_2S exposure but to a lesser extent than HT whereas ASC, Tempol and Trolox, which do not contain sulfhydryl groups, decreased viability or had no effect. These data show that HT can protect cells from sulfide *in vitro* and suggest that sulfide scavenging, rather than free radical scavenging, is the most important mechanism of protection.

Key words: antioxidants, hydrogen sulfide, hypotaurine, invertebrate, oxidative stress.

INTRODUCTION

A variety of marine animals live in habitats such as deep-sea hydrothermal vents, hydrocarbon seeps, mudflats and estuaries where they are exposed to the toxin sulfide (representing the sum of H_2S, HS^- and S^{2-}) either continuously or periodically (Gamenick et al., 1998). In addition to inhibiting cytochrome c oxidase (Dorman et al., 2002; Khan et al., 1990; Nicholls and Kim, 1982) and a variety of other enzymes (Bagarinao, 1992), sulfide may increase oxidative damage *via* the generation of free radicals. Chen and Morris first recognized that the oxidation of sulfide would result in a chain of redox reactions, producing a variety of free radical intermediates, including reactive oxygen species and sulfur-centered free radicals (Chen and Morris, 1972). This was later confirmed experimentally in seawater (Tapley et al., 1999) and in animal tissues *in vitro* (Tapley, 1993; Truong et al., 2006). However, sulfide may also have antioxidant properties: H_2S can directly scavenge reactive oxygen and nitrogen species (Whiteman et al., 2004; Whiteman et al., 2005), and reduce lipid peroxidation in a mouse model of hepatic ischemia-reperfusion injury (Jha et al., 2008). Nonetheless, oxidative stress is increased in at least some marine organisms during sulfide exposure (Abele-Oeschger and Oeschger, 1995; Joyner-Matos et al., 2007; Julian et al., 2005; Tapley, 1993), as well as in mammalian cells exposed to sulfide *in vitro* (Attene-Ramos et al., 2007; Eghbal et al., 2004). Furthermore, environmental conditions other than sulfide, in particular high concentrations of dissolved metals, may contribute to oxidative stress at hydrothermal vents (Bebianno et al., 2005). Animals in sulfidic habitats appear to minimize the toxic effects of sulfide primarily by reducing the free sulfide concentration, especially by enzymatic oxidation of sulfide to thiosulfate and by covalent binding of sulfide with sulfhydryl groups of 'sulfide-binding proteins' (Arp and Childress, 1983; Arp et al., 1984; Arp et al., 1987; Grieshaber and Völkel, 1998; O'Brien and Vetter, 1990).

Hypotaurine (HT), the reduced metabolic precursor of taurine, is an organic osmolyte that is present in high concentrations (as much as 150 mmol kg^{-1} tissue wet mass) within the hemolymph and cells of many marine invertebrates at deep-sea hydrothermal vents and hydrocarbon seeps (Alberic, 1986; Horak et al., 2003; Pruski et al., 2000a; Rosenberg et al., 2006; Yancey, 2005; Yancey et al., 2002b; Yin et al., 2000). HT is an effective hydroxyl radical scavenger in mammalian cells *in vitro* (Aruoma et al., 1988), and HT and its precursors, including cysteamine, cysteinesulphinic acid and cysteic acid, have been proposed to act as antioxidants in mammalian brain, male reproductive tissue and liver cells (Aruoma et al., 1988; Donnelly et al., 2000; Fellman and Roth, 1985; Huxtable, 1992; Schurr and Rigor, 1987). Therefore, HT may reduce sulfide toxicity by scavenging free radicals produced from sulfide oxidation. However, HT can also react with sulfide directly, forming thiotaurine (ThT) *in vitro* (Cavallini et al., 1963). If this reaction occurs *in vivo*, it would scavenge free sulfide and might, therefore, reduce sulfide toxicity by acting as a sulfide 'buffer' (Alberic and Boulegue, 1990; Brand et al., 2007; Joyner et al., 2003; Pruski and Fiala-Medioni, 2003; Rosenberg et al., 2006). In sulfide-exposed animals that lack sulfide-oxidizing endosymbionts, the resulting ThT may be enzymatically recycled back to HT, thereby releasing sulfide at a rate that allows detoxification through other mechanisms (Rosenberg et al., 2006). In sulfide-exposed animals with sulfide-oxidizing endosymbiotic bacteria, the conversion of HT to ThT may provide a mechanism to transport sulfide to the endosymbionts, which would then convert ThT back to sulfide and HT (Pranal et al., 1995; Pruski and Fiala-Medioni, 2003; Pruski et al., 1997; Pruski

et al., 2001; Pruski et al., 2000b). Accordingly, Pranal and colleagues proposed that the ratio of ThT to HT in tissues represents the extent of that animal's recent sulfide exposure (Pranal et al., 1995). This was confirmed by Brand and colleagues and Rosenberg and colleagues for vent species with and without sulfide-oxidizing endosymbionts, respectively (Brand et al., 2007; Rosenberg et al., 2006). Therefore, HT may have two beneficial actions in sulfide-exposed animals: firstly, scavenging free radicals generated by the oxidation of sulfide and secondly, scavenging sulfide by reacting with it to form ThT (Joyner et al., 2003; Pruski et al., 2000b; Yancey et al., 2002b). However, whether HT actually reduces sulfide toxicity and, if so, whether this is primarily achieved by scavenging free radicals or by scavenging sulfide have never been directly tested.

In the present study, we investigated whether HT, ThT, antioxidants containing sulfhydryl groups and antioxidants without sulfhydryl groups can reduce the cytotoxicity of sulfide exposure *in vitro*. Sulfide cytotoxicity was assessed by exposing erythrocytes from the bloodworm *Glycera dibranchiata* Ehlers 1868 (Annelida: Polychaeta: Phyllodocida: Glyceridae) to sulfide for 2 h, followed by the application of vital fluorescent dyes to determine cell viability as indicated by plasma membrane integrity. *G. dibranchiata* inhabits mudflats along the North Atlantic coast of the United States, where it is probably exposed to sulfide. It lacks a vascular system and its coelomic, hemoglobin-containing erythrocytes are circulated to the parapodia for gas exchange (Mangum, 1994) and, therefore, are at risk for sulfide exposure. Body wall and coelomocyte extracts from *G. dibranchiata* have been reported to contain HT but the concentrations were not determined (Costa et al., 1980). However, Yancey indicated that *Glycera* sp. are among the invertebrates for which HT is not a major organic osmolyte in muscle or whole-body tissue (Yancey, 2005; Yancey et al., 2002b). This suggests that if *G. dibranchiata* tissues contain endogenous HT, the concentrations are probably comparatively low. Consequently, an effect of exogenous HT on *G. dibranchiata* cell viability would presumably be independent of any physiological adaptations that might otherwise utilize endogenous HT.

MATERIALS AND METHODS

HT, ThT and antioxidants

The following seven compounds and antioxidants were tested for their ability to reduce the dissolved sulfide concentration and increase cell viability during H_2S exposure: (1) HT (0.5, 5.0, 50 mmol l^{-1}); (2) ThT (0.5, 5.0 mmol l^{-1}); (3) glutathione ethyl ester (GEE, 0.03, 0.3, 3 mmol l^{-1}), a cell-permeant analogue of glutathione that is commonly used at 0.01–2 mmol l^{-1} *in vitro* (e.g. Doolen et al., 2007; Pocernich et al., 2001); (4) N-acetylcysteine (NAC, 0.03, 0.3, 1.0 mmol l^{-1}), a cell-permeant N-acetyl derivative of L-cysteine that is a glutathione precursor and is commonly used at 0.5–20 mmol l^{-1} *in vitro* (e.g. Konarkowska et al., 2005; Yan et al., 2006); (5) L-ascorbic acid (ASC, 0.001, 0.01, 0.1 mmol l^{-1}), i.e. vitamin C, which is cell-impermeant and is commonly used at 0.001–0.1 mmol l^{-1} *in vitro* (e.g. Yedjou et al., 2008); (6) 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 0.03, 0.3, 3.0 mmol l^{-1}), a cell-permeant nitroxide antioxidant that scavenges superoxide radicals and is commonly used at 0.003–10 mmol l^{-1} *in vitro* (e.g. Samuni et al., 2004; Singh et al., 2007); and (7) 6-hydoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®, 0.001, 0.01, 0.1 mmol l^{-1} from a 100 mmol l^{-1} stock solution in ethanol), a water-soluble, cell-permeant analog of α-tocopherol that is commonly used at 0.002–100 mmol l^{-1} *in vitro* with mammalian cells (e.g. Bhattacharya and Lakshmana Rao, 2001; Casado et al., 2007) and mussel hemocytes (Machella et al., 2006), although its aqueous solubility is limited above 1.8 mmol l^{-1} (Wu et al., 1990). All compounds except ThT were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were the highest quality available. ThT was prepared by reacting HT with sulfide as described previously (Cavallini et al., 1963; Joyner et al., 2003).

Erythrocytes

G. dibranchiata were purchased from Harbor Bait Company, Wiscasset, ME, USA, which collected the worms from mudflats during low tide. The worms were maintained in the laboratory unfed in filtered, 15°C chilled seawater for no longer than two weeks before being used. Worms were removed from the aquarium immediately prior to experiments, and the coelomic fluid was drained from an incision in the proboscis and then centrifuged for 18 s at 12,000×***g*** in order to remove the supernatant and overlying white cell layer. The pelleted erythrocytes were further purified by centrifugation for 10 min at 170×***g*** and 10°C into a 25% sucrose–seawater cushion. The erythrocytes were then washed with cold incubation buffer (sterile-filtered seawater with 10 mmol l^{-1} Hepes and 0.1% glucose, pH 7.6, 1000 mosmol kg^{-1}) to remove sucrose residue, and the pelleted erythrocytes were then diluted 140-fold in cold incubation buffer.

H_2S exposure

Sulfide exposures were conducted by exposing a thin layer of incubation buffer, either with or without erythrocytes, to a mixture of H_2S gas in air. This was performed in five gas-tight chambers, each containing a black-wall, clear-bottom 96-well microplate (Corning Life Sciences, Lowell, MA, USA), in which wells contained 50 μl of incubation buffer. An appropriate volume of H_2S gas (from a compressed tank of 99% pure H_2S gas) was then added by syringe to the gas space of each chamber to obtain 0 (control), 0.10, 0.32, 1.0 and 3.2% H_2S in air. After exposure, the sulfide concentration in each well was determined with a Methylene Blue assay (Gilboa-Garber, 1971) modified for 50 μl sample volumes.

To determine the effect of HT and the antioxidants on the dissolved sulfide concentration, 50 μl of incubation buffer containing HT (0.5, 5.0, 50 mmol l^{-1}) or the highest concentrations of GEE, NAC, ASC, Tempol and Trolox were exposed to H_2S for 2 h at 22–24°C, after which the dissolved sulfide concentration was determined, all as described above (note that ThT was not tested in this experiment due to the expense of synthesizing it, given the low probability that it would have any effect on dissolved sulfide).

To determine the effect of sulfide on cell viability, 50 μl of erythrocytes (diluted 1:1400 in incubation buffer) was added to microplate wells and exposed to H_2S gas, as described above, for 2 h at 15°C. This experiment was independently replicated with erythrocytes from three worms, with erythrocyte samples from each worm being exposed to control conditions and each level of H_2S (i.e. a fully balanced design). At each H_2S concentration for each replicate, an additional well containing 50 μl of incubation buffer without erythrocytes was used for the determination of the dissolved sulfide concentration, as described above.

To determine whether the addition of HT, ThT or any of the antioxidants affected cell viability during sulfide exposure, erythrocytes (diluted 1:1400 in incubation buffer) were pre-incubated with HT, ThT or an antioxidant for 1 h at 15°C in the microplates, after which H_2S gas was added, as described above, and the erythrocytes were incubated for an additional 2 h. Following the incubation, the cells were labeled with vital fluorescent dyes and assessed for viability, as described below. This experiment was independently replicated with erythrocytes from five worms, with

erythrocyte samples from each worm being exposed to all concentrations of each compound under control conditions and at each level of H_2S (i.e. a fully balanced design).

Cell viability

To assess erythrocyte viability, a vital fluorescent dye mix was created in incubation buffer by adding calcein [loaded as calcein green acetoxy-methylester, a cell-permeant cytoplasmic stain that labels cells with intact plasma membranes; 10 mol l^{-1} from a 2 mmol l^{-1} stock solution in dimethyl sulfoxide (DMSO)], propidium iodide (PI, a cell-impermeant nucleic acid stain that labels cells with compromised plasma membrane integrity; 5 μg ml^{-1} from a 1 mg ml^{-1} stock solution in H_2O) and Hoechst 33342 (a cell-permeant nuclear stain that is used to identify cells; 2 μg ml^{-1} from a 1 mg ml^{-1} stock solution in H_2O). This dye mix was prepared daily from stock solutions stored at –20°C before use. Immediately after the erythrocytes were exposed to sulfide, as described above, 5 μl dye mix was added to each erythrocyte-containing well in each microplate and the dyes were allowed to load into the cells for 25 min in the dark at room temperature. After dye loading, 100 μl incubation buffer was added to each well, which diluted any unloaded dyes to reduce background fluorescence. All dyes were from Invitrogen Corporation (Carlsbad, CA, USA).

For fluorescence detection of the fluorescent dyes, each microplate was placed on an epifluorescence microscope (Olympus IX-70, Olympus America Inc., Center Valley, PA, USA) with a DAPI/FITC/TRITC triple bandpass polychroic emission filter set (Chroma Technology, Rockingham, VT, USA). The dyes were excited with light from a 100 W mercury bulb through appropriate excitation filters (484/15 nm for calcein, 555/15 nm for PI and 360/40 nm for Hoechst), and digital monochrome images were recorded with a cooled CCD camera (2000R, QImaging, Burnaby, BC, Canada). The images were later analyzed using ImageJ software (v. 1.37c; http://rsb.info.nih.gov/ij/). Cells were placed into three categories based on calcein and PI labeling: (1) cells that were labeled by calcein but not PI were considered viable; (2) cells that were labeled by PI with nuclear-specific staining were considered non-viable; and (3) cells that were labeled by PI with cytoplasmic staining were considered transitional and, although probably lethally injured, were not counted in the statistical analyses. The latter category was never present in erythrocytes from control conditions. Hoechst labeled both viable and non-viable erythrocytes and was used as a nuclear marker.

Statistical analyses

All data are presented as means ±1 standard error of the mean (s.e.m.), unless noted otherwise. The effects of HT and the antioxidants on dissolved sulfide concentration were analyzed by two-factor analysis of variance (ANOVA) followed by the Tukey HSD test (JMP 7.2, SAS Institute, Cary, NC USA). In the graphs, data for PI labeling represent both cytoplasmic and nuclear staining but statistical analysis of PI labeling was restricted to data for nuclear staining to be maximally conservative (see Discussion). The effects of H_2S on the fractions of cells labeled by calcein and PI were each analyzed as a split plot design, with antioxidant (including HT and ThT) as the whole plot factor, H_2S as the split plot factor and the interaction between animal and antioxidant as the error term for the whole plot factor. This design reduces the residual variation resulting from between-worm differences in cell viability. Because the fractions were not normally distributed (i.e. the majority of cells in any well were typically either viable or non-viable), the data were analyzed with a generalized linear mixed model (GLIMMIX, SAS software, SAS Institute). However, this analysis could not be performed for treatments that had zero variance (i.e. when all cells in a treatment were calcein-positive, calcein-negative, PI-positive or PI-negative) and, therefore, tests of statistical significance were limited to 0.10% H_2S for calcein and 0.32% H_2S for PI, which were selected because they are closest to the LD_{50} for each dye (see Results).

RESULTS

Dissolved sulfide

The total dissolved sulfide concentration increased approximately proportionally to the H_2S gas fraction, as would be expected, with 0.10, 0.32, 1.0 and 3.2% H_2S producing 0.18, 0.32, 1.9 and 4.8 mmol l^{-1} of sulfide, respectively (Fig. 1A, blue symbols). The addition of HT significantly reduced the dissolved sulfide concentration (whole model $F_{19,126}$=110, P<0.0001 for sulfide; P=1.0 for HT; P<0.0001 for sulfide×HT), with the addition of 50 mmol l^{-1} of HT decreasing the sulfide concentration by 80% at 1.0% H_2S and 86% at 3.2% H_2S (Fig. 1A, red symbols). By contrast, adding the highest concentration of the antioxidants did not significantly affect the dissolved sulfide concentration (Fig. 1B–F), with the exception of 3.0 mmol l^{-1} Tempol at 1.0% H_2S, which reduced the sulfide concentration by 50%. The general effect of HT was concentration-dependent and showed a similar trend at each H_2S percentage (Fig. 1G), consistent with simple kinetics and the absence of saturation.

Sulfide toxicity

G. dibranchiata erythrocytes exposed to control conditions (air) showed cytoplasmic calcein labeling but no PI labeling (Fig. 2A), consistent with viable cells that have an intact plasma membrane. Exposure to 0.10%–3.2% H_2S gas (in air) caused a dose-dependent decrease in labeling by calcein and increase in nuclear labeling by PI (Fig. 2B–E). This is consistent with a progressive loss of plasma membrane integrity leading to a net outward diffusion of calcein and an inward diffusion of PI (and its subsequent labeling of nucleic acids). Quantification of the number of cells labeled by each dye showed that H_2S exposure produced a significant decrease in the fraction of cells labeled with calcein green (Fig. 2F) (green circles; $F_{4,8}$=21.1, P=0.0003, N=3) with the calcein-positive fraction decreasing from 0.92±0.03 in the controls to 0.01±0.01 at 3.2% H_2S (P=0.0005). Similarly, H_2S exposure produced a significant increase in the fraction of cells labeled by PI (Fig. 2F, red circles) ($F_{4,8}$=83.5, P<0.0001, N=3) with the total PI-positive fraction increasing from 0.04±0.03 in the controls to 0.99±0.01 at 3.2% H_2S (P<0.0001), and with the subset of nuclear PI-positive cells increasing from 0.04±0.03 in the controls to 0.55±0.20 at 3.2% H_2S (Fig. 2F, red triangles) ($F_{4,8}$=9.93, P<0.003, N=3). At the highest sulfide concentration, PI labeling was almost exclusively nuclear whereas cytoplasmic PI labeling (whether alone or with nuclear labeling) was more common at intermediate sulfide concentrations (data not shown). Based on the calcein, nuclear PI, and total PI labeling, the LD_{50} for H_2S appeared closest to 0.32%.

HT and ThT

Addition of HT and ThT to the incubation buffer did not affect cell viability under control conditions (i.e. the absence of H_2S), indicating that neither of these compounds was toxic on its own. Addition of HT increased the viability of cells exposed to H_2S, as evident from a general increase in calcein labeling and decrease in PI labeling (Fig. 3, HT panels A–D). As the concentration of HT increased, the fraction of cells labeled with calcein significantly increased (Fig. 3,

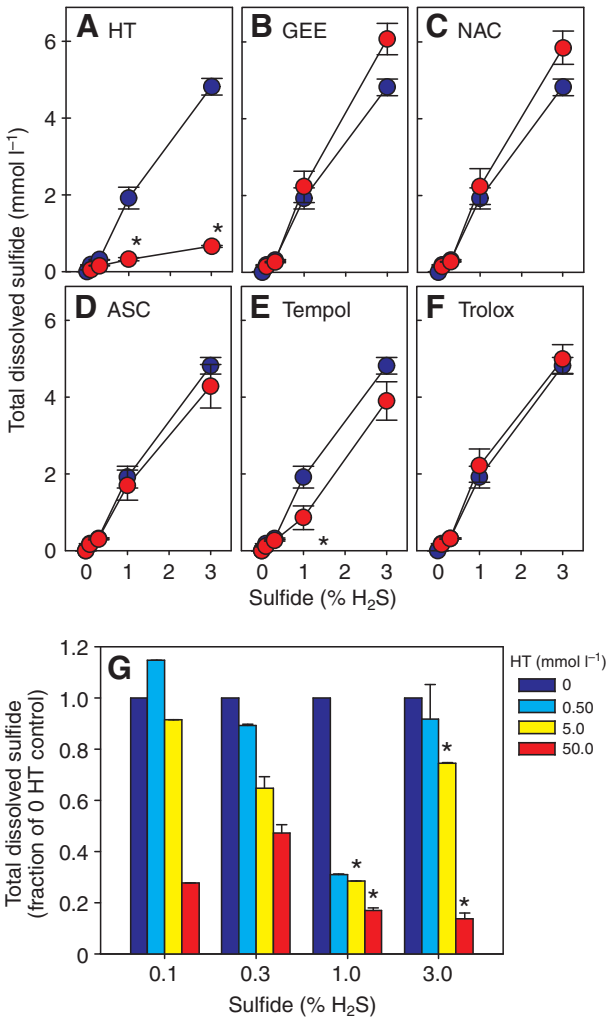

Fig. 1. Comparison of total dissolved sulfide concentration following 2 h exposure to 0–3.2% H_2S in cell-free incubation buffer alone (A–F, blue symbols, N=15, with the same data set shown in each plot) or in cell-free incubation buffer with added hypotaurine (HT) or antioxidants (A–F, red symbols). (A) 50 mmol l^{-1} HT (hypotaurine), (N=3–9). (B) 3 mmol l^{-1} GEE (glutathione ethyl ester) (N=6). (C) 1.0 mmol l^{-1} NAC (N-acetylcysteine) (N=6). (D) 0.10 mmol l^{-1} ASC (L-ascorbic acid) (N=6). (E) 3.0 mmol l^{-1} Tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl) (N=6). (F) 0.10 mmol l^{-1} Trolox (6-hydoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (N=6). Data are means ± s.e.m. (G) Comparison of total dissolved sulfide concentration following 2 h exposure to 0–3.2% H_2S in cell-free incubation buffer alone (0 HT) and with 0.50, 5.0 and 50 mmol l^{-1} HT. For each H_2S percentage, the sulfide concentration is expressed relative to the concentration in the absence of HT. Data are means ± 1 standard deviation. For all plots, asterisks represent significant differences from the dissolved sulfide concentration in incubation buffer alone at the same H_2S percentage.

HT panel E) ($F_{3,11}$=38.4, P<0.0001, N=4) whereas the fraction of cells showing nuclear PI labeling significantly decreased (Fig. 3, HT panel F) ($F_{3,11}$=3.87, P<0.041, N=4). For example, adding 50 mmol l^{-1} of HT (the highest concentration tested) increased the fraction of viable cells from 0.01 to 0.84 at 0.32% H_2S, from 0.00 to 0.79 at 1.0% H_2S, and from 0.02 to 0.21 at 3.2% H_2S. To determine the statistical significance of adding HT, we performed a GLIMMIX analysis at 0.10% H_2S for calcein labeling and 0.32% H_2S for nuclear PI labeling (Table 1). These two H_2S concentrations were selected because higher and lower concentrations tended to produce zero variance (i.e. all cells were positive or negative for one of the dyes at a particular H_2S concentration in at least one replicate) and, therefore, did not allow GLIMMIX analysis. At 0.10% H_2S, calcein labeling was significantly increased at all concentrations of HT (0.5, 5.0 and 50 mmol l^{-1}) (Table 1) and at 0.32% H_2S, nuclear PI labeling was significantly decreased at 5.0 and 50 mmol l^{-1} HT but not 0.50 mmol l^{-1} HT (Table 1).

In contrast to the effect of HT, adding increasing concentrations of ThT generally decreased labeling by calcein and increased labeling by PI in cells exposed to H_2S (Fig. 3, ThT panels A–D). Adding ThT at 0.50 and 5.0 mmol l^{-1} significantly decreased the viability of cells exposed to H_2S, based on both calcein labeling (Fig. 3, ThT panel E; Table 1) ($F_{2,5}$=49.9, P<0.0005, N=3) and PI labeling (Fig. 3, ThT panel F; Table 1) ($F_{2,5}$=16.0, P<0.006, N=3). For example, the addition of 5 mmol l^{-1} of ThT at 0.10% H_2S decreased calcein labeling from 0.95 to 0.40 whereas the addition of 5 mmol l^{-1} of ThT at 0.32% H_2S increased nuclear PI labeling from 0.12 to 0.39.

Antioxidants

None of the antioxidants affected cell viability under control conditions, indicating that these were not toxic on their own. Generally, adding GEE increased labeling by calcein and decreased labeling by PI in cells exposed to H_2S (Fig. 4 GEE, panels A–D). Adding GEE at 0.030 and 0.30 mmol l^{-1} but not 3.0 mmol l^{-1}, significantly increased the viability of cells exposed to H_2S, based on both calcein labeling (Fig. 4, GEE panel E; Table 1) ($F_{3,15}$=10.6, P<0.0005, N=4) and PI labeling (Fig. 4, GEE panel F; Table 1) ($F_{3,15}$=6.88, P<0.004, N=4). Similarly, the addition of increasingly higher concentrations of NAC increased labeling by calcein and decreased labeling by PI in cells exposed to H_2S (Fig. 4, NAC panels A–D). All concentrations of NAC (0.030, 0.30, 1.0 mmol l^{-1}) significantly increased cell viability, based on both calcein labeling (Fig. 4, NAC panel E; Table 1) ($F_{3,13}$=17.4, P<0.0001, N=4) and PI labeling (Fig. 4, NAC panel F; Table 1) ($F_{3,13}$=43.6, P<0.0001, N=4).

The addition of higher concentrations of ASC, Tempol or Trolox either had no effect or decreased the viability of *G. dibranchiata* erythrocytes exposed to H_2S. Adding increasingly higher concentrations of ASC generally decreased labeling by calcein and increased labeling by PI in cells exposed to H_2S (Fig. 4, ASC panels A–D). Adding ASC at 0.010 and 0.10 mmol l^{-1} significantly decreased the viability of cells exposed to H_2S, based on both calcein labeling (Fig. 4, ASC panel E; Table 1) ($F_{3,10}$=114, P<0.0001, N=4) and PI labeling (Fig. 4, ASC panel F; Table 1) ($F_{3,10}$=49.1, P<0.0001, N=4). Similarly, the addition of higher concentrations of Tempol generally decreased labeling by calcein and increased labeling by PI in cells exposed to H_2S (Fig. 4, Tempol panels A–D). Adding Tempol at 0.30 and 3.0 mmol l^{-1} significantly decreased the viability of cells exposed to H_2S, based on both calcein labeling (Fig. 4, Tempol panel E; Table 1) ($F_{3,15}$=112, P<0.0001, N=4) and PI labeling (Fig. 4, Tempol panel F; Table 1) $F_{3,15}$=15.1, P<0.0001, N=4). Addition of Trolox did not substantially change calcein labeling or PI labeling in cells exposed to H_2S (Fig. 4, Trolox panels A–D) and, therefore, did not affect cell viability (Fig. 4, Trolox panel E–F; Table 1).

DISCUSSION

In previous studies, *in vitro* exposure of *G. dibranchiata* erythrocytes to sulfide for 1 h caused dose-dependent mitochondrial depolarization with an EC_{50} of approximately 0.5–0.8 mmol l^{-1}, increased cellular oxidative stress and increased superoxide

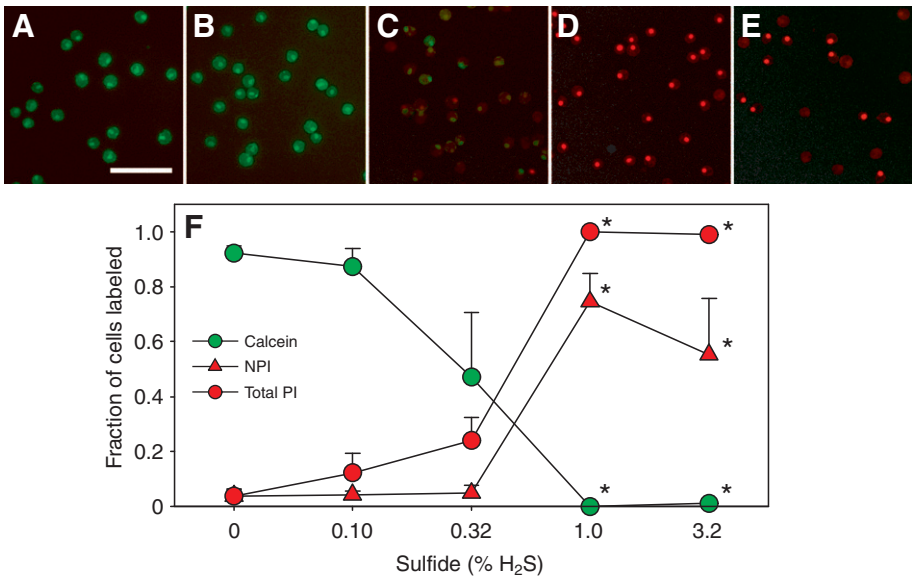

Fig. 2. Viability of *G. dibranchiata* erythrocytes exposed to H_2S *in vitro*. (A–E) Epifluorescence micrographs of cells exposed to control conditions (A) or H_2S at 0.10% (B), 0.32% (C), 1.0% (D) and 3.2% (E) for 2 h, followed by labeling with calcein (green) and propidium iodide (PI; red). Scale bar=100 μm. (F) Quantitative analysis of calcein and PI labeling following H_2S exposure. 'Calcein' (green circles) represents cells with cytoplasmic calcein labeling; 'NPI' (red triangles) represents cells with nuclear PI labeling; 'Total PI' (red circles) represents cells with nuclear or cytoplasmic PI labeling. Asterisks indicate a significant difference from control conditions by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test, *N*=3.

production (Julian et al., 2005); *in vivo* exposure of *G. dibranchiata* worms to sulfide for 24 h caused a dose-dependent increase in PI labeling of erythrocytes and a decrease in cell proliferation at sulfide concentrations above 0.5 mmol l^{-1} (Hance et al., 2008); and sulfide exposure caused oxidative damage to RNA and DNA in *G. dibranchiata* erythrocytes exposed to sulfide *in vitro* and in erythrocytes and body wall of *G. dibranchiata* exposed to sulfide *in vivo* (Joyner-Matos et al., 2008). However, following *in vivo* exposure, *G. dibranchiata* showed no change in gross morphology or behavior at sulfide concentrations up to 1 mmol l^{-1}, and short-term survival of the animals was not affected at sulfide concentrations as high as 2.4 mmol l^{-1} (Hance et al., 2008). Consequently, although *G. dibranchiata* can clearly be classified as 'sulfide-tolerant' based on survival during sulfide exposure, the erythrocytes show evidence of cytotoxicity and oxidative damage both *in vivo* and *in vitro*. These findings are consistent with sulfide exposure causing increased free radical production and oxidative stress (Abele-Oeschger and Oeschger, 1995; Attene-Ramos et al., 2007; Chen and Morris, 1972; Eghbal et al., 2004; Joyner-Matos et al., 2006; Tapley, 1993; Tapley et al., 1999; Truong et al., 2006) and leads to the hypothesis that appropriate antioxidants could increase the viability of cells exposed to sulfide.

To assess erythrocyte viability in the present study, we determined whether the cells became labeled with the fluorescent dyes calcein

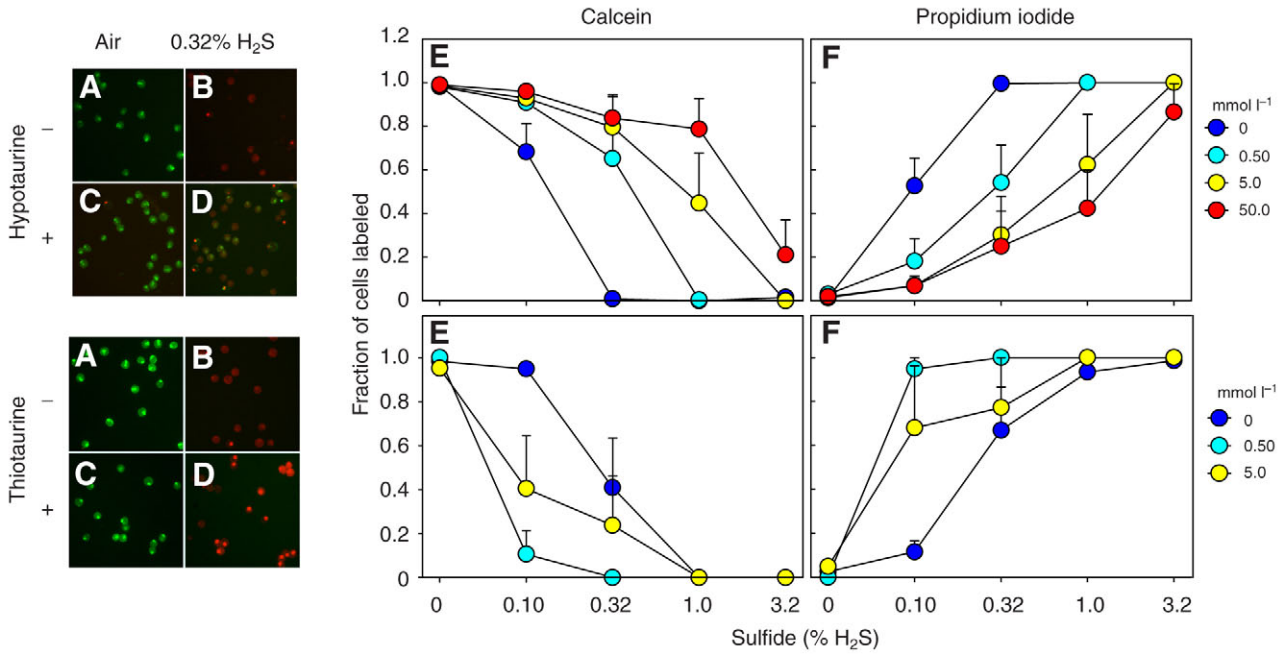

Fig. 3. Effects of hypotaurine and thiotaurine on the viability of *G. dibranchiata* erythrocytes exposed to H_2S *in vitro*. (A–D) Epifluorescence micrographs of cells in incubation buffer that were exposed for 2 h to air (A) and 0.32% H_2S (B), and cells in incubation buffer with hypotaurine (50 mmol l^{-1}) or thiotaurine (5.0 mmol l^{-1}) that were exposed for 2 h to air (C) or 0.32% H_2S (D), followed by labeling with calcein (green) and propidium iodide (PI; red). (E–F) Quantitative analysis of calcein labeling (E) and total PI labeling (F) in cells incubated with various concentrations of hypotaurine (0–50 mmol l^{-1}) or thiotaurine (0–5 mmol l^{-1}) and exposed for 2 h to 0–3.2% H_2S. *N*=5. Results of statistical analysis presented in Table 1.

Table 1. Statistical analysis of cell viability in *G. dibranchiata* erythrocytes exposed to 0.10% or 0.32% H_2S gas for 2 h in incubation buffer with one of the following compounds: hypotaurine (HT), thiotaurine (ThT), glutathione ethyl ester (GEE), N-acetylcysteine (NAC), Tempol or Trolox

	Relative difference (↓ or ↑) and statistical significance (*P*) of change in labeling (*vs* control)					
	Calcein (0.10% H_2S)			PI (0.32% H_2S)		
Compound (mmol l^{-1})	Low conc.	Medium conc.	High conc.	Low conc.	Medium conc.	High conc.
HT	↑	↑	↑	↓	↓	↓
(0.50, 5.0, 50.0)	***P*<0.0001**	***P*<0.0001**	***P*<0.0001**	*P*=0.12	***P*=0.019**	***P*=0.013**
ThT	↓	↓		↑	↑	
(0.50, 5.0)	***P*=0.0002**	***P*=0.0004**	–	***P*=0.0023**	***P*=0.011**	–
GEE	↑	↑	↑	↓	↓	↓
(0.03, 0.30, 3.0)	***P*=0.0037**	***P*=0.0001**	*P*=0.26	***P*=0.0096**	***P*=0.0015**	***P*=0.018**
NAC	↑	↑	↑	↓	↓	↓
(0.030, 0.30, 1.0)	***P*=0.0006**	***P*=0.0002**	***P*<0.0001**	***P*<0.0001**	***P*<0.0001**	***P*<0.0001**
ASC	↓	↓	↓	↑	↑	↑
(0.0010, 0.010, 0.10)	*P*=0.73	***P*<0.0001**	***P*<0.0001**	*P*=0.21	***P*=0.0039**	***P*<0.0001**
Tempol	↓	↓	↓	↑	↑	↑
(0.030, 0.30, 3.0)	*P*=0.80	***P*<0.0001**	***P*<0.0001**	*P*=0.27	***P*=0.0005**	***P*=0.030**
Trolox	↓	↑	↑	↑	↓	↑
(0.0010, 0.010, 0.10)	*P*=0.19	*P*=0.19	*P*=0.53	*P*=0.73	*P*=1.0	*P*=0.083

Each compound was tested at three concentrations (low, medium, high), with the exception of ThT, which had only two concentrations. After H_2S exposure, cells were incubated with calcein and PI, and the fraction of cells labeled with each dye was compared with the fraction labeled with that dye in the absence of H_2S, with ↑ and ↓ indicating increased and decreased labeling compared to the control, respectively. To be maximally conservative, analysis of PI was restricted to nuclear labeling. *P*-values indicate statistical significance based on GLIMMIX analysis, with $P<0.05$ indicated by bold letters.

and PI after H_2S exposure. Calcein (or other similar cell-permeant fluorophores) and PI (or other similar cell-impermeant fluorophores) have long been used together as 'live/dead stains' in mammalian cells *in vitro* (Dive et al., 1990) but these have rarely been used together in marine invertebrates. However, hemocyte viability has been assessed in bivalves and crustaceans using calcein alone (Cardenas et al., 2004; Marin et al., 2004) and PI alone (Hegaret et al., 2003; Lee et al., 2001; Xue et al., 2001), and in *G. dibranchiata* erythrocytes *in vivo* using PI alone (Hance et al., 2008). We found that under control conditions *in vitro*, 100% of *G. dibranchiata* erythrocytes were labeled by calcein but were not labeled by PI whereas at 3% H_2S (approximately 5 mmol l^{-1} dissolved sulfide), 99% of the cells were labeled by PI but were not labeled by calcein. Cytoplasmic PI labeling, whether alone or with nuclear labeling, was more common at intermediate sulfide concentrations whereas PI labeling of cells exposed to the highest H_2S concentration was almost exclusively nuclear. This is consistent with previous studies indicating that non-nuclear PI labeling represents an intermediate membrane permeability state (Dive et al., 1990). Nonetheless, to be maximally conservative, statistical analyses of PI labeling were performed with data for nuclear PI labeling, for which the association with loss of cell viability is least ambiguous. These results are consistent with H_2S exposure compromising plasma membrane integrity, whether directly or indirectly. A half-maximal effect of H_2S on cell viability (LD_{50}) was seen at 0.32% H_2S, which produces approximately 0.3 mmol l^{-1} dissolved sulfide. Interestingly, this is similar to the EC_{50} of 0.5–0.8 mmol l^{-1} sulfide for erythrocyte mitochondrial depolarization *in vitro* (Julian et al., 2005) and the erythrocyte cell death threshold of 0.5 mmol l^{-1} sulfide *in vivo* (Hance et al., 2008). This suggests a common mechanism of toxicity at moderate sulfide concentrations, which has yet to be determined. Between-worm variability was evident in the effect of sulfide on cell viability. For the statistical analyses, this residual variation was reduced by the split plot design, in which samples of cells from each animal were exposed to every H_2S concentration (and at every concentration of HT, ThT and the antioxidants). The source of this inter-individual variation is unknown but such differences in the response of cells to sulfide *in vitro* could produce variation in whole-animal sulfide tolerance, which would be likely to affect features of populations such as the distribution within a sulfidic habitat.

HT has been hypothesized to protect deep-sea animals from sulfide toxicity by scavenging free radicals generated from sulfide oxidation and/or by scavenging sulfide (Alberic, 1986; Alberic and Boulegue, 1990; Horak et al., 2003; Pruski et al., 1997; Pruski et al., 2000b; Rosenberg et al., 2006; Yancey, 2005; Yancey et al., 2002b; Yin et al., 2000) but whether HT reduces sulfide toxicity and, if so, by what mechanism, had never been tested. We found that incubation buffer containing HT had up to 86% less dissolved sulfide after 2 h exposure to H_2S than incubation buffer without HT, and that HT increased the viability of *G. dibranchiata* erythrocytes exposed to H_2S for 2 h. For example at 1% H_2S in the absence of HT, no cells were calcein-positive, whereas 83% of cells were calcein-positive at 1% H_2S with 50 mmol l^{-1} HT. The effect on PI-labeling was similar but less dramatic. Overall, 50 mmol l^{-1} HT shifted the LD_{50} from 0.32% H_2S to 1–3% H_2S, with lower HT concentrations having a smaller but still significant effect. Considered alone, the cell viability data do not distinguish whether the mechanism of protection is *via* scavenging free radicals, scavenging sulfide or both. However, the positive effect of HT on cell viability was roughly correlated with its negative effect on dissolved sulfide concentration. This suggests, but does not prove, that sulfide scavenging is the most important factor in the ability of HT to reduce sulfide toxicity.

Because the ability of HT to reduce the dissolved sulfide concentration was observed in cell-free incubation buffer, it was independent of enzymatic activity. It would be valuable to test whether this effect is stronger in tissues and, in particular, whether tissues of sulfide-adapted animals that accumulate HT have specific enzymatic pathways that promote sulfide scavenging by HT. Because we did not measure HT or ThT concentrations, we also do not know the kinetics, equilibrium constant or other characteristics of the reaction between sulfide and HT but, clearly, these would be

valuable to obtain, especially in tissues. Interestingly, although HT is assumed to be cell-permeant in its zwitterionic form (Alvarez and Storey, 1983), it is typically concentrated primarily within cells, with lower concentrations in hemolymph (Fiess et al., 2002; Pruski et al., 2000a; Yin et al., 2000), which indicates that it is not distributed strictly by diffusion. It is unknown how HT was ultimately distributed across the erythrocyte plasma membrane in our experiments but it is reasonable to assume that HT reduced the

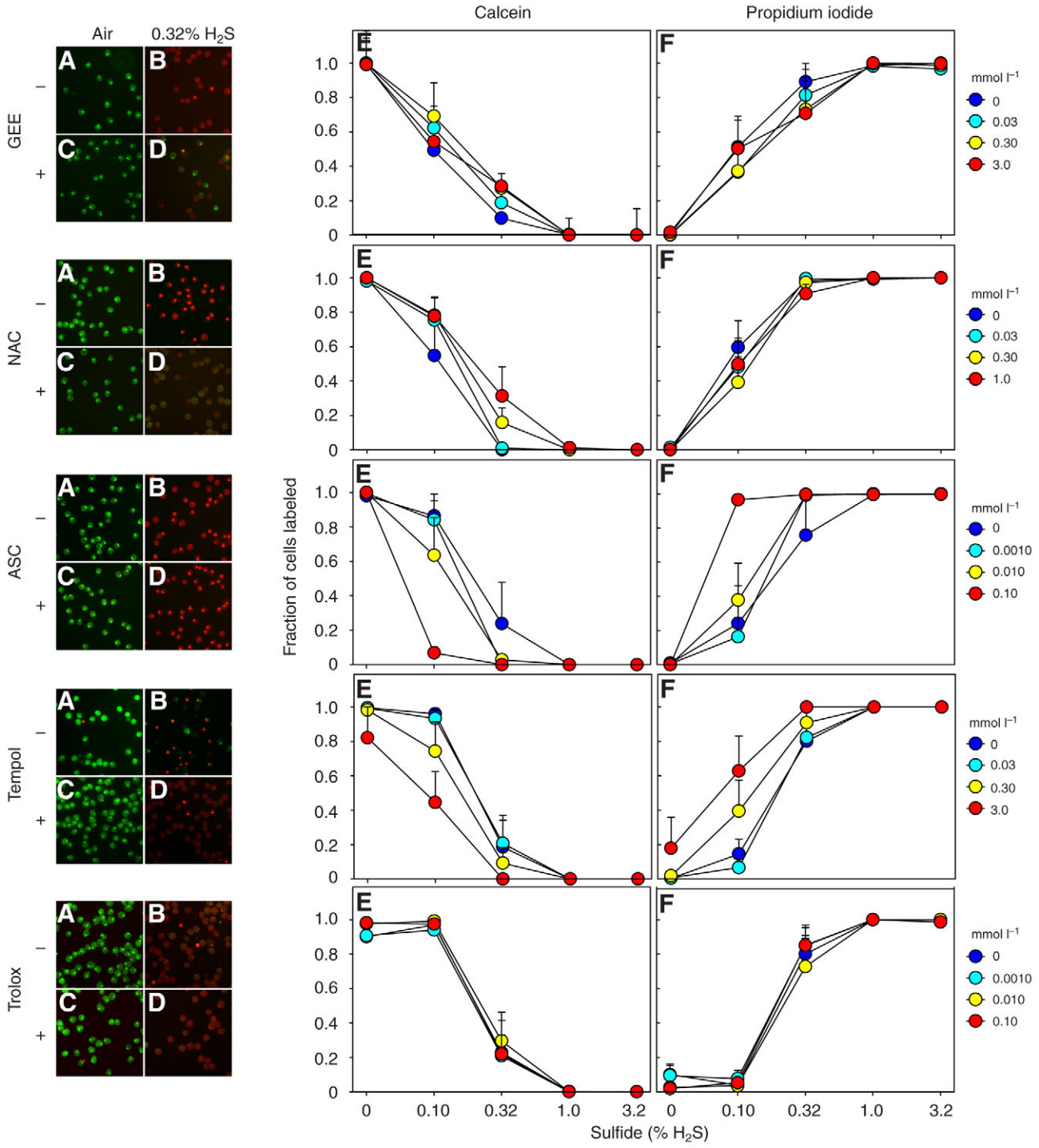

Fig. 4. Effects of antioxidants on the viability of *G. dibranchiata* erythrocytes exposed to H_2S *in vitro*. (A–D) Epifluorescence micrographs of cells in incubation buffer that were exposed for 2 h to air (A) and 0.32% H_2S (B), and cells in incubation buffer with GEE (glutathione ethyl ester) (3 mmol l^{-1}), NAC (N-acetylcysteine) (1.0 mmol l^{-1}), ASC (L-ascorbic acid) (0.10 mmol l^{-1}), Tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl) (3.0 mmol l^{-1}) or Trolox (6-hydoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (0.10 mmol l^{-1}) that were then exposed for 2 h to air (C) or 0.32% H_2S (D), followed by labeling with calcein (green) and propidium iodide (PI; red). (E–F) Quantitative analysis of calcein labeling (E) and total PI labeling (F) in cells incubated with various concentrations of antioxidant (0–3.0 mmol l^{-1} GEE, 0–1 mmol l^{-1} NAC, 0–0.1 mmol l^{-1} ASC, 0–3.0 mmol l^{-1} Tempol or 0–0.1 mmol l^{-1} Trolox) and exposed for 2 h to 0–3.2% H_2S. *N*=5. Results of statistical analysis presented in Table 1.

intracellular sulfide concentration as well as the extracellular sulfide concentration.

As the reaction product of sulfide and HT, ThT has no further ability to bind sulfide. However, if ThT is an antioxidant (Cavallini and Tentori, 1960) it may still reduce sulfide toxicity by binding free radicals from sulfide oxidation. Therefore, we anticipated that ThT would at worst have no effect on cell viability during H_2S exposure and at best might have a moderate beneficial effect. However, while ThT did not affect the viability of cells under control conditions (and was therefore not toxic itself), it decreased cell viability during H_2S exposure by as much as 83% (at 0.5 mmol l^{-1} ThT and 0.10% H_2S). The mechanism by which ThT increased H_2S toxicity is unknown but any antioxidant capacity it might have had was not sufficient to protect the cells. Furthermore, this raises the question of whether, in cells incubated with HT during sulfide exposure, gradual accumulation of ThT begins to reduce cell viability. This exemplifies the importance of recycling of ThT to HT, whether by sulfide-oxidizing endosymbionts or by specific enzymes (Brand et al., 2007; Rosenberg et al., 2006).

To further explore whether free radical scavenging alone could decrease the toxicity of sulfide in *G. dibranchiata* erythrocytes, we tested whether the antioxidants GEE, NAC, ASC, Tempol and Trolox could reduce the dissolved sulfide concentration in cell-free buffer exposed to H_2S and increase the viability of cells exposed to H_2S. Other than ASC, these antioxidants are all cell-permeant. Unlike HT, none of the antioxidants significantly decreased the dissolved sulfide concentration in incubation buffer exposed to H_2S but all concentrations of GEE and NAC (0.03–3 and 0.03–1 mmol l^{-1}, respectively) increased the fraction of viable cells. This suggests that the beneficial action of GEE and NAC was at least partially *via* free radical scavenging, although an alternative explanation is that GEE and NAC reduced free sulfide primarily intracellularly, which would not have been detected in our experiments. GEE is converted within cells to the antioxidant reduced glutathione (Halliwell and Gutteridge, 2007), which, upon becoming oxidized, can react with sulfide, forming glutathione persulfide (Rohwerder and Sand, 2003; Smith and Gosselin, 1966). NAC reacts with reactive oxygen species to produce cysteine (Aruoma et al., 1988) and stimulates intracellular glutathione synthesis (Moldeus et al., 1986) but it also contains a sulfhydryl group. The remaining antioxidants, all of which do not contain a sulfhydryl group, either reduced the viability of cells exposed to H_2S or had no effect. ASC readily oxidizes to dehydroascorbic acid, reducing the oxidative loss of sulfide *in vitro* (Glaister et al., 1984). All concentrations of ASC (0.001–0.1 mmol l^{-1}) decreased cell viability. The superoxide dismutase-mimetic Tempol is a catalytic scavenger of superoxide anions *in vitro*. Tempol at lower concentrations (0.03–0.3 mmol l^{-1}) had no effect on cell viability but 3 mmol l^{-1} Tempol decreased cell viability in the presence of H_2S (e.g. by as much as 77% at 0.10% H_2S), consistent with a report that Tempol can be deleterious at concentrations higher than 0.05 mmol l^{-1} (Lewinska et al., 2008). Trolox, which inhibits membrane lipid peroxidation and protein carbonylation (Halliwell and Gutteridge, 2007), had no effect on cell viability at any concentration (0.001–0.1 mmol l^{-1}) but it is important to note that we used comparatively low concentrations so it remains possible that higher concentrations would have been more effective. Interestingly, the persulfide dithiothreitol (DTT) is an antioxidant that contains two sulfhydryl groups and has been reported to reduce the lethality of H_2S exposure in rats, presumably by reducing the free sulfide concentration (Reiffenstein et al., 1992). We tested the ability of 0.05–5.0 mmol l^{-1} DTT to increase cell viability during H_2S exposure and observed a similar effect to that of GEE and NAC; however, the highest DTT concentration interfered with the loading, retention or fluorescence of the dyes through an unknown mechanism and therefore the DTT data were excluded.

The ability of HT to reduce sulfide toxicity was much greater than that of GEE and NAC but the maximum concentration of HT we used was 16-times and 50-times higher than that of GEE and NAC, respectively. HT is highly biocompatible, and the HT concentrations we used were within the range of tissue concentrations reported for many animals from hydrothermal vents, although it is somewhat difficult to make comparisons with the literature because the methods used to measure and express the HT concentration have varied. For example, when measured as HT per tissue wet mass, reported HT concentrations are 63–152 mmol kg^{-1} in several tubeworms (Yin et al., 2000) and 59–63 mmol kg^{-1} in two gastropods (Rosenberg et al., 2006). When measured as HT per tissue dry mass, reported concentrations are 7.2–112 mmol kg^{-1} in a large variety of bivalves and vestimentiferans (Fiess et al., 2002; Pruski et al., 2000a). Based on these values, Yancey estimated cell solute HT concentrations of 20–60 mmol l^{-1} HT for *Calyptogena* sp. and 320–360 mmol l^{-1} HT for *Riftia pachyptila* (Yancey, 2005).

Conclusion

HT, and to a lesser extent the sulfhydryl-containing antioxidants GEE and NAC, increase the viability of *G. dibranchiata* erythrocytes exposed to H_2S *in vitro*. The data support the hypothesis that HT can protect tissues of deep-sea, sulfide-adapted animals from sulfide cytotoxicity and suggest that sulfide scavenging rather than free radical scavenging is the most important mechanism of protection, although this does not rule out free radical scavenging as an additional protective mechanism. There are no reports of shallow water invertebrates with high HT concentrations (Fiess et al., 2002; Yancey, 2005; Yancey et al., 2002a) but to our knowledge animals in shallow sulfidic habitats have not been specifically investigated. Consequently, it is still unknown whether HT or similar compounds may reduce sulfide toxicity in *G. dibranchiata* or other invertebrates living in characteristically sulfidic habitats such as mudflats and mangroves. Finally, it is evident that H_2S, from both exogenous and endogenous sources, has a variety of physiological actions (Szabó, 2007) and, therefore, it would be valuable to determine the extent to which HT or compounds with sulfhydryl groups may modulate the activity of H_2S in vertebrate tissues.

LIST OF ABBREVIATIONS

ASC	L-ascorbic acid
DTT	dithiothreitol
GEE	glutathione ethyl ester
HT	hypotaurine
NAC	N-acetylcysteine
PI	propidium iodide
Tempol	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl
ThT	thiotaurine
Trolox	6-hydoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

J.A.O. and J.M.O. contributed equally to this manuscript. This research was supported by NSF IBN-0422139 to D.J. The authors thank Meghan Brennan, University of Florida Department of Statistics, for providing consultation on the statistical analyses. All experiments complied with the requirements of the University of Florida Institutional Animal Care and Use Committee.

REFERENCES

Abele-Oeschger, D. and Oeschger, R. (1995). Hypoxia-induced autooxidation of haemoglobin in the benthic invertebrates *Arenicola marina* (polychaeta) and *Astarte borealis* (bivalvia) and the possible effects of sulphide. *J. Exp. Mar. Biol. Ecol.* **187**, 63-80.

Alberic, P. (1986). Occurrence of thiotaurine and hypotaurine in the tissues of *Riftia pachyptila* (Pogonophora, Vestimentifera). *C R Seances Acad. Sci. III* **302**, 503.
Alberic, P. and Boulegue, J. (1990). Unusual amino-compounds in the tissues of *Calyptogena phaseoliformis* (Japan Trench): possible link to symbiosis. *Prog. Oceanogr.* **24**, 89-101.
Alvarez, J. and Storey, B. (1983). Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol. Reprod.* **29**, 548-555.
Arp, A. J. and Childress, J. J. (1983). Sulfide binding by the blood of the hydrothermal vent tube worm *Riftia pachyptila*. *Science* **219**, 295-297.
Arp, A. J., Childress, J. J. and Fisher, C. R. J. (1984). Metabolic and blood gas transport characteristics of the hydrothermal vent bivalve *Calyptogena magnifica*. *Physiol. Zool.* **57**, 648-662.
Arp, A. J., Childress, J. J. and Vetter, R. D. (1987). The sulphide-binding protein in the blood of the vestimentiferan tube-worm, *Riftia pachyptila*, is the extracellular hemoglobin. *J. Exp. Biol.* **128**, 139-158.
Aruoma, O., Halliwell, B., Hoey, B. and Butler, J. (1988). The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem. J.* **256**, 251-255.
Attene-Ramos, M., Wagner, E., Gaskins, H. and Plewa, M. (2007). Hydrogen sulfide induces direct radical-associated DNA damage. *Mol. Cancer Res.* **5**, 455-459.
Bagarinao, T. (1992). Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquat. Toxicol.* **24**, 21-62.
Bebianno, M. J., Company, R., Serafim, A., Camus, L., Cosson, R. P. and Fiala-Medoni, A. (2005). Antioxidant systems and lipid peroxidation in *Bathymodiolus azoricus* from Mid-Atlantic Ridge hydrothermal vent fields. *Aquat. Toxicol.* **75**, 354-373.
Bhattacharya, R. and Lakshmana Rao, P. V. (2001). Pharmacological interventions of cyanide-induced cytotoxicity and DNA damage in isolated rat thymocytes and their protective efficacy *in vivo*. *Toxicol. Lett.* **119**, 59-70.
Brand, G., Horak, R., Le Bris, N., Goffredi, S., Carney, S., Govenar, B. and Yancey, P. (2007). Hypotaurine and thiotaurine as indicators of sulfide exposure in bivalves and vestimentiferans from hydrothermal vents and cold seeps. *Mar. Ecol. Evol. Persp.* **28**, 208-218.
Cardenas, W., Dankert, J. and Jenkins, J. (2004). Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides. *Fish Shellfish Immunol.* **17**, 223-233.
Casado, M., Cecchini, A., Simao, A., Oliveira, R. and Cecehini, R. (2007). Free radical-mediated pre-hemolytic injury in human red blood cells subjected to lead acetate as evaluated by chemiluminescence. *Food Chem. Toxicol.* **45**, 945-952.
Cavallini, D. and Tentori, L. (1960). Inability of thiotaurine to protect mice against ionizing radiation. *Nature* **186**, 254.
Cavallini, D., Mondovi, B. and de Marco, C. (1963). Hypotaurine and thiotaurine. *Biochem. Prep.* **10**, 72-75.
Chen, K. Y. and Morris, J. C. (1972). Oxidation of sulfide by O_2: catalysis and inhibition. *J. Sanit. Eng. Div. Proc. Am. Soc. Civ. Eng.* **98**, 215-227.
Costa, C. J., Pierce, S. K. and Warren, M. K. (1980). The intracellular mechanism of salinity tolerance in polychaetes-volume regulation by isolated *Glycera dibranchiata* red coelomocytes. *Biol. Bull.* **159**, 626-638.
Dive, C., Watson, J. and Workman, P. (1990). Multiparametric analysis of cell membrane permeability by two colour flow cytometry with complementary fluorescent probes. *Cytometry* **11**, 244-252.
Donnelly, E., McClure, N. and Lewis, S. (2000). Glutathione and hypotaurine *in vitro*: effects on human sperm motility DNA integrity and production of reactive oxygen species. *Mutagenesis* **15**, 61-68.
Doolen, J. F., Geddes, G. C. and Blackstone, N. W. (2007). Multicellular redox regulation in an early-evolving animal treated with glutathione. *Physiol. Biochem. Zool.* **80**, 317-325.
Dorman, D., Moulin, F., McManus, B., Mahle, K., James, R. and Struve, M. (2002). Cytochrome oxidase inhibition induced by acute hydrogen sulfide inhalation: correlation with tissue sulfide concentrations in the rat brain, liver, lung, and nasal epithelium. *Toxicol. Sci.* **65**, 18-25.
Eghbal, M. A., Pennefather, P. S. and O'Brien, P. J. (2004). H_2S cytotoxicity mechanism involves reactive oxygen species formation and mitochondrial depolarisation. *Toxicology* **203**, 69-76.
Fellman, J. and Roth, E. (1985). The biological oxidation of hypotaurine to taurine: hypotaurine as an antioxidant. *Prog. Clin. Biol. Res.* **179**, 71-82.
Fiess, J., Hudson, H., Hom, J., Kato, C. and Yancey, P. (2002). Phosphodiester amine, taurine and derivatives, and other osmolytes in Vesicomyid bivalves: correlations with depth and symbiont metabolism. *Cah. Biol. Mar.* **43**, 337-340.
Gamenick, I., Vismann, B., Grieshaber, M. K. and Giere, O. (1998). Ecophysiological differentiation of *Capitelle capitata* (Polychaeta): sibling species from different sulfidic habitats. *Mar. Ecol. Prog. Ser.* **175**, 155-166.
Gilboa-Garber, N. (1971). Direct spectrophotometric determination of inorganic sulfide in biological materials and in other complex mixtures. *Anal. Biochem.* **43**, 129-133.
Glaister, M. G., Moody, G. J. and Thomas, J. D. R. (1984). The stability of sulphide anti-oxidant buffer. *Anal. Chim. Acta* **165**, 113-119.
Grieshaber, M. K. and Völkel, S. (1998). Animal adaptations for tolerance and exploitation of poisonous sulfide. *Annu. Rev. Physiol.* **60**, 33-53.
Halliwell, B. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.
Hance, J. M., Andrzejewski, J. E., Predmore, B. L., Dunlap, K. J., Misiak, K. L. and Julian, D. (2008). Cytotoxicity from sulfide exposure in a sulfide-tolerant marine invertebrate. *J. Exp. Mar. Biol. Ecol.* **359**, 102-109.
Hegaret, H., Wikfors, G. and Soudant, P. (2003). Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation. II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. *J. Exp. Mar. Biol. Ecol.* **293**, 249-265.
Horak, R., Rosenberg, N., Hudson, H., Lee, R. and Yancey, P. (2003). Occurrence of thiotaurine and hypotaurine in hydrothermal vent and cold seep species without endosymbionts. *Integr. Comp. Biol.* **43**, 1043.
Huxtable, R. (1992). Physiological actions of taurine. *Physiol. Rev.* **72**, 101-163.
Jha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A. and Lefer, D. J. (2008). Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *Am. J. Physiol. Heart Circ. Physiol.* **295**, H801-H806.
Joyner, J. L., Peyer, S. M. and Lee, R. W. (2003). Possible roles of sulfur-containing amino acids in a chemoautotrophic bacterium-mollusc symbiosis. *Biol. Bull.* **205**, 331-338.
Joyner-Matos, J., Downs, C. A. and Julian, D. (2006). Increased expression of stress proteins in the surf clam *Donax variabilis* following hydrogen sulfide exposure. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* **145**, 245-257.
Joyner-Matos, J., Chapman, L., Downs, C., Hofer, T., Leeuwenburgh, C. and Julian, D. (2007). Stress response of a freshwater clam along an abiotic gradient: too much oxygen may limit distribution. *Funct. Ecol.* **21**, 344-355.
Joyner-Matos, J., Predmore, B. L., Stein, J. R., Leeuwenburgh, C. and Julian, D. (2008). Hydrogen sulfide induces oxidative damage to RNA and DNA in a sulfide-tolerant marine invertebrate. *Physiol. Biochem. Zool.* (in press).
Julian, D., April, K. L., Patel, S., Stein, J. R. and Wohlgemuth, S. E. (2005). Mitochondrial depolarization following hydrogen sulfide exposure in erythrocytes from a sulfide-tolerant marine invertebrate. *J. Exp. Biol.* **208**, 4109-4122.
Khan, A. A., Schuler, M. M., Prior, M. G., Yong, S., Coppock, R. W., Florence, L. Z. and Lillie, L. E. (1990). Effects of hydrogen sulfide exposure on lung mitochondrial respiratory chain enzymes in rats. *Toxicol. Appl. Pharmacol.* **103**, 482-490.
Konarkowska, B., Aitken, J., Kistler, J., Zhang, S. and Cooper, G. (2005). Thiol reducing compounds prevent human amylin-evoked cytotoxicity. *FEBS J.* **272**, 4949-4959.
Lee, Y., Sob, B. and Wu, J. (2001). Quantitative assessment of phagocytic activity of hemocytes in the prawn, *Penaeus merguiensis*, by flow cytometric analysis. *Cytometry* **43**, 82-85.
Lewinska, A., Wnuk, M., Slota, E. and Bartosz, G. (2008). The nitroxide antioxidant Tempol affects metal-induced cyto- and genotoxicity in human lymphocytes *in vitro*. *Mutat. Res.* **649**, 7-14.
Machella, N., Battino, M., Pisanelli, B. and Regoli, F. (2006). Influence of the SCGE protocol on the amount of basal DNA damage detected in the Mediterranean Mussel, *Mytilus galloprovincialis*. *Environ. Mol. Mutagen.* **47**, 579-586.
Mangum, C. P. (1994). Multiple sites of gas-exchange. *Am. Zool.* **34**, 184-193.
Marin, M., Legros, H., Poret, A., Leboulenger, F. and Le Foll, F. (2004). Cell responses to xenobiotics: comparison of MCF7 multi-drug- and mussel blood cell multi-xenobiotic-defense mechanisms. *Mar. Environ. Res.* **58**, 209-213.
Moldeus, P., Cotgreave, I. A. and Berggren M. (1986). Lung protection by a thiol-containing antioxidant – N-acetylcysteine. *Respiration 50 Supplement 1*, 31-42.
Nicholls, P. and Kim, J. K. (1982). Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system. *Can. J. Biochem.* **60**, 613-623.
O'Brien, J. and Vetter, R. D. (1990). Production of thiosulphate during sulphide oxidation by mitochondria of the symbiont-containing bivalve *Solemya reidi*. *J. Exp. Biol.* **149**, 133-148.
Pocernich, C., Cardin, A., Racine, C., Lauderback, C. and Butterfield, D. (2001). Glutathione elevation and its protective role in acrolein-induced protein damage in synaptosomal membranes: relevance to brain lipid peroxidation in neurodegenerative disease. *Neurochem. Int.* **39**, 141-149.
Pranal, V., Fialamedioni, A. and Colomines, J. (1995). Amino-acid and related compound composition in two symbiotic Mytilid species from hydrothermal vents. *Mar. Ecol. Prog. Ser.* **119**, 155-166.
Pruski, A. and Fiala-Medioni, A. (2003). Stimulatory effect of sulphide on thiotaurine synthesis in three hydrothermal-vent species from the East Pacific Rise. *J. Exp. Biol.* **206**, 2923-2930.
Pruski, A., Fiala-Medioni, A. and Colomines, J. (1997). High amounts of sulphur amino acids in three symbiotic mytilid bivalves from deep benthic communities. *C R Seances Acad. Sci. III* **320**, 791-796.
Pruski, A., Fiala-Medioni, A., Fisher, C. and Colomines, J. (2000a). Composition of free amino acids and related compounds in invertebrates with symbiotic bacteria at hydrocarbon seeps in the Gulf of Mexico. *Mar. Biol.* **136**, 411-420.
Pruski, A. M., Fiala-Médioni, A., Prodon, R. and Colomines, J.-C. (2000b). Thiotaurine is a biomarker of sulfide-based symbiosis in deep-sea bivalves. *Limnol. Oceanogr.* **45**, 1860-1867.
Pruski, A., De Wit, R. and Fiala-Medioni, A. (2001). Carrier of reduced sulfur is a possible role for thiotaurine in symbiotic species from hydrothermal vents with thiotrophic symbionts. *Hydrobiologia* **461**, 9-13.
Reiffenstein, R. J., Hulbert, W. C. and Roth, S. H. (1992). Toxicology of hydrogen sulfide. *Annu. Rev. Pharmacol. Toxicol.* **32**, 109-134.
Rohwerder, T. and Sand, W. (2003). The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp. *Microbiology* **149**, 1699-1710.
Rosenberg, N., Lee, R. and Yancey, P. (2006). High contents of hypotaurine and thiotaurine in hydrothermal-vent gastropods without thiotrophic endosymbionts. *J. Exp. Zool. Part A Comp. Exp. Biol.* **305A**, 655-662.
Samuni, A., DeGraff, W., Cook, J., Krishna, M., Russo, A. and Mitchell, J. (2004). The effects of antioxidants on radiation-induced apoptosis pathways in TK6 cells. *Free Radic. Biol. Med.* **37**, 1648-1655.
Schurr, A. and Rigor, B. (1987). The mechanism of neuronal resistance and adaptation to hypoxia. *FEBS Lett.* **224**, 4-8.
Singh, I., Sullivan, P. and Hall, E. (2007). Peroxynitrite-mediated oxidative damage to brain mitochondria: protective effects of peroxynitrite scavengers. *J. Neurosci. Res.* **85**, 2216-2223.
Smith, R. and Gosselin, R. (1966). On the mechanism of sulfide inactivation by methemoglobin. *Toxicol. Appl. Pharmacol.* **8**, 159-172.
Szabó, C. (2007). Hydrogen sulphide and its therapeutic potential. *Nat. Rev. Drug Discov.* **6**, 917-935.
Tapley, D. (1993). Sulfide-dependent oxidative stress in marine invertebrates, especially thiotrophic symbioses. PhD thesis, University of Maine, Orono, ME, USA.

Tapley, D., Buettner, G. and Shick, J. (1999). Free radicals and chemiluminescence as products of the spontaneous oxidation of sulfide in seawater, and their biological implications. *Biol. Bull.* **196**, 52-56.

Truong, D. H., Eghbal, M. A., Hindmarsh, W., Roth, S. H. and O'Brien, P. J. (2006). Molecular mechanisms of hydrogen sulfide toxicity. *Drug Metab. Rev.* **38**, 733-744.

Whiteman, M., Armstrong, J. S., Chu, S. H., Jia-Ling, S., Wong, B. S., Cheung, N. S., Halliwell, B. and Moore, P. K. (2004). The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J. Neurochem.* **90**, 765-768.

Whiteman, M., Cheung, N. S., Zhu, Y. Z., Chu, S. H., Siau, J. L., Wong, B. S., Armstrong, J. S. and Moore, P. K. (2005). Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem. Biophys. Res. Commun.* **326**, 794-798.

Wu, T., Hashimoto, N., Wu, J., Carey, D., Li, R., Mickle, D. and Weisel, R. (1990). The cytoprotective effect of Trolox demonstrated with three types of human cells. *Biochem. Cell Biol.* **68**, 1189-1194.

Xue, Q., Renault, T. and Chilmonczyk, S. (2001). Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. *Fish Shellfish Immunol.* **11**, 557-567.

Yan, S. K., Chang, T., Wang, H., Wu, L., Wang, R. and Meng, Q. H. (2006). Effects of hydrogen sulfide on homocysteine-induced oxidative stress in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **351**, 485-491.

Yancey, P. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819-2830.

Yancey, P. H., Blake, W. R. and Conley, J. (2002a). Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* **133**, 667-676.

Yancey, P. H., Blake, W. R. and Conley, J. (2002b). Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* **133**, 667-676.

Yedjou, C., Rogers, C., Brown, E. and Tchounwou, P. (2008). Differential effect of ascorbic acid and n-acetyl-L-cysteine on arsenic trioxide-mediated oxidative stress in human leukemia (HL-60) cells. *J. Biochem. Mol. Toxicol.* **22**, 85-92.

Yin, M., Palmer, H. R., Fyfe-Johnson, A. L., Bedford, J. J., Smith, R. A. J. and Yancey, P. H. (2000). Hypotaurine, N-methyltaurine, taurine, and glycine betaine as dominant osmolytes of vestimentiferan tubeworms from hydrothermal vents and cold seeps. *Physiol. Biochem. Zool.* **73**, 629-637.