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Regulation of glycolytic enzymes in the marine invertebrate *Halicryptus spinulosus* (Priapulida) during environmental anoxia and exposure to hydrogen sulfide

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Abstract. A particularly strong reduction of metabolic activity is a precondition for long-term survival of *Halicryptus spinulosus* von Siebold under anoxic habitat conditions because of its relatively low fuel reserves (mainly glycogen). The present study analyses the mechanism of this metabolic slow-down. For this purpose the effects of environmental anoxia and exposure to hydrogen sulfide on the activity and selected kinetic properties of glycolytic enzymes [glycogen phosphorylase (GP), pyruvate kinase (PK)] and the concentrations of fructose-2,6-bisphosphate in the body wall of *H. spinulosus* were analysed. Anoxia and hydrogen sulfide exposure stimulated modifications of the properties of the enzymes, in both cases due to probable covalent modification of the enzyme proteins. Under both conditions phosphorylase activity was depressed by about ⅓, the result of changes in the percentage of enzyme in the active *a*-form as well as the total amount of enzyme activity expressed ($a+b$). Effects of anoxia on the properties of pyruvate kinase included reduced V_{max}, decreased $S_{0.5}$ for phosphoenolpyruvate, changes in K_a for fructose-1,6-bisphosphate (an initial decrease was followed by a later increase). The I_{50} for *L*-alanine of PK was extremely reduced under anoxia and showed an even greater sensitivity to the presence of hydrogen sulfide. Anoxia stimulated a slight reduction in the content of fructose-2,6-bisphosphate, whereas exposure to hydrogen sulfide caused a dramatic decrease of this allosteric activator of phosphofructokinase. The study gives evidence that mechanisms of glycolytic rate depression are conserved within a wide variety of vertebrate and invertebrate phyla. With two exceptions (fructose-2,6-bisphosphate levels and alanine inhibition of PK) the responses to hydrogen sulfide were the same as those to anoxia, suggesting that at a metabolic level, the consequences of each stress on energy metabolism are similar.

Introduction

The priapulid worm *Halicryptus spinulosus* has an outstanding ability to survive long periods of environmental anoxia (Oeschger and Theede 1986). Many marine invertebrates from intertidal areas are often exposed to oxygen deficiency and are able to cope with it for short periods between the tides. *H. spinulosus* however, a sublittoral species found in Kiel Bight, must often endure prolonged oxygen deficiency in its natural habitat (Oeschger and Theede 1988), and belongs to those marine invertebrates exhibiting the highest survival times under anoxia (Theede et al. 1969, Theede 1984). Oxygen deficiency in *H. spinulosus* is caused not only by the hydrographic conditions in its habitat, but also by the worms behaviour as they burrow deeply into the anoxic sediment. During long-term anaerobiosis worms rely mainly on fermentative pathways of anaerobic ATP production, as can be seen from the accumulation of anaerobic end products such as succinate, acetate, and propionate (Oeschger 1990).

The sediments of the deeper parts of Kiel Bight contain hydrogen sulfide, as do the lower parts of the water column after severe periods of oxygen deficiency. Minute concentrations of hydrogen sulfide are extraordinarily toxic to most organisms, because this substance interferes with various enzymes e.g. cytochrome *c* oxidase – an important mitochondrial enzyme of the electron transport chain. Also, cleavages of disulfide bonds are a likely consequence of sulfide inhibition (National Research Council 1979). *Halicryptus spinulosus* turns completely black when exposed to hydrogen sulfide (Oeschger and Schmaljohann 1988), a reaction which might be involved in a sulfide detoxification process.

Anoxia-induced metabolic rate depression has been clearly demonstrated in a number of species, most studies being on marine molluscs (de Zwaan and Wijsman 1976, Famme et al. 1981, Shick et al. 1983, Shumway et al. 1983, Plaxton and Storey 1985, Storey 1985b, 1988a, Stickle et al. 1989, Oeschger 1990). One of the most frequently documented effects of anoxia on metabolism is

the stable modification of regulatory enzymes of glycolysis to create less active enzyme forms in anoxia. Covalent modification by protein phosphorylation or dephosphorylation reactions affects three key regulatory enzymes of glycolysis: glycogen phosphorylase, phosphofructokinase (PFK) and pyruvate kinase (PK) to coordinate glycolytic rate depression (Siebenaller 1979, Holwerda et al. 1983, Storey 1984, 1988a, Plaxton and Storey 1985, Michaelidis et al. 1988, Storey and Storey 1990). Do similar mechanisms of glycolytic rate control also occur for *Halicryptus spinulosus*? The present study analyses the effects of anoxic stress on the activities and properties of glycogen phosphorylase and PK from body wall of *H. spinulosus*. In addition, the effects of long-term anoxia on the muscle concentrations of fructose-2,6-bisphosphate, a powerful activator of PFK (Hers and Van Schaftingen 1982), were determined. Equivalent analyses were also carried out on animals given long-term exposure to hydrogen sulfide.

To our knowledge this is the first investigation on the influence of hydrogen sulfide on glycolytic enzymes in a marine invertebrate.

Materials and methods

Worms

Specimens of *Halicryptus spinulosus* von Siebold were dredged with a naturalist's triangular dredge in Kiel Bight (Western Baltic Sea) in August 1989. They were kept unfed in aerated seawater at 9.5 °C ± 0.5° and 22‰ S in original sediment from their habitat. The same conditions were applied during experimental anoxic and hydrogen sulfide exposure in August/September 1989. Anoxia was achieved by bubbling pure N_2 through 1 to 2 l of seawater for at least 2 to 4 h prior to incubation. For hydrogen sulfide experiments, crystals of $Na_2S \times 7$ to 9 H_2O were added to the incubation flasks to a final concentration of 0.2 m*M* H_2S. Hydrogen sulfide may occur in different ionic forms, depending on the pH of the medium, and this study refers to "hydrogen sulfide" as the sum of H_2S, HS^- and S^{2-}. At physiological pH (around 7.4) H_2S and HS^- are the prevailing species. Prior to the incubation of worms in flasks, the pH of the seawater was adjusted to about 7.5 with 1 *M*, HCl, and small amounts of original sediment were added.

After timed intervals of exposure to anoxia or hydrogen sulfide worms were quickly tested for life signs by mechanically stimulating the body wall. Tissues were then quickly dissected out, blotted, immediately frozen in liquid nitrogen and stored at −75 °C. Biochemicals and coupling enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. and Boehringer Mannheim, Montreal.

Enzyme preparations

For the estimation of glycogen phosphorylase activity, frozen tissue samples (approximately 100 mg) were homogenized (1:4 W/V) in ice-cold imidazole-HCl buffer (50 m*M*, pH 7.0) containing 100 m*M* NaF, 5 m*M* EDTA, 5 m*M* EGTA, 100 m*M* NaCl, 2 m*M* DTT (dithiothriethol) and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) with an Ultra Turrax tissue homogenizer. Particulate matter in the homogenate was allowed to settle without centrifugation for at least 30 min (on ice), and then enzyme activity was measured in the supernatant. Homogenates were not centrifuged because phosphorylase activity can be lost when enzyme-glycogen complexes are sedimented.

For studies of pyruvate kinase, tissue samples were extracted in imidazole HCl-buffer (50 m*M*, pH 7.0) containing 100 m*M* NaF, 5 m*M* EDTA, 5 m*M* EGTA, 30 m*M* 2-mercaptoethanol and 0.1 m*M* PMSF. Homogenates were centrifuged at 27 000 × *g* at 4 °C for 20 min. The supernatants were desalted by centrifugation through a Sephadex G-25 column (1 ml gel per 0.1 ml homogenate), previously equilibrated in homogenization buffer, to remove low molecular weight compounds.

Enzyme assays

Glycogen phosphorylase

Assay conditions for glycogen phosphorylase *a* were 50 m*M* potassium phosphate buffer (pH 7.0), 2 mg/ml^{-1} glycogen (previously dialyzed), 0.4 m*M* NADP, 10 μ*M* glucose-1,6-P_2, 0.25 m*M* EDTA, 15 m*M* $MgCl_2$, with excess dialysed phosphoglucomutase and glucose-6-P dehydrogenase. For measurements of total phosphorylase, 1.6 m*M* AMP proved to be optimal for activating phosphorylase *b* and was added to the assay mixture.

Pyruvate kinase

Conditions for maximal enzyme activity were 50 m*M* imidazole-HCl buffer, pH 7.0, 5 m*M* (PEP), 5 m*M* ADP, 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.2 m*M* NADH and 1 unit (U) lactate dehydrogenase per ml assay buffer.

One unit of enzyme activity is defined either as the amount producing 1 μmol glucose-1-P (for phosphorylase), or utilizing 1 μmol PEP (for PK) min^{-1} at 23 °C. Kinetic constants were determined as follows: $S_{0.5}$ values for PEP and ADP were determined from Hill plots using experimentally determined V_{max} values: I_{50} values for *L*-alanine were estimated after Job et al. (1978) from plots of *v* versus [inhibitor] at subsaturating PEP concentration (2 m*M*), values for K_a were determined from plots of *v* versus [activator] at subsaturating PEP concentration (0.2 m*M*). All assays were performed at 23 °C using a Gilson recording UV-spectrophotometer.

Fructose-2,6-bisphosphate (F-2,6-P_2) determination

Frozen tissue samples (50 to 100 mg) were homogenized 1:10 W/V in hot 50 m*M* NaOH (80 °C) followed by incubation for 10 min at 80 °C. Homogenates were centrifugated at 12 000 × *g* and the supernatant used for determination of F-2,6-P_2 concentrations. Samples were assayed for F-2, 6-P_2 content by the method of Van Schaftingen (1984), which relies on the ability of F-2,6-P_2 to activate potato tuber pyrophosphate-linked phosphofructokinase (PPi-PFK). F-2,6-P_2 in sample aliquots (50 to 100 μl) was determined by comparison with a standard curve of F-2,6-P_2 activation of PPi-PFK.

Results

Glycogen phosphorylase

The influence of anoxia and exposure to hydrogen sulfide on properties of glycogen phosphorylase from the body wall muscle of *Halicryptus spinulosus* is shown in Fig. 1.

During long-term anaerobiosis the total activity of glycogen phosphorylase (*a* + *b*) dropped significantly, compared to control worms. There was no significant difference in enzyme activity between specimens incubated under anoxia and in hydrogen sulfide after 5 and 10 d respectively. In addition, the percentage of phosphory-

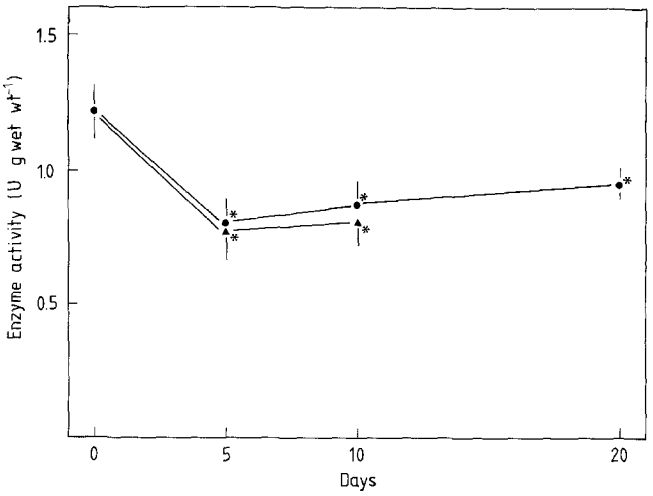

Fig. 1. *Halicryptus spinulosus*. Effect of anoxia (●) and exposure to hydrogen sulfide (▲) on glycogen phosphorylase from the body wall muscle of worms. Values are means ± SEM; $n=4$ to 5 determinations on tissues from separate specimens. Activity of total phosphorylase is in μmol glucose-1-P produced min^{-1} g^{-1} wet wt determined in the presence of 1.6 m*M* AMP. * Significantly different from corresponding aerobic value by the Student's *t*-test, $p<0.05$

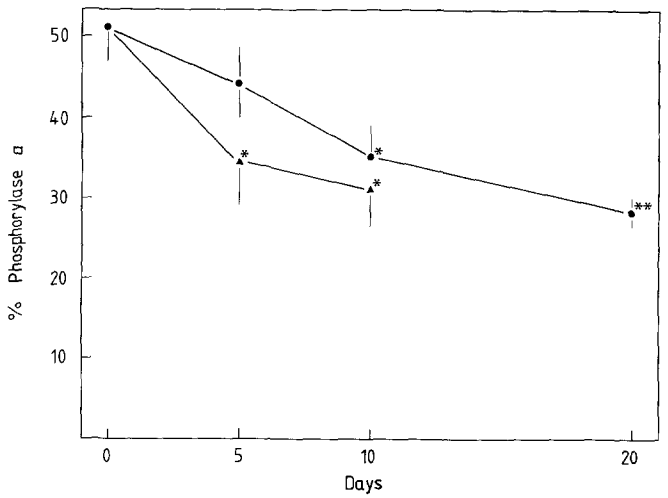

Fig. 2. *Halicryptus spinulosus*. Effect of anoxia and exposure to hydrogen sulfide on the percentage of phosphorylase in the active *a*-form in the body wall muscle of worms. * Significantly different from corresponding aerobic value by the Student's *t*-test, $p<0.05$; ** $p<0.005$. For further details see Fig. 1

Table 1. *Halicryptus spinulosus*. Kinetic properties of pyruvate kinase from body wall muscle of worms. Aerobic values versus different periods of long-term anaerobiosis and exposure to hydrogen sulfide are shown. Values are means ± SEM; $n=3$ to 6 determinations on separate specimens. Effector constants were determined at subsaturating levels of PEP near $S_{0.5}$, for K_a (fructose-1,6-P_2) concentrations were 0.2 m*M* PEP. Values in parentheses refer to the fold activation by optimal levels of fructose-1,6-P_2. Asterisks refer to values significantly different from corresponding control value by Student's *t*-test at $p<0.05$ (*) and $p<0.005$ (**). NE: no effect of alanine in amounts of up to 50 m*M* on PK from aerobic muscle

Parameter	Aerobic exposure	Anaerobic exposure			Hydrogen sulfide exposure	
		5 d	10 d	20 d	5 d	10 d
V_{max} (U g^{-1} wet wt)	3.08 ± 0.15	1.27 ± 0.16*	1.61 ± 0.27*	0.84 ± 0.19**	1.28 ± 0.13**	1.47 ± 0.15*
$S_{0.5}$ PEP (m*M*)	0.47 ± 0.03	0.26 ± 0.03*	0.20 ± 0.04*	0.18 ± 0.06*	0.24 ± 0.03**	0.10 ± 0.01**
$S_{0.5}$ ADP (m*M*)	0.26 ± 0.02	0.31 ± 0.02	0.20 ± 0.04	0.28 ± 0.05	0.30 ± 0.03	0.34 ± 0.04
K_a fructose-1,6-P_2 (m*M*)	1.56 ± 0.35 (2.7)	2.59 ± 0.38 (2.9)	1.47 ± 0.17 (2.2)*	1.38 ± 0.21 (2.2)*	2.09 ± 0.40 (3.1)*	1.01 ± 0.12 (2.3)*
I_{50} *L*-alanine (m*M*)	NE	0.29 ± 0.06	0.19 ± 0.03	0.23 ± 0.02	0.20 ± 0.02	0.14 ± 0.01

lase in the active *a*-form also declined over the course of anoxia or hydrogen sulfide exposure (Fig. 2). After 20 d of anoxia the percent *a* had decreased to nearly half of the aerobic value. There were no significant differences between anoxia and hydrogen sulfide exposure with respect to their effects on percent *a*.

A concentration of 5 m*M* caffeine, as well as concentrations of 25 m*M* propionate, succinate, acetate, or alanine, added to assays had no effect on the measured phosphorylase activities.

Pyruvate kinase

Selected properties of PK from body wall muscle of aerobic, anoxia and H_2S-exposed *Halicryptus spinulosus*, are shown in Table 1. V_{max} of PK decreased significantly by more than two thirds after 20 d of anoxia, and by about 50% during 10 d exposure to hydrogen sulfide.

As a result of anoxia and H_2S exposure, $S_{0.5}$ of PEP decreased more than two and four-fold, respectively, whereas the $S_{0.5}$ for ADP was unaffected. Changes in $S_{0.5}$ PEP were largely complete after 5 d of experimental exposure, but a continuing decline was noted with longer exposure times to anoxia or hydrogen sulfide.

Neither experimental condition resulted in any clear change in the K_a for fructose-1,6-P_2 and aspartate, an activator of PK in some marine molluscs, produced no effect at all at concentrations of up to 50 m*M*.

L-Alanine had no inhibitory effect on PK from aerobic worms at levels as high as 50 m*M*. However, under anoxia or exposure to hydrogen sulfide, the enzyme showed very strong inhibition by alanine with I_{50} values of 0.14 to 0.29 m*M*. Indeed, when the anoxic form of PK

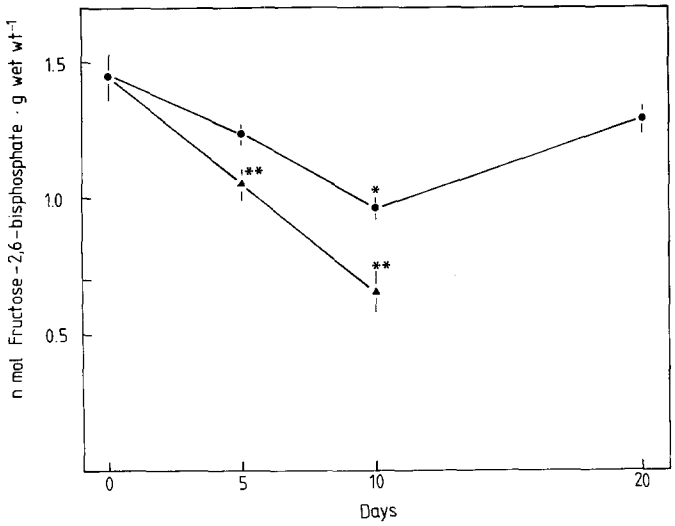

Fig. 3. *Halicryptus spinulosus*. Effect of anoxia (●) and exposure to hydrogen sulfide (▲) on fructose-2,6-P_2 content of the body wall muscle. Values (in nmol g^{-1} wet wt) are means ± SEM, $n = 5$ to 8 determinations on tissues from separate specimens. * Significantly different from corresponding aerobic values by the Student's *t*-test, $p < 0.005$; ** significantly different from aerobic values ($p < 0.005$). Hydrogen sulfide-exposed worms are also significantly different from corresponding anoxic ones

was assayed in the presence of 1 to 5 m*M* alanine concentrations (equivalent to in vivo levels of alanine in *Halicryptus spinulosus*), enzyme activity was reduced to virtually zero. Other possible inhibitors, each tested at concentrations of up to 50 m*M*, had no effect on the enzyme; these included ATP and the metabolites produced by the animals during anaerobiosis, succinate, acetate, and propionate.

Fructose-2,6-bisphosphate

Concentrations of the PFK activator, F-2,6-P_2, decreased in body wall over the course of anoxia (Fig. 3). During incubation in hydrogen sulfide, the effect was more pronounced, levels of F-2,6-P_2 dropping to about one-half the levels in aerobic controls (Fig. 3).

Discussion

Effects of anoxia

Anaerobiosis of intertidal marine invertebrates has been studied extensively, but there is only very limited information on metabolic adaptations during long-term anaerobiosis. Environmental anoxia caused by hydrographic conditions may last for several weeks in the deeper parts of Kiel Bight. *Halicryptus spinulosus*, is able to withstand anoxia for prolonged periods, thus constitutes an excellent experimental animal for studies of the effects of long-term anoxia. Glycogen is the main fuel during anaerobiosis in *H. spinulosus* and its anaerobic metabolism has been described in a previous study (Oeschger 1990).

In order to cope with long-term environmental anoxia, marine invertebrate facultative anaerobes depress their metabolic rate frequently to levels only 5 to 10% of the corresponding aerobic rate at the same temperature (de Zwaan and Wijsman 1976, Famme et al. 1981, Stickle et al. 1989). Without this adaptation anoxia could be lethal within a relatively short time, so adverse environmental oxygen conditions require a quick and direct response by the worms affected. A very effective method of accomplishing metabolic arrest appears to be the modification of key regulatory enzymes to reduce their activity state during anoxia (Storey 1985b, 1988a).

Studies on the gastropod mollusc *Busycotypus canaliculatum* have revealed three key mechanisms for coordinated control of glycolytic rate depression: covalent modification of enzymes via phosphorylation or dephosphorylation, fructose-2,6-bisphosphate regulation of phosphofructokinase (the rate limiting enzyme in glycolysis), and the reversible association of enzymes with particle-bound multi-enzyme complexes (Plaxton and Storey 1984, 1986, Storey 1985a, b, 1988b). The present study indicates that stable posttranslational enzyme modifications also occur in *Halicryptus spinulosus* in response to anoxia. During anoxia in *H. spinulosus* both the total activity of GP and the percentage of the enzyme in the active *a*-form decreased. The reduced rate of glycogenolysis that this implies is consistent with a profound metabolic rate depression; thus, glycogen use is reduced despite the dependence in anoxia on carbohydrate fermentation as the main means of ATP production.

There was also strong evidence for anoxia-induced modification of PK in *Halicryptus spinulosus*. The enzyme showed significant changes in its properties with anoxia exposure: diminished V_{max}, decreased $S_{0.5}$ for PEP and a strongly reduced I_{50} for alanine. Anoxia-induced phosphorylation of PK in molluscs typically increases $S_{0.5}$ for PEP (i.e. affinity for substrate decreases); this has been seen in *Mytilus edulis* (Holwerda et al. 1983), in *Busycotypus canaliculatum* (Plaxton and Storey 1984), and *Arctica islandica* (Oeschger and Storey unpublished). However, a similar small decrease in $S_{0.5}$ PEP, as noted here, has also been reported for muscular body wall tissue of the sea anemone *Metridium senile* (Michaelidis and Storey 1990). Much more important to PK control in anoxia, however, is the dramatic change in I_{50} alanine of the enzyme. The aerobic enzyme was unaffected by this amino acid but the anoxic enzyme form was strongly inhibited with I_{50} values (0.14 to 0.29 m*M*), far below the normal range of alanine concentrations in vivo (up to 5 m*M*). In addition, alanine accumulates as an anaerobic end product early in anoxia in this species, which would further compound the inhibitory influence of alanine on PK. Indeed, the activity of the anoxic enzyme form was virtually undetectable in the presence of physiological levels (1 to 5 m*M*) of alanine. Thus, the anoxia-induced modification of PK, by probable phosphorylation of the enzyme protein, creates as its primary result an enormously increased sensitivity to alanine inhibition and this would dramatically reduce PK activity at physiological alanine levels. Furthermore with K_a values of 1 to 2 m*M*

for fructose-1,6-bisphosphate, *H. spinulosus* PK is only weakly sensitive to this activator and at levels of fructose-1,6-bisphosphate that are typically physiological, there may be little or no reversal of alanine inhibition. Thus, PK can be rapidly and effectively shut down in the anoxic animal in order to direct glycolytic flux into the synthesis of organic acid end products.

Effect of hydrogen sulfide

Halicryptus spinulosus is regularly found burrowed deeply into anoxic sediment. Unlike other marine invertebrates, it also remains active under anoxia (Oeschger 1990), even when confronted with hydrogen sulfide. The ability of this species to survive these conditions raises questions about the impact of hydrogen sulfide on enzyme function. This particularly concerns the glycolytic enzymes, which are important for maintaining energy supply under aerobic, as well as anaerobic conditions.

The impact of H_2S exposure on *Halicryptus spinulosus* was very similar to the effects of anoxia on the worms. The total activity of glycogen phosphorylase, as well as the percentage of the active *a*-form decreased under H_2S incubation, virtually paralleling the changes in anoxia. This guarantees a similar basal energy flux when the worms are exposed to anoxia or to hydrogen sulfide. Both anoxia and H_2S exposure also reduced fructose-2,6-P_2 levels in *H. spinulosus* muscle but the effect was more pronounced in the H_2S-exposed specimens. This might indicate a particular influence of hydrogen sulfide on the enzyme of fructose-2,6-bisphosphate and, as a result may contribute to a further reduction of PFK activity and glycolytic activity overall under H_2S exposure. Hydrogen sulfide exposure, had similar, but not identical effects on PK activity and kinetic properties as were seen in anoxia. In both cases, V_{max} was reduced to a similar extent and I_{50} for alanine was similar. There were no clear cut effects of either stress on $S_{0.5}$ ADP or K_a fructose-1,6-bisphosphate. However, H_2S exposure resulted in a greater reduction of $S_{0.5}$ PEP than occurred in anoxia.

In comparing the overall effect of anoxia and exposure to hydrogen sulfide in *Halicryptus spinulosus* metabolism, there were no substantially different effects of the two stresses on the properties of glycolytic enzymes or fructose-2,6-bisphosphate levels. This suggests that the H_2S stress is functionally the same as anoxia stress in its consequences for metabolism and metabolic regulation. Hydrogen sulfide exposure, therefore, had no additional or specific effects on glycolytic enzymes.

The question as to why anoxia and exposure to hydrogen sulfide show similar responses in *Halicryptus spinulosus* remains unanswered. It is not yet known to what degree hydrogen sulfide enters the worms. Unlike molluscs, the worms have no shells to protect them from hydrogen sulfide. Worms turn completely black when exposed to hydrogen sulfide (Oeschger and Schmaljohann 1988), and after a few days of H_2S incubation the coelomic fluid of the worms also begins to turn black (Oeschger unpublished observation). This indicates that *H. spinulosus* probably relies on its own sulfide detoxifying systems, as has been reported for some marine invertebrates from sulfide-rich environments (Powell and Somero 1986, Powell and Arp 1989).

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