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# Enzymatic hydrogen sulfide production in marine invertebrate tissues

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## Abstract

At least some mammalian tissues produce H<sub>2</sub>S in vitro from L-cysteine at rates sufficient to have physiological effects. To determine whether tissues of macrofaunal invertebrates have the same capacity, we measured H<sub>2</sub>S production in tissue homogenates of the Manila clam *Tapes philippinarum* and the lugworm *Arenicola marina*. Tissue homogenates from both animals produced significant quantities of H<sub>2</sub>S gas upon addition of L-cysteine and the enzyme cofactor pyridoxal-5-phosphate (10 mmol l<sup>-1</sup> and 2 mmol l<sup>-1</sup>, respectively), while only tissues from *T. philippinarum* produced measurable H<sub>2</sub>S in the absence of added substrate or cofactor. In *T. philippinarum* tissues, H<sub>2</sub>S production was completely inhibited by the cystathionine β-synthase (CBS) inhibitor aminooxyacetic acid (AOAA), suggesting that the majority of H<sub>2</sub>S production was via CBS pathways, while in *A. marina* body wall, AOAA inhibited only half of the total H<sub>2</sub>S production, indicating that the CBS pathway was not the only major source of H<sub>2</sub>S production. H<sub>2</sub>S production in tissues of *T. philippinarum* but not *A. marina* was doubled by the addition of a second thiol substrate (2.5 mmol l<sup>-1</sup> 2-mercaptoethanol), suggesting the presence of an 'activated serine sulfhydrase pathway', which had previously been demonstrated only in some microfauna. © 2002 Elsevier Science Inc. All rights reserved.

**Keywords:** Cysteine; H<sub>2</sub>S; *Tapes philippinarum*; *Arenicola marina*; Serine sulfhydrase

## 1. Introduction

Hydrogen sulfide (H<sub>2</sub>S) can be produced in animal tissues by endogenous enzymes and non-enzymatic pathways via reduction of thiols and thiol-containing molecules. H<sub>2</sub>S is a well-known toxin that poisons isolated mitochondria at low micromolar concentrations via reversible inhibition of cytochrome *c* oxidase (Nicholls, 1975; National Research Council, 1979). Therefore, until recently it had been assumed that H<sub>2</sub>S concentrations in animal tissues must be very low. However, recent studies of mammalian tissue have shown that H<sub>2</sub>S

concentrations may be considerable, with measured concentrations of up to 50–160 μmol l<sup>-1</sup> H<sub>2</sub>S in rat, human and bovine brain tissue (Goodwin et al., 1989; Warenycia et al., 1989). Endogenous H<sub>2</sub>S can be formed enzymatically from L-cysteine, and Abe and Kimura (1996) proposed that H<sub>2</sub>S formed in this way could function as an intracellular messenger. Consistent with this hypothesis, exogenously applied H<sub>2</sub>S has a number of physiological effects on mammalian tissue in vitro, including facilitation of long-term potentiation (Abe and Kimura, 1996), modulation of smooth muscle tone and nitric oxide activity (Hosoki et al., 1997), induction of cyclic AMP and modulation of NMDA receptors (Kimura, 2000), and decreased release of corticotropin-releasing hormone (Dello Russo et al., 2000).

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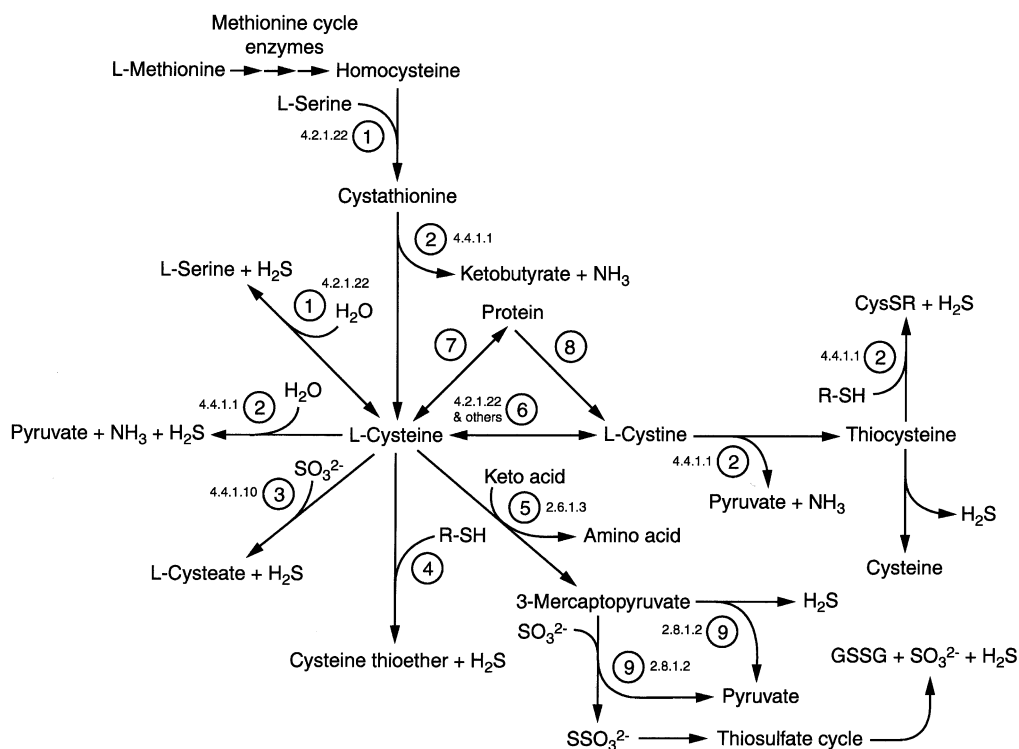


Fig. 1. Representative enzymatic pathways for production of  $H_2S$  from L-cysteine in animals: (1) cystathionine  $\beta$ -synthase (EC 4.2.1.22); (2) cystathionine- $\gamma$ -lyase (EC 4.4.1.1); (3) cysteine lyase (EC 4.4.1.10); (4) activated L-serine sulfhydrase (known only for some microfauna; Walker and Barrett, 1997); (5) cysteine aminotransferase (EC 2.6.1.3); (6) enzymatic and non-enzymatic disulfide interchange reactions (forming L-cysteine) and various thiol oxidases (forming L-cysteine); (7) ribosomal protein synthesis; (8) formation of protein disulfide bonds; and (9)  $\beta$ -mercaptopyruvate sulfurtransferase (EC 2.8.1.2). Pathways are based Cavallini et al. (1962), Roy and Trudinger (1970), Griffith (1987), Walker and Barrett (1997) and Dello Russo et al. (2000).

### 1.1. Pathways for $H_2S$ production

In mammalian tissues,  $H_2S$  is produced from L-cysteine through at least four pathways (Fig. 1). In the first pathway, L-cysteine is hydrolyzed by cystathionine  $\beta$ -synthase (CBS, EC 4.2.1.22, also called serine sulfhydrase), producing equimolar amounts of  $H_2S$  and L-serine. In the second pathway, two L-cysteine molecules dimerize to form cystine, which is transformed into thiocysteine, pyruvate and  $NH_3$  by cystathionine- $\gamma$ -lyase (CSE, EC 4.4.1.1). The thiocysteine can then undergo one of two reactions to form  $H_2S$ : CSE can catalyze the reaction of thiocysteine with other thiol compounds (e.g. glutathione or cysteine) to form  $H_2S$  and CysSR, or thiocysteine can form cysteine and  $H_2S$ , possibly non-enzymatically (Cavallini et al., 1962). In the third pathway, cysteine aminotransferase (CAT, EC 2.6.1.3) catalyzes the reaction of L-cysteine with a ketoacid (e.g.  $\alpha$ -ketoglutarate) to form 3-mercaptopyruvate

and an amino acid (e.g. L-glutamate). The 3-mercaptopyruvate can then be desulfurated by 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2) to form  $H_2S$  and pyruvate, or, in the presence of  $SO_3^{2-}$ , thiosulfate and pyruvate. In the 'thiosulfate cycle', the thiosulfate reacts with reduced glutathione (GSH) to produce  $H_2S$ ,  $H_2SO_3$  and oxidized glutathione (GSSG). In the fourth reaction, cysteine lyase (EC 4.4.1.10) can convert L-cysteine and sulfite to L-cysteate and  $H_2S$ . The cofactor pyridoxal-5'-phosphate (PLP) is required by CBS, CAT, CSE and cysteine lyase, whereas MPST is zinc-dependent.

A fifth  $H_2S$  production pathway, termed the activated L-serine sulfhydrase reaction (Braunstein et al., 1971), has been identified in some microfauna (see Walker and Barrett, 1997 for a review), including some nematodes (Walker and Barrett, 1992; Walker et al., 1992), parasitic protozoa (Thong and Coombs, 1985a), trichomonads (Thong and Coombs, 1985b), and a cestode (Gom-

ez-Bautista and Barrett, 1988). In this pathway, a serine sulfhydrase catalyzes the reaction of L-cysteine with a second thiol compound (e.g. 2-mercaptoethanol) to form the respective cysteine thioether and H<sub>2</sub>S (reaction #4 in Fig. 1). The serine sulfhydrase has yet to be fully characterized, but in at least some nematodes it appears to be a variant form of CBS (Walker et al., 1992). Although low levels of activated L-serine sulfhydrase activity were initially reported in highly purified mammalian hepatic CBS (Braunstein et al., 1971), subsequent studies have failed to detect it (see Thong and Coombs, 1985a,b; Gomez-Bautista and Barrett, 1988; Walker and Barrett, 1997 for a review). To our knowledge, no studies have demonstrated an activated L-serine sulfhydrase pathway in macrofaunal invertebrates.

### 1.2. Objectives

If H<sub>2</sub>S does have a physiological role in mammals, then it is likely to have a physiological role in invertebrates as well. Therefore, it is also likely that invertebrate tissues have enzymatic mechanisms for H<sub>2</sub>S production similar to those that have been demonstrated in mammals. In this study, we measured the production of H<sub>2</sub>S in vitro from tissue homogenates of two marine invertebrates: the Manila clam *Tapes philippinarum* and the lugworm *Arenicola marina*. To better understand whether the biochemical pathways used for H<sub>2</sub>S synthesis are similar to those shown to be important in mammals, we measured H<sub>2</sub>S production in the presence of specific inhibitors and activators of the most well-characterized H<sub>2</sub>S production pathways.

## 2. Methods

### 2.1. Collection and maintenance of animals

*Tapes philippinarum* were purchased from a local market in San Francisco, California. In the laboratory, they were maintained in glass dishes in a recirculating, aerated seawater system at 13–15 °C and 31–33 ppt, and they were fed commercial invertebrate food three times per week. The clams were used within 1 week of purchase, and they were allowed to depurate for 48 h before the beginning of an experiment. *Arenicola marina* were collected by hand during low tide from Zierikzee, The Netherlands. In the laboratory, they

were maintained in a recirculating, aerated sea water system at 15 °C and 35 ppt, in which they were allowed to burrow in mud. The worms typically ingested the mud under these conditions, but they were otherwise not fed. The worms were used up to 4 weeks after collection.

### 2.2. Measurement of H<sub>2</sub>S production in homogenates

Production of H<sub>2</sub>S was measured using the techniques described previously (Siegel, 1965; Stipanuk and Beck, 1982; Abe and Kimura, 1996). On each day of an experiment, one or more animals were dissected, and the tissues were pooled, separated into tissue type (e.g. into gill tissue, body wall tissue, etc., as noted) and placed in ice-cold 50 mmol l<sup>-1</sup> potassium phosphate buffer (pH 6.8) until all dissections were completed (always less than 1 h). Tissues were then blotted dry and chopped into small pieces. Each tissue sample was then weighed and homogenized with an equal volume of 100 mmol l<sup>-1</sup> potassium phosphate buffer (pH 7.4) for 60–90 s using either a borosilicate micro tissue grinder (Radnoti Glass Technology Inc., Monrovia, CA, USA) for *T. philippinarum* tissues, or an electric homogenizer (Ultra-Turrax T-25, IKA Werke, Staufen, Germany) for *A. marina* tissues. The liquid portion of the homogenate was then removed and placed on ice. Additional phosphate buffer was then added to the remaining tissue and homogenization was continued until the solution was smooth. This remaining homogenate was then combined with the initial portion and additional phosphate buffer to bring the final volume to approximately 12 times the original tissue volume. A 1.0–3.0-ml aliquot of this homogenate solution was then placed in the outer well of a 25-ml glass flask containing a center well (Warburg flasks were used, although the side-arms were unnecessary).

After the tissue homogenate was added to the outer well, the center well was filled with 0.5-ml 1% (w/v) zinc acetate and a piece of filter paper (Whatman No. 1) that had been cut in half and folded into a fan shape. At this time, the desired substrates, inhibitors and cofactors (see below) were added to the outer well. The flask was then flushed with N<sub>2</sub> gas, sealed with a septum stopper or glass stopper, and then incubated on a shaker at 15 °C for 24 h. After incubation, 1.5-ml 50% (w/v) trichloroacetic acid was added to the outer

well to stop enzyme activity and convert  $S^{2-}$  and  $HS^-$  to  $H_2S$ . The flask was then incubated for an additional hour to allow the remaining  $H_2S$  to volatilize from the incubation medium and become trapped on the filter paper. The filter paper was then removed from the center well and placed in a test tube containing 3.5 ml of de-ionized water, 0.4 ml of 20 mmol  $l^{-1}$  *N,N*-dimethyl-*p*-phenylenediamine oxalate in 7.2 M HCl and 0.4 ml of 30 mmol  $l^{-1}$   $FeCl_3$  in 1.2 M HCl. The test tube was then gently vortexed and incubated for 30 min at room temperature. Absorbance of the solution in the test tube was read with a spectrophotometer at 670 nm. Samples with an absorbance greater than 1 were diluted 10-fold with water and the absorbance was re-measured. Absorbance measurements were calibrated against a standard curve generated from rinsed crystals of  $Na_2S \cdot 9 H_2O$  in deoxygenated, de-ionized water, which was mixed directly with zinc acetate and assayed as above. To determine the sensitivity and accuracy of the  $H_2S$  measurement procedure, a known amount of  $H_2S$  ( $Na_2S$  dissolved in phosphate buffer) was added to each flask in place of homogenate, and this was allowed to incubate at 15 °C for 24 h. At the end of incubation, the zinc acetate-containing filter paper strips were assayed for  $H_2S$ . Recovery of  $H_2S$  was  $59.2 \pm 0.82\%$  with 400 nmol of added  $Na_2S$ , and  $32.6 \pm 10.5\%$  with 50 nmol of added  $Na_2S$  ( $n=3$  for each).  $H_2S$  production rates in this manuscript are not corrected for partial recovery, so all values are almost certainly underestimates.

### 2.3. Characterization of $H_2S$ production pathways

To determine to what extent the five known cysteine metabolism pathways contributed to  $H_2S$  production, the assays were run with combinations of the following thiol substrates: L-cysteine (10 mmol  $l^{-1}$ ), 2-mercaptoethanol (2.5 mmol  $l^{-1}$ )  $\alpha$ -ketoglutarate (5 mmol  $l^{-1}$ ), and reduced glutathione (GSH; 5 mmol  $l^{-1}$ ). To further characterize the specific enzyme pathways, aminooxyacetic acid (AOAA; 10  $\mu$ M to 10 mmol  $l^{-1}$ ) was used to inhibit CBS, and DL-proparagylglycine (PGly; 0.5 and 5.0 mmol  $l^{-1}$ ) was used to inhibit CSE. Pyridoxal-5'-phosphate (PLP; 2 mmol  $l^{-1}$ ) was added to most flasks as an enzyme cofactor. When inhibitors were used, they were added to the homogenates 5 min before the flask was sealed. Most flasks with homogenate contained 10 mmol

$l^{-1}$  of L-cysteine and 2 mmol  $l^{-1}$  of PLP, and we refer to this as the 'standard conditions'. Concentrations of substrates, inhibitors, and PLP were based on previous studies by Abe and Kimura (1996), Hosoki et al. (1997) and Walker and Barrett (1992). To determine the rate of  $H_2S$  production as a function of total tissue protein concentration, homogenates of *T. philippinarum* tissues were centrifuged at  $12\,500 \times g$  for 15 min, after which the total protein concentration of each supernatant was measured using a standard Lowry assay. The supernatants were then assayed for  $H_2S$  production using the standard conditions, with the addition of 2.5 mmol  $l^{-1}$  of 2-mercaptoethanol. All chemicals and reagents were from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 2.4. Data analysis

Data are presented as means  $\pm$  1 S.D. Some samples represent pooled tissues from more than one individual animal, in which case both the number of animals in the pool and the number of independent replicate tests are noted. In general, experiments were designed such that (1) a single tissue homogenate was aliquoted into 10 or more different flasks, with each flask receiving a different experimental treatment (e.g. a different combination of substrates); or (2) homogenates of different tissues from the same animal (or pool of animals) were placed in different flasks but all were subjected to the same experimental treatment. In both cases, statistical comparisons between treatments were performed with repeated-measures ANOVA, followed by the Tukey multiple comparisons post-test, or by a paired *t*-test (as indicated in the text). When these tests were not appropriate, comparisons between groups were performed with standard ANOVA followed by the Tukey post-test. Probabilities less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. $H_2S$ production of specific tissues

Tissue homogenates from *T. philippinarum* produced  $H_2S$ , to varying degrees, even in the absence of added substrates or cofactors (Fig. 2, solid bars).  $H_2S$  production was highest in gut ( $0.030 \pm 0.011$  nmol  $H_2S$   $g^{-1}$   $min^{-1}$ ), followed

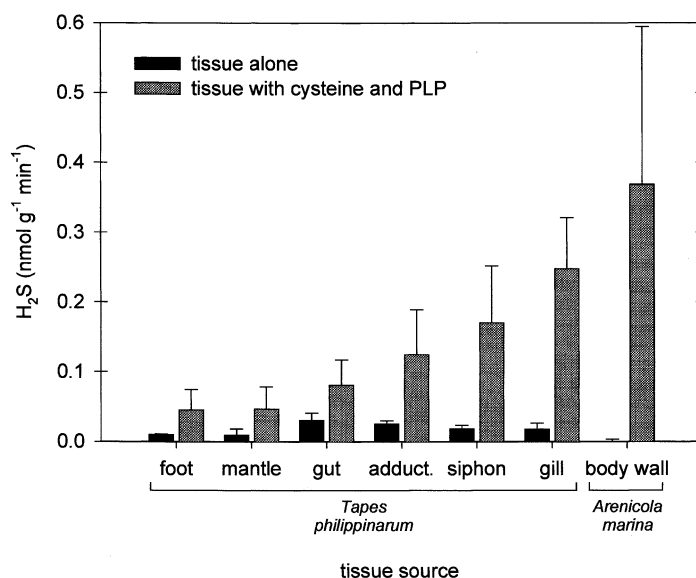


Fig. 2. H<sub>2</sub>S production rates (nmol H<sub>2</sub>S per gram tissue wet weight per minute) in tissue homogenates from *Tapes philippinarum* (foot, mantle, gut, adductor muscle, siphon and gill) and *Arenicola marina* (body wall). Homogenates were tested alone in buffer (filled bars) or with 10 mmol l<sup>-1</sup> L-cysteine and 2 mmol l<sup>-1</sup> PLP (gray bars). Data represent the mean ± S.D. (*N*=4 for *T. philippinarum*, *N*=5 for *A. marina*). For all tissues, H<sub>2</sub>S production in the presence of L-cysteine and PLP was significantly higher than in buffer alone (*P*<0.05).

by adductor muscle, siphon and gill, and then foot and mantle, with the latter two producing approximately one-third as much H<sub>2</sub>S as gut and adductor muscle. Negligible H<sub>2</sub>S was produced by *A. marina* body wall tissue in the absence of added substrates (<0.001 nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>). To determine the H<sub>2</sub>S production rates of specific tissues in the presence of added L-cysteine and PLP, *T. philippinarum* foot, mantle, gut, adductor muscle, siphon and gill were homogenized and assayed separately with 10 mmol l<sup>-1</sup> of L-cysteine and 2 mmol l<sup>-1</sup> of PLP, which we refer to as 'standard conditions' (Fig. 2, gray bars). Homogenates of all tissues showed large increases in H<sub>2</sub>S production upon addition of L-cysteine and PLP, compared with homogenate alone. H<sub>2</sub>S production was over fivefold higher in gill tissue (0.25 ± 0.07 nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>; the highest activity of *T. philippinarum* tissues) than in foot or mantle tissues (0.045 ± 0.029 and 0.047 ± 0.032 nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>, respectively; these tissues had the lowest activity). The differences between H<sub>2</sub>S production by gill tissue and those of foot, mantle or gut tissue were statistically significant (*P*<0.05). H<sub>2</sub>S production by *A. marina* body wall tissue (0.37 ± 0.22 nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>, *n*=5), which was the only tissue from *A. marina*

tested, was not significantly higher than that of *T. philippinarum* tissue gill or siphon. For both *T. philippinarum* and *A. marina*, H<sub>2</sub>S production in flasks containing homogenate under standard conditions was approximately 10-fold higher than that of flasks containing the same concentrations of PLP and L-cysteine in phosphate buffer but lacking tissue homogenate (10.3× for *T. philippinarum*, 9.4× for *A. marina*). Aquarium sediment added to a flask under standard conditions in the absence of tissue did not produce H<sub>2</sub>S (data not shown), confirming that the H<sub>2</sub>S production capacity of tissues was not due to contamination with sediment bacteria. To confirm that a 24-h incubation was appropriate for assaying H<sub>2</sub>S production, homogenates of *T. philippinarum* siphon, gill, and adductor muscle were assayed under standard conditions with incubation periods of 1, 3, 6, 12 and 24 h. While the H<sub>2</sub>S concentrations after only 1, 3 and 6 h were at or below the threshold of detection, the calculated rates of H<sub>2</sub>S production at 12 and 24 h were not significantly different from each other (*P*=0.90 by *t*-test, *n*=3; i.e. the total sulfide produced at 24 h was twice that at 12 h), indicating that both time points were equivalent for the assay.

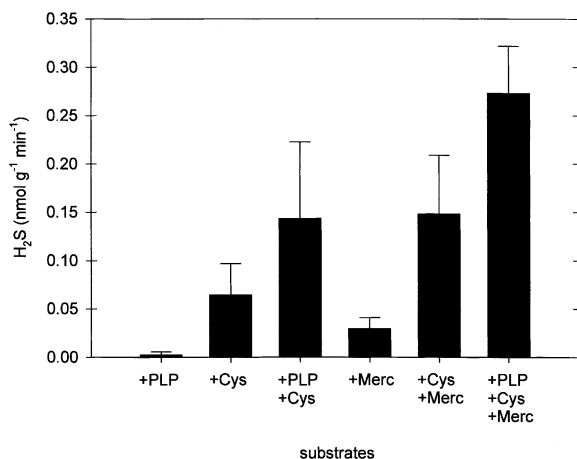


Fig. 3. H<sub>2</sub>S production rates (nmol H<sub>2</sub>S per g tissue wet weight per minute) in combined homogenates of siphon, gill and adductor muscle from *Tapes philippinarum*. H<sub>2</sub>S production was measured in the absence or presence of PLP (2 mmol l<sup>-1</sup>), L-cysteine (Cys, 10 mmol l<sup>-1</sup>) and 2-mercaptoethanol (Merc, 2.5 mmol l<sup>-1</sup>). Background H<sub>2</sub>S production (production of H<sub>2</sub>S from substrates and/or the cofactor PLP in the absence of tissue) was subtracted from the mean for each combination. Data represent the mean  $\pm$  S.D. of four trials (bars 1, 2, 4 and 5) or five trials (bars 3 and 6).

### 3.2. Characterization of enzymatic pathways

Combined homogenates of gill, siphon and adductor muscle from *T. philippinarum* were assayed with and without PLP and thiol substrates to confirm that H<sub>2</sub>S production was due to PLP-dependent enzymes acting on L-cysteine (Fig. 3). Addition of L-cysteine to the homogenate increased H<sub>2</sub>S production 30-fold compared with homogenate containing only PLP ( $P < 0.01$ ), while addition of PLP and L-cysteine increased H<sub>2</sub>S production 60-fold compared with homogenate containing only PLP ( $P = 0.012$ ) and 2.2-fold compared with L-cysteine alone ( $P$  not significant at 0.077). H<sub>2</sub>S production in *A. marina* body wall homogenate had a similar effect of PLP and L-cysteine addition (data not shown). The activity of *A. marina* body wall homogenates was assayed with 5 mmol l<sup>-1</sup> of GSH or 5 mmol l<sup>-1</sup> of  $\alpha$ -ketoglutarate added to the standard conditions to estimate the contribution of the thiosulfate cycle and the CAT pathway, respectively, to H<sub>2</sub>S production. Neither GSH nor  $\alpha$ -ketoglutarate caused an increase in H<sub>2</sub>S production ( $n = 2$  for each; these were not tested in *T. philippinarum* tissues).

To determine whether the addition of a second thiol substrate increased H<sub>2</sub>S production, 2-mer-

captoethanol was added to tissue homogenates of *T. philippinarum* and *A. marina*, both with and without L-cysteine and PLP. In *T. philippinarum*, addition of 2.5 mmol l<sup>-1</sup> 2-mercaptoethanol to the standard assay conditions (10 mmol l<sup>-1</sup> L-cysteine and 2 mmol l<sup>-1</sup> PLP) increased H<sub>2</sub>S production by 80% in combined homogenates of siphon, gill and adductor muscle compared with tissues under standard conditions without 2-mercaptoethanol ( $0.29 \pm 0.049$  nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup> vs.  $0.14 \pm 0.079$  nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>, respectively;  $n = 5$ ,  $P = 0.007$ , Fig. 3). In homogenates of *A. marina* body wall, on the other hand, addition of 2-mercaptoethanol to standard assay conditions had no effect on H<sub>2</sub>S production ( $0.27 \pm 0.18$  nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup> vs.  $0.37 \pm 0.23$  nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup> for standard assay conditions,  $P = 0.55$ ,  $n = 4$ , data not shown). The addition of 2-mercaptoethanol had no effect on H<sub>2</sub>S production in control flasks containing PLP and L-cysteine in phosphate buffer, but lacking tissue homogenate. When measured as mass H<sub>2</sub>S produced per mass total protein per unit time, the mean H<sub>2</sub>S production rate of *T. philippinarum* tissues was  $12 \pm 4.3$  nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup> under standard conditions, with added 2.5 mmol l<sup>-1</sup> 2-mercaptoethanol. Activity was highest in the gut, followed by gill (18 and 14 nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup>, respectively) and lowest in siphon (5.0 nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup>).

### 3.3. Effects of inhibitors

Upon addition of the CSE inhibitor PGly to combined homogenates of *T. philippinarum* gill, siphon and adductor muscle under standard conditions, H<sub>2</sub>S production was not significantly affected, regardless of PGly concentration ( $63 \pm 5.3\%$  of standard H<sub>2</sub>S production with 0.5 mmol l<sup>-1</sup> PGly,  $P = 0.089$ , and  $66 \pm 14\%$  of standard H<sub>2</sub>S production with 5 mmol l<sup>-1</sup> PGly,  $P = 0.13$ ,  $n = 3$ ; Fig. 4). The addition of 2-mercaptoethanol along with PGly had no additional effect ( $P = 0.56$  and  $P = 0.99$  for 0.5 and 5 mmol l<sup>-1</sup> PGly, respectively). In contrast, the addition of the CBS inhibitor AOAA to *T. philippinarum* homogenates under standard conditions produced a dose dependent inhibition of H<sub>2</sub>S production, with complete inhibition at the highest AOAA concentration ( $65 \pm 8.2\%$  inhibition with 10  $\mu$ M AOAA,  $P = 0.020$ , and  $100 \pm 0.4\%$  inhibition with 100  $\mu$ M AOAA,  $P = 0.002$ ;  $n = 3$ ). The

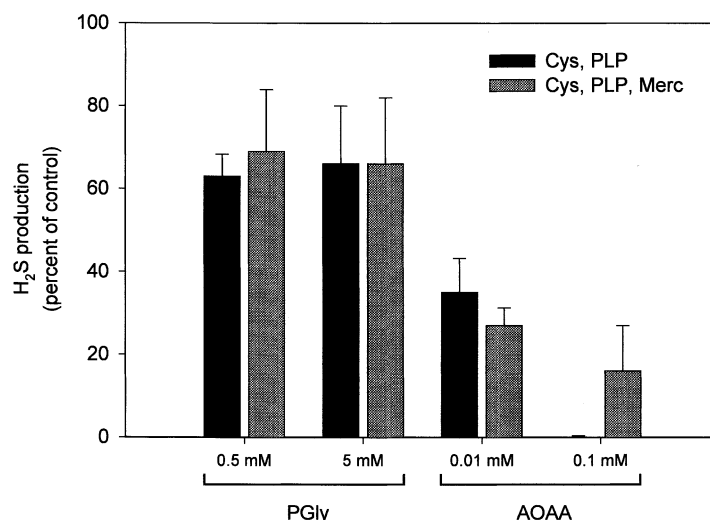


Fig. 4. Effect of DL-proparaglyglycine (PGly; 0.5 and 5.0 mmol l<sup>-1</sup>) and aminoxyacetic acid (AOAA; 0.01 and 0.1 mmol l<sup>-1</sup>) on H<sub>2</sub>S production in combined homogenates of *T. philippinarum* gill, siphon and adductor muscle. PGly is an inhibitor of the enzyme CSE, while AOAA is an inhibitor of the enzyme CBS. L-Cysteine (Cys; 10 mmol l<sup>-1</sup>) and pyridoxal-5-phosphate (PLP; 2 mmol l<sup>-1</sup>) were present in all homogenates, while some experiments also contained 2-mercaptoethanol (Merc; gray bars, 2.5 mmol l<sup>-1</sup>). H<sub>2</sub>S production rates are the percentage of the control production rate for that homogenate under the same assay conditions (i.e. with added L-cysteine and PLP alone, or with 2-mercaptoethanol, L-cysteine and PLP) but in the absence of an inhibitor. Data represent the mean ± S.D. of three trials, with three replicates in each trial.

addition of 2-mercaptoethanol along with 100 μM AOAA allowed H<sub>2</sub>S production to persist at 16 ± 11% of control ( $P < 0.001$ ,  $n = 3$ ). In homogenates of *A. marina* body wall under standard conditions, the addition of even 10 mmol l<sup>-1</sup> AOAA (i.e. 100 times the concentration sufficient to completely inhibit *T. philippinarum* tissue) caused a reduction in H<sub>2</sub>S production of only 44%, which was not statistically significant due to high variability ( $P = 0.36$ ,  $n = 5$ ).

#### 4. Discussion

##### 4.1. Capacity for H<sub>2</sub>S production

In the absence of added substrate and co-factor, homogenates of *T. philippinarum* adductor muscle, siphon and gill produced, on average, 0.023 nmol H<sub>2</sub>S g<sup>-1</sup> min<sup>-1</sup>, which would correspond to roughly 1.4 μmol H<sub>2</sub>S l<sup>-1</sup> h<sup>-1</sup> in those tissues *in vivo*. Given that the efficiency of the H<sub>2</sub>S assay was probably less than 33% at such low H<sub>2</sub>S concentrations, the actual H<sub>2</sub>S production rate is likely to be at least three-fold higher. Furthermore, even under the anaerobic incubation conditions of the assay, some conversion of H<sub>2</sub>S to non-volatile thiols may have occurred. Therefore, in tissues of

*T. philippinarum*, even in the absence of added substrate and co-factors, H<sub>2</sub>S may be produced *in vivo* at rates that would previously have been considered toxic to aerobic respiration. It is important to note, however, that since these studies used homogenates of animal tissues, the capacity for H<sub>2</sub>S production in whole tissues, in which intra- and inter-cellular regulatory mechanisms and compartmentalization of reactants are all intact, is still unknown.

In tissues of both *T. philippinarum* and *A. marina*, the addition of L-cysteine and PLP greatly increased H<sub>2</sub>S production compared with tissue homogenate alone (e.g. *T. philippinarum* gill = 14 ×, *A. marina* body wall = 1900 ×). Addition of PLP without L-cysteine did not increase H<sub>2</sub>S production, but addition of L-cysteine without PLP increased H<sub>2</sub>S production to approximately half that of L-cysteine and PLP together (*T. philippinarum* = 45%, *A. marina* = 66%). These observations suggest that PLP-dependent enzymatic pathways associated with L-cysteine metabolism are responsible for H<sub>2</sub>S production in these tissues under experimental conditions, as has been shown previously for rat and guinea pig homogenates (Abe and Kimura, 1996; Hosoki et al., 1997). When incubated with identical concentrations of

L-cysteine and PLP, but with the addition of 2.5 mmol l<sup>-1</sup> 2-mercaptoethanol, and when calculated as mass H<sub>2</sub>S produced per mass total protein per unit time, the H<sub>2</sub>S production rate of *T. philippinarum* combined gill, siphon and adductor homogenates at 15 °C (12 nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup>) was comparable to H<sub>2</sub>S production rates at 37 °C of rat brain homogenate (22.6 nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup>, Abe and Kimura, 1996) and guinea pig thoracic aorta, ileum and portal vein homogenates (up to 34 nmol H<sub>2</sub>S g protein<sup>-1</sup> min<sup>-1</sup>, Hosoki et al., 1997). Given the temperature difference, the capacity for enzymatic H<sub>2</sub>S production appears to be similar between mammals and invertebrates.

#### 4.2. Pathways for H<sub>2</sub>S production

A direct pathway for production of H<sub>2</sub>S from L-cysteine is by hydrolysis to serine and H<sub>2</sub>S via the enzyme CBS. When the CBS inhibitor AOAA (100 µmol l<sup>-1</sup>) was added to homogenates of *T. philippinarum* tissue containing added L-cysteine and PLP, H<sub>2</sub>S production was completely inhibited. The addition of the CSE inhibitor PGly, on the other hand, reduced H<sub>2</sub>S production in the same tissues by only 35%. CSE can generate H<sub>2</sub>S through two pathways: an elimination reaction of L-cysteine that produces pyruvate, NH<sub>3</sub> and H<sub>2</sub>S, and a desulfhydration reaction of cystine that produces thiocysteine, pyruvate and NH<sub>3</sub>. That AOAA produced complete inhibition of H<sub>2</sub>S production in *T. philippinarum* tissues and PGly produced only partial inhibition, suggests that the majority of H<sub>2</sub>S production is via CBS pathways. This is similar to previous data from rat brain homogenates, in which CBS inhibitors suppressed H<sub>2</sub>S production by 100%, while CSE inhibitors suppressed H<sub>2</sub>S production by only 15% (Abe and Kimura, 1996). Interestingly, in *A. marina* body wall, AOAA inhibited only approximately half of the total H<sub>2</sub>S production, suggesting that the CBS pathway is not the major source of H<sub>2</sub>S production in this tissue.

That AOAA produced complete inhibition of H<sub>2</sub>S production under standard conditions in *T. philippinarum*, but not *A. marina* suggests that specific differences exist in L-cysteine metabolism between these two animals. Because the addition of α-ketoglutarate to *A. marina* body wall homogenates did not cause an effect on H<sub>2</sub>S production, the primary pathway for H<sub>2</sub>S production in this

tissue is probably not the CAT pathway. Furthermore, the absence of an effect upon the addition of GSH indicates that it is not the thiosulfate pathway. In guinea pig, Hosoki et al. (1997) demonstrated that CBS is expressed only in the ileum, while CSE is expressed in the ileum, portal vein and thoracic aorta. As would be expected, the CSE inhibitors PGly and β-cyano-L-alanine strongly suppressed H<sub>2</sub>S production in the portal vein and thoracic aorta, while the CBS inhibitor AOAA suppressed H<sub>2</sub>S production in the ileum. We did not directly determine whether CBS and CSE were differentially expressed between different tissues in either *T. philippinarum* or *A. marina*.

#### 4.3. Evidence for activated L-serine sulfhydrase pathway

The addition of 2.5 mmol l<sup>-1</sup> 2-mercaptoethanol to *T. philippinarum* tissue homogenates under standard conditions increased H<sub>2</sub>S production by 80%. This enhancement was completely inhibited by 0.5 and 5.0 mmol l<sup>-1</sup> PGly and 10 µmol l<sup>-1</sup> AOAA, although some activity was seen with 100 µmol l<sup>-1</sup> AOAA. Enhancement by 2-mercaptoethanol did not occur in homogenates of *A. marina* body wall. Based on the pathways shown in Fig. 1, additional thiol substrate could enhance H<sub>2</sub>S production via two pathways: (1) by reacting with thiocysteine (which, in our system, would be derived from L-cysteine via L-cystine); and (2) by reacting with L-cysteine to form cysteine thioether (the 'activated serine sulfhydrase' pathway). Cavallini et al. (1962) demonstrated that H<sub>2</sub>S production by purified rat liver CSE (with L-cysteine as the substrate) was inhibited by 90% upon the addition of 1 mmol l<sup>-1</sup> 2-mercaptoethanol. This was attributed to 2-mercaptoethanol maintaining L-cysteine in a reduced state. Therefore, it is unlikely that the increase in H<sub>2</sub>S production seen after addition of 2-mercaptoethanol in *T. philippinarum* homogenates was from a reaction with thiocysteine, since this would have first required the formation of L-cystine from L-cysteine. Furthermore, 2-mercaptoethanol in the absence of added L-cysteine was not sufficient to cause significant H<sub>2</sub>S production, and H<sub>2</sub>S production in the presence of 2-mercaptoethanol and L-cysteine was enhanced by approximately two-fold by the addition of PLP. These findings indicate that H<sub>2</sub>S production was enzymatic, dependent on L-cysteine as a substrate, and did not proceed via the

formation of L-cystine. Based on known pathways, this suggests that 2-mercaptoethanol enhanced H<sub>2</sub>S production by increasing the formation of cysteine thioether in the 'activated serine sulfhydrase pathway'.

In the nematode *Nippostrongylus brasiliensis*, the native molecular weight of the activated L-serine sulfhydrase enzyme is approximately 70 kDa, whereas the molecular weight of that animal's CBS is 288 kDa (Walker et al., 1992), which is similar to that of CBS purified from fresh rat liver (290 kDa). It is not known whether the activated serine sulfhydrase enzyme is similar to CBS in its sensitivity to AOAA or in being dependent on PLP, although the latter is likely. If an activated L-serine sulfhydrase pathway is present in *T. philippinarum*, its activity in vivo would require suitable second thiol substrates, which we provided in vitro with the addition of 2-mercaptoethanol (similar results were seen upon addition of dithiothreitol; data not shown). However, the nature of these thiols in vivo, and whether they exist in sufficient concentrations in *T. philippinarum* tissues to contribute significantly to H<sub>2</sub>S production, is unknown. The apparent absence of an activated serine sulfhydrase activity in *A. marina* body wall suggests that specific differences in putative H<sub>2</sub>S production pathways, exist between these two animals.

#### 4.4. Potential roles of endogenous H<sub>2</sub>S

Abe and Kimura (1996) first proposed that H<sub>2</sub>S is an intracellular messenger in mammals. Chemically, H<sub>2</sub>S has many properties in common with nitric oxide (NO), which is a well-known intracellular messenger that is conserved across the major systematic groups (Moroz, 2001, for review). For example, both can be synthesized enzymatically from L-amino acids (i.e. L-arginine for NO and L-cysteine for H<sub>2</sub>S), both are small, gaseous molecules that are permeable through cell membranes, and both can be rapidly oxidized in vivo (especially by heme groups). Furthermore, NO, which is highly reactive, exerts its effects through direct covalent modification of target molecules. The same could be true of H<sub>2</sub>S and its dissociation products, since they are also highly reactive.

Searcy and Lee (1998) demonstrated that human erythrocytes can reduce S<sub>8</sub> to H<sub>2</sub>S using reducing equivalents from glucose oxidation, and they pro-

posed three potential roles for H<sub>2</sub>S that did not include a function as an intracellular messenger: methemoglobin salvaging, facilitation of NADH and NADPH oxidation, and shuttling of electrons, both within and between cells. This last role is especially interesting, since H<sub>2</sub>S oxidation can be coupled to ATP synthesis in *A. marina* (Völkel and Grieshaber, 1996) and at least three other H<sub>2</sub>S-adapted animals: the symbiont-containing clam *Solemya reidi* (Powell and Somero, 1986), the estuarine California killifish *Fundulus parvipinnis* (Bagarinao and Vetter, 1990), and the estuarine mussel *Geukensia demissa* (Doeller et al., 1999; Parrino et al., 2000). Because the homogenates in our study had no added substrates for glycolysis, it is unlikely that H<sub>2</sub>S production was via the glycolysis-dependent pathways described by Searcy and Lee (1998). Nonetheless, the same potential roles for H<sub>2</sub>S would still exist, in which case oxidation of L-cysteine-derived H<sub>2</sub>S could be coupled to ATP production by the mitochondria of some animals.

We cannot make broad conclusions about differences in H<sub>2</sub>S production pathways in marine invertebrates based on a study of two animals from different phyla. A variety of physiological adaptations have been elucidated in *A. marina* that allow it to tolerate frequent exposure to H<sub>2</sub>S in its natural environment (Grieshaber and Völkel, 1998 for review). In contrast to *A. marina*, *T. philippinarum* is typically found on sand-gravel beaches where the H<sub>2</sub>S concentration is presumably low by comparison, so it would not be expected to have extensive mechanisms for tolerating H<sub>2</sub>S exposure (although this has never been tested). We do not know whether this difference in environmental H<sub>2</sub>S exposure is related to the observed differences in H<sub>2</sub>S production pathways. Nonetheless, any physiological role for endogenous H<sub>2</sub>S production would certainly be impacted by the presence of environmental H<sub>2</sub>S.

In previous studies, detection of H<sub>2</sub>S was reported in the tissues of *A. marina* (Hauschild and Grieshaber, 1997; Wohlgemuth et al., 2000) and three other H<sub>2</sub>S-adapted marine invertebrates: the isopod *Saduria entomon* (Vismann, 1991), the priapulid worm *Halicryptus spinulosus* (Oeschger and Vetter, 1992) and the marsh clam *Geukensia demissa* (Doeller et al., 2001), as well as the blue mussel *Mytilus edulis* (Doeller et al., 2001). In those studies, the authors were studying the accumulation or oxidation of H<sub>2</sub>S in tissues of animals

experimentally exposed to H<sub>2</sub>S, and it was the 'control' tissues from unexposed animals that unexpectedly showed the presence of H<sub>2</sub>S. Concentrations of H<sub>2</sub>S from tissues of animals kept in H<sub>2</sub>S-free conditions were reported only for *A. marina*, with mean values of 24.2 and 27.2 μmol l<sup>-1</sup> (Hauschild and Grieshaber, 1997; Wohlgemuth et al. 2000, respectively), and for *M. edulis*, with a mean value of approximately 18 μmol l<sup>-1</sup> (Doeller et al., 2001, and J.E. Doeller, personal communication). It was proposed in those studies that the appearance of H<sub>2</sub>S was an experimental artifact caused by protein mercapto groups in the tissues reacting with the bromobimane reagent used for the assay. Our assay method, in contrast, measured only volatilized H<sub>2</sub>S, so such interference from proteins would have been unlikely. We suggest, therefore, that at least some of the H<sub>2</sub>S detected in the previous studies was not an experimental artifact, but was free H<sub>2</sub>S produced enzymatically.

## 5. Conclusion

In this study, we have shown that tissue homogenates from the Manila clam, *T. philippinarum*, produce H<sub>2</sub>S even in the absence of added substrate or co-factor, and that all *T. philippinarum* tissue homogenates and body wall tissue homogenates from the lugworm, *A. marina*, produce significant quantities of H<sub>2</sub>S gas upon the addition of L-cysteine and PLP. In *T. philippinarum* tissues, H<sub>2</sub>S production was completely inhibited by AOAA, suggesting that the majority of H<sub>2</sub>S production occurs via CBS pathways. In *A. marina* body wall, AOAA inhibited only approximately half of the total H<sub>2</sub>S production, suggesting that the CBS pathway is not the only major source of H<sub>2</sub>S production in this tissue. H<sub>2</sub>S production in tissue homogenates of *T. philippinarum*, but not *A. marina*, was enhanced by the addition of a second thiol substrate, suggesting the presence of an 'activated serine sulfhydrylase pathway', which had previously been demonstrated only in some microfauna (Walker and Barrett, 1997). Whether H<sub>2</sub>S production in the tissues of invertebrates has a physiological function remains to be demonstrated, but the previously suggested roles of intracellular messenger (e.g. Abe and Kimura, 1996) and electron acceptor (e.g. Powell and Somero, 1986; Searcy and Lee, 1998) are particularly worthy of further investigation.

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## References

- Abe, K., Kimura, H., 1996. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.* 16, 1066–1071.
- Bagarinao, T., Vetter, R.D., 1990. Oxidative detoxification of sulfide by mitochondria of the California killifish *Fundulus parvipinnis* and the speckled sanddab *Citharichthys stigmæus*. *J. Comp. Physiol. B* 160, 519–527.
- Braunstein, A.E., Goryachenkova, E.V., Tolosa, E.A., Willhardt, I.H., Yefremova, L.L., 1971. Specificity and some other properties of liver serine sulphhydrylase: evidence for its identity with cystathionine β-synthase. *Biochim. Biophys. Acta.* 242, 247–260.
- Cavallini, D., Modovi, B., De Marco, C., Scioscia-Santoro, A., 1962. Inhibitory effect of mercaptoethanol and hypotaurine on the desulfuration of cysteine by cystothionase. *Arch. Biochem. Biophys.* 96, 456–457.
- Dello Russo, C., Tringali, G., Ragazzoni, E., Maggiano, N., Menini, E., Vairano, M., Preziosi, P., Navarra, P., 2000. Evidence that hydrogen sulphide can modulate hypothalamo-pituitary-adrenal axis function: in vitro and in vivo studies in the rat. *J. Neuroendocrinol.* 12, 225–233.
- Doeller, J.E., Gaschen, B.K., Parrino, V., Kraus, D.W., 1999. Chemolithoheterotrophy in a metazoan tissue: sulfide supports cellular work in ciliated mussel gills. *J. Exp. Biol.* 202, 1953–1961.
- Doeller, J.E., Grieshaber, M.K., Kraus, D.W., 2001. Chemolithoheterotrophy in a metazoan tissue: thiosulfate production matches ATP demand in ciliated mussel gills. *J. Exp. Biol.* 204, 3755–3764.
- Gomez-Bautista, M., Barrett, J., 1988. Cysteine metabolism in the cestode *Hymenolepis diminuta*. *Parasitology* 97, 149–159.
- Goodwin, L.R., Francom, D., Dicken, F.P., et al., 1989. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. *J. Anal. Toxicol.* 13, 105–109.
- Grieshaber, M.K., Völkel, S., 1998. Animal adaptations for tolerance and exploitation of poisonous sulfide. *Ann. Rev. Physiol.* 60, 33–53.
- Griffith, O.W., 1987. Mammalian sulfur amino acid metabolism: an overview. *Meth. Enzymol.* 143, 366–376.
- Hauschild, K., Grieshaber, M.K., 1997. Oxygen consumption and sulfide detoxification in the lugworm *Arenicola marina* (L.) at different ambient oxygen partial pressures and sulfide concentrations. *J. Comp. Physiol. B.* 167, 378–388.

- Hosoki, R., Matsuki, N., Kimura, H., 1997. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys. Res. Comm.* 237, 527–531.
- Kimura, H., 2000. Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem. Biophys. Res. Comm.* 267, 129–133.
- Moroz, L.L., 2001. Gaseous transmission across time and species. *Am. Zool.* 41, 304–320.
- National Research Council, Subcommittee on Hydrogen Sulfide, Division of Medical Sciences, 1979. *Hydrogen Sulfide*. Baltimore MD: University Park Press.
- Nicholls, P., 1975. The effect of sulfide on cytochrome-aa3 isosteric and allosteric shifts of the reduced  $\alpha$ -peak. *Biochim. Biophys. Acta* 396, 24–35.
- Oeschger, R., Vetter, R.D., 1992. Sulfide detoxification and tolerance in *Halicryptus spinulosus* (Priapulida): a multiple strategy. *Mar. Ecol. Prog. Ser.* 86, 167–179.
- Parrino, V., Kraus, D.W., Doeller, J.E., 2000. ATP production from the oxidation of sulfide in gill mitochondria of the ribbed mussel *Geukensia demissa*. *J. Exp. Biol.* 203, 2209–2218.
- Powell, M., Somero, G.N., 1986. Hydrogen sulfide oxidation is coupled to oxidative phosphorylation in mitochondria of *Solemya reidi*. *Science* 233, 563–566.
- Roy, A.B., Trudinger, P.A., 1970. *The Biochemistry of Inorganic Compounds of Sulphur*. Cambridge Univ. Press, pp. 399.
- Searcy, D.G., Lee, S.H., 1998. Sulfur reduction by human erythrocytes. *J. Exp. Zool.* 282, 310–322.
- Siegel, L.M., 1965. A direct microdetermination for sulfide. *Anal. Biochem.* 11, 126–132.
- Stipanuk, M.H., Beck, P.W., 1982. Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem. J.* 206, 267–277.
- Thong, K.-W., Coombs, G.H., 1985a. Homocysteine desulphurase activity in trichomonads. *IRCS Med. Sci.* 13, 493–494.
- Thong, K.-W., Coombs, G.H., 1985b. L-Serine sulphhydrase activity in trichomonads. *IRCS Med. Sci.* 13, 495–496.
- Vismann, B., 1991. Physiology of sulfide detoxification in the isopod *Saduria* (Mesidotea) *entomon*. *Mar. Ecol. Prog. Ser.* 76, 283–293.
- Völkel, S., Grieshaber, M.K., 1996. Mitochondrial sulfide oxidation in *Arenicola marina*: Evidence for alternative electron pathways. *Eur. J. Biochem.* 235, 231–237.
- Walker, J., Barrett, J., 1992. Biochemical characterisation of the enzyme responsible for 'activated L-serine sulphhydrase' activity in nematodes. *Exp. Parasitol.* 74, 205–215.
- Walker, J., Barrett, J., 1997. Parasite sulphur amino acid metabolism. *Int. J. Parasitol.* 27, 883–897.
- Walker, J., Barrett, J., Thong, K.-W., 1992. The identification of a variant form of cystathionine  $\beta$ -synthase in nematodes. *Exp. Parasitol.* 75, 415–424.
- Warencya, M.W., Goodwin, L.R., Benishin, C.G., et al., 1989. Acute hydrogen sulfide poisoning: demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels. *Biochem. Pharmacol.* 38, 973–981.
- Wohlgemuth, S.E., Taylor, A.C., Grieshaber, M.K., 2000. Ventilatory and metabolic responses to hypoxia in the lugworm *Arenicola marina* (L.). *J. Exp. Biol.* 203, 3177–3188.