

ADAPTATIONS TO SULFIDE BY HYDROTHERMAL VENT ANIMALS: SITES AND MECHANISMS OF DETOXIFICATION AND METABOLISM

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ABSTRACT

The detoxification and metabolism of sulfide were studied in three symbiont-containing invertebrates from the deep-sea hydrothermal vents: the tube worm, *Riftia pachyptila*; the clam, *Calyptogena magnifica*; and the mussel, *Bathymodiolus thermophilus*. Sulfide oxidizing activities, due to specific sulfide oxidase enzymes, were found in all tissues, with the greatest activities occurring in the symbiont-containing tissues: the trophosome of the tube worm and the gills of the bivalves. Sulfide oxidase activity was correlated with the bacterial content of the tissues. The sulfide oxidases in the outer cell layer(s) of symbiont-free tissues, e.g., body wall muscle of *Riftia* and foot and mantle of the bivalves, may detoxify sulfide as soon as it enters the body. Sulfide entering the blood in *Riftia* and *Calyptogena* may be bound by sulfide-binding factors that transport sulfide to the symbionts and protect against sulfide inhibition of aerobic respiration [via effects on the cytochrome-c oxidase (CytOx) system]. Sulfide strongly inhibited the CytOx systems of these animals, but this inhibition was offset by the addition to the CytOx assay mixture of blood of *Riftia* or *Calyptogena*. Reduced sulfur compounds, sulfide, sulfite, and thiosulfate, were effective in stimulating ATP synthesis in homogenates of symbiont-containing tissues. The most effective reduced sulfur compound varied among the three symbioses.

INTRODUCTION

Symbioses between marine invertebrates and sulfur-metabolizing bacteria have been discovered in a variety of habitats that are rich in hydrogen sulfide, including the deep-sea hydrothermal vents and many shallow habitats rich in decomposing organic materials (Cavanaugh *et al.*, 1981; Felbeck, 1981, 1983; Felbeck *et al.*, 1981; Felbeck and Somero, 1982; Cavanaugh, 1983; Fisher and Hand, 1984; Powell and Somero, 1985). These discoveries have raised two major questions concerning the metabolism of sulfide and other reduced sulfur compounds in these organisms. First, how are aerobic metabolic processes maintained in the face of sulfide concentrations that are high enough to poison completely the cytochrome-c oxidase (CytOx) systems of organisms from sulfide-free environments? The discovery that animals from these sulfide-rich habitats contain CytOx activities comparable to those found in related species from low-sulfide environments (Hand and Somero, 1983) suggests that sulfide detoxification is an important adaptation in these species (*cf.*, Powell and Somero, 1983, 1985).

A second question about the roles of reduced sulfur compounds in these symbioses concerns where and how these energy-rich molecules are metabolized to allow efficient exploitation of their bond energy. In the gutless protobranch clam, *Solemya reidi*, which occurs in shallow water sulfide-rich habitats (pulpmill effluent areas and sewage

outfall zones), the initial metabolism of sulfide in the symbiont-containing gills appears to occur not in the bacterial symbionts, but rather in specific organelles of animal origin we have termed sulfide oxidizing bodies (Powell and Somero, 1985). It is not known whether the initial step(s) in sulfide oxidation in other symbioses between marine invertebrates and sulfur bacteria occur in these organelles or, instead, within the bacterial endosymbionts. The type of reduced sulfur compound supplied to the bacteria by the host may influence the types of metabolic pathways present in the symbiont and the net ATP yield available from oxidation of sulfur compounds.

During the Galapagos '85 Expedition (March 1985) to the hydrothermal vent site located at the Galapagos Rift (0°N, 86°W) at a depth of approximately 2500 m, we examined several aspects of the sulfide metabolizing and detoxifying mechanisms of three symbiont-containing species that contribute importantly to the vent biomass: the vestimentiferan tube worm, *Riftia pachyptila* (Phylum Pogonophora); the vesicomid clam, *Calyptogena magnifica*; and the mussel, *Bathymodiolus thermophilus*. We sought to determine for each species the sulfide oxidizing potentials of symbiont-containing and symbiont-free tissues; whether this oxidation was due to specific enzymes (sulfide oxidases; Powell and Somero, 1985); what the aerobic potentials of the species' tissues were and how aerobic respiration was protected from sulfide; and how effective sulfide and other reduced sulfur compounds (sulfite and thiosulfate) were in driving ATP synthesis in the symbiont-containing tissues of the species. Our results show that the species differ in their capacities for sulfide oxidation, in their capacities for aerobic respiration, in the mechanisms used for protecting aerobic respiration from poisoning by sulfide, and in the types of reduced sulfur compounds that are most effective in stimulating ATP generation. Thus, while these three symbioses share major features in common, they also differ strikingly in the mechanisms used to detoxify and exploit sulfide.

MATERIALS AND METHODS

Experimental animals

Organisms were collected during the 1985 expedition to the Galapagos Rift "Rose Garden" hydrothermal vent site (Hessler and Smithey, 1983). The three species studied, *Riftia pachyptila*, the large vestimentiferan tube worm (Jones, 1981); *Calyptogena magnifica*, a vesicomid clam (Boss and Turner, 1980); and *Bathymodiolus thermophilus*, the vent mussel (Kenk and Wilson, 1985) are endemic to the vents and are the dominant contributors to the faunal biomass at the Rose Garden site. Specimens were retrieved using the submersible DSRV Alvin operated by the Woods Hole Oceanographic Institution. Freshly collected animals were placed in a thermally insulated container held in the front basket of the submersible and brought to the surface within 2–3 h of collection. Animals were not held at *in situ* pressures (approx. 250 atm) during ascent, but the temperature of the container stayed below approximately 15°C, a temperature readily tolerated by the animals, which experience temperatures of between 2 and approximately 20°C *in situ* (Hessler and Smithey, 1983). The animals were immediately placed in 4°C seawater upon retrieval and held in a refrigerated (approx. 4°C) van on board the RV Melville.

For most experiments tissue samples were dissected from the freshly retrieved samples within 1–3 h of their arrival at the surface. For some studies tissues were removed from animals that had been held at *in situ* pressures for one to several days after capture.

Preparation of solutions of sulfide

At pH values near neutrality, sulfide exists primarily as HS^- and H_2S ; only small amounts of S^{2-} are present. We use the term "sulfide" to refer to all sulfide species in solution.

All sulfide solutions were freshly prepared immediately before an experiment, and used within 1–3 h of preparation. To prepare a stock solution, a freshly washed crystal of sodium sulfide was weighed using the motion-compensated shipboard balance system designed by Childress and Mickel (1980), and dissolved immediately in distilled water.

Enzyme assays

Sulfide oxidation was assayed photometrically (at 578 nm) by following the reduction by sulfide of the artificial electron acceptor benzyl viologen (BV) using the assay system of Powell and Somero (1985). The other enzymes studied, cytochrome-c oxidase (CytOx), citrate synthase (CS), and malate dehydrogenase (MDH), were assayed according to the methods of Hand and Somero (1983). The standard assay pH for CytOx was 6.0; different pH values were used under certain circumstances, as indicated in the figures. Partial purification of CytOx was performed as described in Powell and Somero (1983). All enzymatic activities were measured at 20°C. Activities are expressed as international units (umoles substrate converted to product per minute) per g fresh weight (gFW) of tissue.

Microscopy

Sectioning of tissues and staining for sulfide oxidizing activity were performed as described in Powell and Somero (1985), except that the staining buffer for trophosome tissue was 80% artificial seawater (ASW) buffered with 0.2 M glycine, pH 9.0, containing 5 mM sulfide and 2 mM benzyl viologen. One hundred percent ASW was made of NaCl (0.47 M), KCl (10 mM), CaCl_2 (10 mM), and MgCl_2 (50 mM).

Heating of enzyme preparations

The thermal stabilities of the sulfide oxidizing activities were studied by heating unfractionated homogenates of the tissues (trophosome and body wall of *Riftia*, and gills of *Calyptogena* and *Bathymodiolus*) at 90°C for 10 min, and then assaying for sulfide oxidizing activity.

Determining bacterial counts in homogenates

Connective tissue and blood vessels were removed as completely as possible from the tissue samples before use. Tissues were homogenized gently with a Dounce homogenizer, employing three strokes by hand with a loose pestle, and three strokes by hand with a tight pestle. The homogenization buffer was ASW buffered with 20 mM HEPES, pH 7.4. *Riftia pachyptila* trophosome samples were counted immediately using a hemocytometer; the large size of the bacteria (2–6 μm) permitted this method of counting. To verify that this cell counting method was accurate, samples of trophosome homogenates were fixed in 5% formalin in ASW HEPES. These samples were stained with D.A.P.I. and counted by epifluorescence microscopy upon return to Scripps. Epifluorescence counts of these trophosome samples confirmed that the earlier hemocytometer counts were accurate.

Size of bacteria and bacterial composition of tissues

Gill tissues were fixed and embedded for microscopy as described by Powell and Somero (1985). One to five μm thick sections were cut, and the size of the bacteria was measured by light microscopy. Tissue composition (% bacteria) was estimated according to the following formula: (% bacteria = # of bacteria per gram fresh weight (gFW) \times mass of bacteria \times 100). Mass of bacteria = density of bacteria \times volume of bacteria. Density of bacteria was assumed to be 1.0 g/c.c volume of bacteria = $\frac{4}{3} \times \pi \times (\text{radius of bacteria})^3$, (all bacteria were approximately spherical).

ATP synthesis studies

Tissues were homogenized in either 20 mM potassium phosphate buffer, pH 7.4 (hypoosmotic buffer for lysis of bacteria), or ASW/Hepes, pH 7.4 (isosmotic buffer to prevent bacterial lysis). Samples were examined by phase contrast microscopy for intactness of the bacteria. Reactions were performed at 20°C. The reaction mixture (total volume 2.2 ml), contained 1 mM of the reduced sulfur compound serving as the substrate (experimental) or no substrate (control), and homogenate volumes containing 0.022–0.20 gFW tissue. The reaction buffer was 100 mM potassium phosphate buffer, pH 7.4 (lysed samples), or ASW/Hepes, pH 7.4 (intact samples). Homogenate and buffer were mixed and allowed to equilibrate for 2 min at 20°C. For the 0 min time point, 0.4 ml of the reaction mixture was removed and quenched with an equal volume of 1.5 M HClO₄ with rapid mixing; the substrate (sulfate, sulfite, or thiosulfate) was added after quenching. Substrate was then added to the rest of the mixture, and 0.4 ml of the complete reaction mixture was added to each of four test tubes, which were held at 20°C for different lengths of time. At the times indicated in Figure 6, the 0.4 ml aliquots were quenched with an equal volume of 1.5 M HClO₄. The quenched samples were held for 10 min at room temperature to allow complete denaturation of the proteins in the sample. The precipitated samples were next placed on ice for 10 min, and then centrifuged for 5 min at 10,000 \times g. The supernatant was neutralized with cold 2 M KOH/1 M triethanolamine, left on ice for at least 10 min or frozen for up to 6 hours, and centrifuged for 5 min at 10,000 \times g. The supernatant was then assayed immediately for ATP concentration with firefly luciferase as described (Karl and Holm-Hansen, 1978).

RESULTS

Sulfide oxidation activities

Using the benzyl viologen assay for measuring photometrically the oxidation of sulfide, we detected sulfide oxidizing activities in all of the tissues that were examined (Table I). Activities were highest in the symbiont-containing tissues: the gills of the bivalves and the trophosome of *Riftia*. The latter tissue contained by far the highest levels of sulfide oxidizing activity, and this activity varied along the length of the trophosome in a consistent manner, increasing from top to bottom. The gills of *Bathymodiolus* contained the lowest levels of sulfide oxidizing activity of any symbiont-containing tissue.

The sulfide oxidizing activities listed in Table I represent averages for the entire tissue sample used in the homogenate, and do not indicate whether activity was uniformly distributed throughout a tissue or localized in a precise region. To investigate the localization of sulfide oxidizing activity we used the benzyl viologen histochemical staining procedure.

TABLE I

Sulfide oxidizing activities of tissues of vent invertebrates

Sample	Units \times gFW ⁻¹
<i>Riftia pachyptila</i>	
trophosome	
top	22.04 \pm 7.14 (12)
middle	31.66 \pm 8.80 (8)
bottom	40.31 \pm 12.30 (13)
plume	0.24 \pm 0.09 (8)
body wall	0.44 \pm 0.20 (10)
<i>Bathymodiolus thermophilus</i>	
gill	0.98 \pm 0.33 (21)
foot	0.18 \pm 0.05 (7)
mantle	0.41 \pm 0.11 (7)
<i>Calyptogena magnifica</i>	
gill	6.06 \pm 2.09 (22)
foot	0.58 \pm 0.14 (8)
mantle	0.95 \pm 0.41 (5)

Sulfide oxidizing activities of supernatants were measured using the benzyl viologen photometric assay system, and are expressed as units of activity per g fresh weight of tissue. Values expressed as mean \pm standard deviation (number of individuals).

Figure 1 illustrates the staining patterns observed in trophosome and body wall of *Riftia* and in foot and mantle of *Calyptogena*. Foot and mantle of *Bathymodiolus* exhibited the same staining patterns found for *Calyptogena* (data not shown). In trophosome all staining was associated with the bacterial endosymbionts. Bacteria were always present in interconnected sausage-shaped lobes. Layers of green structure always surrounded the lobes of bacteria, but the thickness of the green layers was variable; approximately average thickness is shown here. These green layers were responsible for the striking intense green color of trophosome tissue. The green structures were present within animal cells. The bacteria appeared to be endosymbionts, *i.e.*, they were within animal cells. In the body wall muscle of *Riftia* and the foot and mantle tissues of the bivalves—tissues which lack symbionts—the sulfide oxidizing activity was located in the outer cell layers of the tissue, *i.e.*, in the cells in direct contact with the sulfide-laden seawater. So localized, the sulfide oxidizing activity may serve as a “peripheral defense” by rapidly oxidizing any sulfide that penetrates the outer body surface.

Because of the very small size of the bacterial symbionts of the bivalves, histochemical staining of gill tissue could not reveal the precise localization of the sulfide oxidizing activities. Sulfide oxidizing activity did co-purify with the bacteria in tissue fractionation studies employing differential centrifugation (authors' unpub. obs.). However, the bacterial pellets also contained fragments of mitochondria, so we could not determine unambiguously where the sulfide oxidizing activities were localized (see Discussion).

The oxidation of hydrogen sulfide can be catalyzed by a variety of factors besides specific sulfide oxidase (SOx) enzymes like those found in the clam, *Solemya reidi* (Powell and Somero, 1985). Three types of evidence suggest that the sulfide oxidizing activities found in the symbiont-containing and symbiont-free tissues of these animals were specific sulfide oxidase enzymes. First, the sulfide oxidizing activities were significantly higher than those characteristic of sulfide oxidation due to non-specific factors

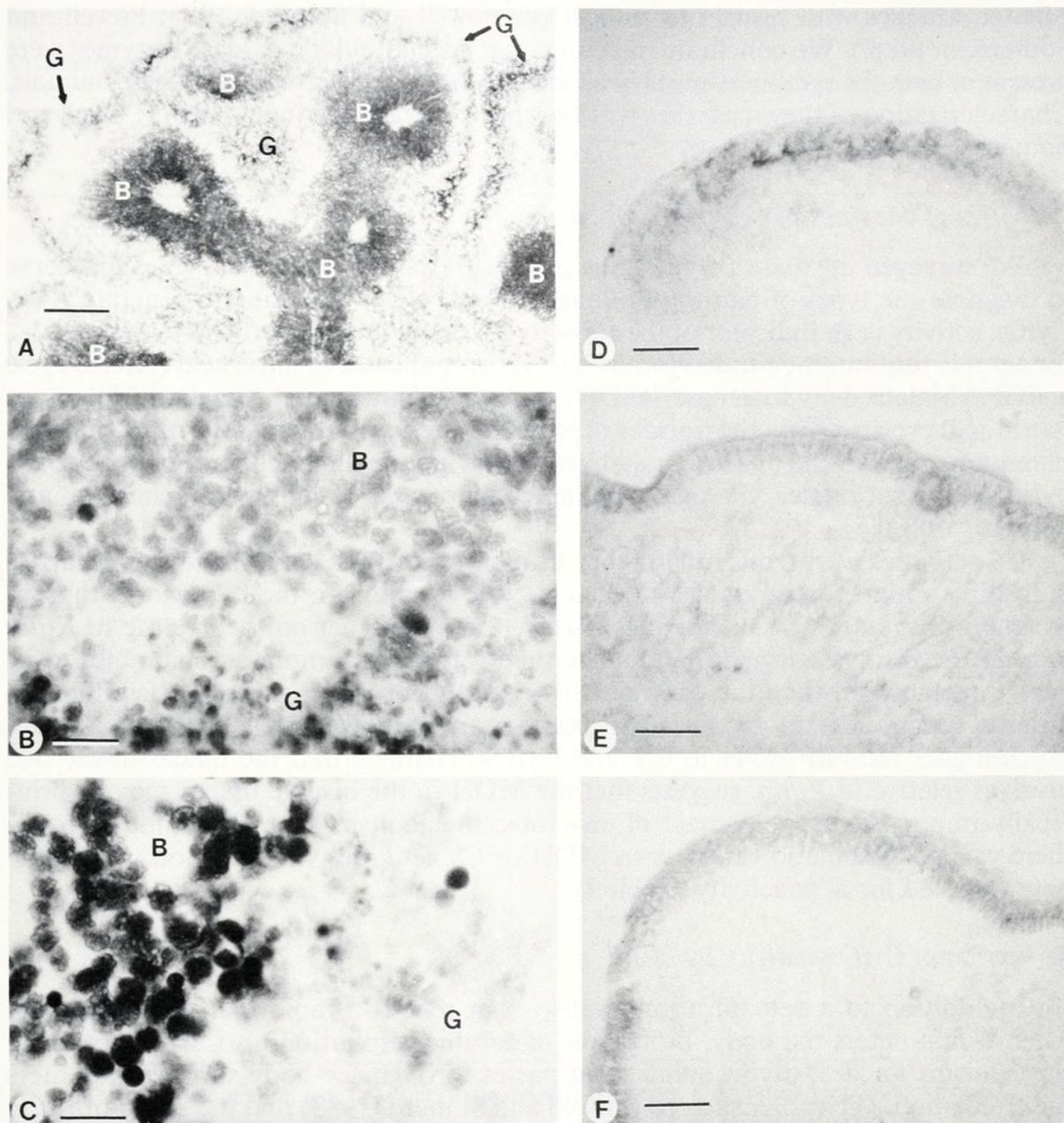


FIGURE 1. Light micrographs of transverse sections through tissues of vent animals. Sections were unstained (A, B), or stained for sulfide oxidation activity (C-F). (A) Low magnification view of unstained *Riftia pachyptila* trophosome tissue. B indicates bacteria and G indicates green structures. Bacteria were filled with translucent sulfur globules (see Fig. 1B), and appear dark at low magnification. Scale bar = 100 μm . (B) High magnification view of unstained trophosome tissue. Bacteria were filled with translucent sulfur globules. Scale bar = 10 μm . (C) High magnification view of trophosome tissue stained for sulfide oxidase activity. Bacteria were stained dark purple (positive for sulfide oxidase activity). Green structures were unstained. Some sulfur globules are still visible. Scale bar = 10 μm . (D) *Riftia pachyptila* body wall tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Scale bar = 40 μm . (E) *Calyptogena magnifica* mantle tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Scale bar = 40 μm . (F) *Calyptogena magnifica* foot tissue stained for sulfide oxidase activity. The surface layer of cells stained positively. Controls (benzyl viologen reagent but no sulfide) did not exhibit any staining. Scale bar = 40 μm . Abbreviations: B = bacteria, G = green structures.

(*cf.*, Powell and Somero, 1985). Second, we found that the activities were heat labile, with rapid loss of sulfide oxidizing activity occurring during incubation at 90°C (see Materials and Methods; data not shown). Third, the activities displayed Michaelis-

Menten kinetics with respect to sulfide (*cf.*, Powell and Somero, 1985; Powell and Somero, *in prep.*). We conclude, therefore, that specific sulfide oxidase enzymes were present in both the symbiont-containing and the symbiont-free tissues of these animals. Characterization of these enzymes will be reported in a subsequent paper (Powell and Somero, *in prep.*).

Activities of enzymes of energy metabolism

We surveyed the tissues of the three species for the activities of enzymes that serve to diagnose the types of pathways of energy metabolism operating to generate ATP. CytOx activity is an indicator of the capacity for electron transport chain activity, *i.e.*, for aerobic respiration; citrate synthase (CS) is an indicator of citric acid (Krebs) cycle activity. Malate dehydrogenase (MDH) is involved in several metabolic functions [citric acid cycle activity; the transfer of reducing equivalents across the mitochondrial membrane; and in the anaerobic pathway involving the channeling of phosphoenolpyruvate to succinate, via oxaloacetate, malate, and fumarate (Hochachka and Somero, 1984)].

CS activities were uniformly higher in tissues of *Riftia* than in the two bivalves, indicating a higher capacity for citric acid cycle function in the tube worm than in the clam or mussel. CytOx activities also were much higher on the average in *Riftia* than in the bivalves, suggesting that the tube worm has a more aerobic poise to its energy metabolism than the clam or mussel. MDH activities varied by less than an order of magnitude among all of the tissues examined (Table II). The finding that a much higher ratio of MDH to CS (and CytOx) characterized the tissues of the two bivalves relative to *Riftia*, suggests that the MDH in the bivalve tissues may be principally important in the context of anaerobic metabolism. In trophosome of *Riftia* there was no systematic variation in MDH or CS activities with position, unlike the pattern noted for SOx activity (Table I).

Protection of CytOx activity by blood

In addition to a detoxification strategy that relies on rapid oxidation of sulfide when it first enters the body, protection of aerobic respiration also may depend on mechanisms for detoxifying sulfide that passes through the body surface and enters the circulation. We earlier showed (Powell and Somero, 1983) that the blood of *Riftia* was capable of offsetting the inhibition of CytOx activity caused by sulfide. To investigate protection by blood in more detail we further characterized the protective effects of *Riftia* blood and also studied blood of the clam, *Calyptogena*.

The importance of mechanisms for protecting CytOx activity from inhibition by sulfide is emphasized by the data in Figure 2, which show that the CytOx systems of *Riftia* and *Bathymodiolus* are highly sensitive to sulfide, as is the CytOx of *Calyptogena* (see Fig. 3). The CytOx systems of *Riftia* and *Bathymodiolus* show identical sensitivities to sulfide (pH 6.0 data), with virtually complete inhibition occurring by sulfide concentrations of 20–50 μM . The sensitivity of CytOx to sulfide is strongly dependent on pH, and the stronger effects noted at pH 6.0 suggest that H_2S is the inhibitory form of sulfide.

Bloods of *Riftia* and *Calyptogena* were able to counteract the inhibition of CytOx activity by sulfide (Fig. 3). The addition of small quantities of freshly collected blood to the CytOx assay system reduced the inhibitory effects of sulfide on the activities of the enzymes of *Riftia* and *Calyptogena*. The protective effects of blood of *Riftia* appear from the data in Figure 3 to be greater than those of *Calyptogena* blood. However,

TABLE II

Enzymatic activities in tissues of three vent invertebrates

Sample	Units \times gFW ⁻¹			
	MDH	CS	CytOx	SOx
<i>Riftia pachyptila</i>				
plume	28.1	4.29	8.32	nd
vestmentum	40.4	2.53	2.54	nd
body wall	63.6	10.00	1.27	nd
trophosome				
top-1	29.7	1.53	nd	31.7
top-2	30.2	0.65	nd	33.8
top-3	21.3	1.88	nd	19.7
bottom-1	41.8	1.23	nd	49.7
bottom-2	23.9	0.71	nd	56.4
bottom-3	24.8	1.12	nd	56.1
<i>Bathymodiolus thermophilus</i>				
gill-1	41.8	0.02	0.89	nd
gill-2	38.0	0.02	1.54	nd
adductor-1	54.3	0.02	0.41	nd
adductor-2	39.0	0.03	0.27	nd
foot-1	35.7	0.02	0.12	nd
foot-2	33.4	0.02	0.22	nd
mantle-1	27.8	0.01	0.14	nd
mantle-2	25.8	0.01	0.08	nd
<i>Calymene magnifica</i>				
gill-1	39.2	0	0.41	nd
gill-2	37.1	nd	nd	5.15
gill-3	42.5	nd	nd	8.94
gill-4	32.0	nd	nd	8.67
gill-5	34.8	nd	nd	6.63
gill-6	34.1	nd	nd	5.29
adductor	116.9	0.41	0.11	nd
foot	25.1	0.18	0	nd
mantle	23.2	0.12	0	nd

"nd" means not determined. "0" refers to no detectable activity. Each numbered tissue refers to a tissue sample from a different individual.

the protocols used for obtaining the CytOx systems of the two species (plume of *Riftia* and heart of *Calymene*) differed, and the sensitivity of CytOx to sulfide is strongly dependent on the extent to which the enzyme is purified (Powell and Somero, 1983). The purification scheme used for the *Riftia* enzyme inactivated the enzyme from *Calymene*, so we were forced to use a crude homogenate to assay the CytOx activity of clam heart. Therefore, the apparent difference in protective ability between the two bloods may, in fact, be entirely a reflection of the state of purity of the CytOx preparations. Therefore, the data in Figure 3 should be taken only as evidence that both bloods can protect CytOx, and not as quantitative estimates of this protective capacity.

The abilities of blood (*Riftia*) to protect the CytOx system from poisoning by sulfide were dependent on the time of addition of blood to the CytOx assay mixture, and the length of the period during which blood, sulfide, and the enzyme were allowed to equilibrate (Fig. 4). At a given concentration of sulfide, the degree of inhibition of CytOx increased with time of exposure to sulfide ("sulfide equilibration" data in Fig.

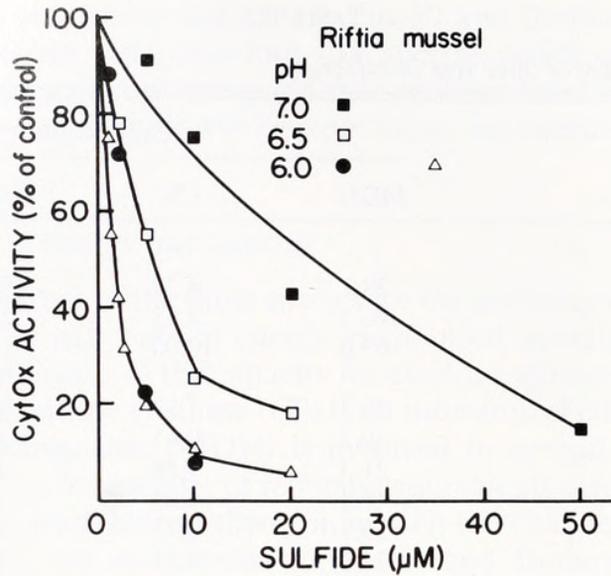


FIGURE 2. Inhibition of CytOx activity of *Riftia* plume tissue and *Bathymodiolus* mantle by sulfide. The pH of assay buffer was varied as indicated. The controls were the activities of the enzyme preparations without addition of sulfide. For *Riftia* CytOx, a twice acid-precipitated preparation was used (Powell and Somero, 1983). For *Bathymodiolus* a crude homogenate was used.

4). When a sulfide-inhibited sample of CytOx was incubated for increasing amounts of time with *Riftia* blood, the reversal of inhibition was enhanced ("blood equilibration" data in Fig. 4). These data indicated that inhibition by sulfide is reversible, and that two equilibria are involved in establishing the effects of sulfide on CytOx: one between sulfide and the enzyme, and one between sulfide and the blood factor that binds sulfide.

The protection afforded by blood (*Riftia*) was a function of pH (Fig. 5). At pH 7.4 the protective effect was much higher than at pH 6.0, a finding which may reflect the pH sensitivity of binding of sulfide to the protective factor in blood (see Discussion). The data in Figure 5 were obtained with blood samples containing different concentrations of sulfide (see legend to Fig. 5). Equilibration of blood with high concentrations

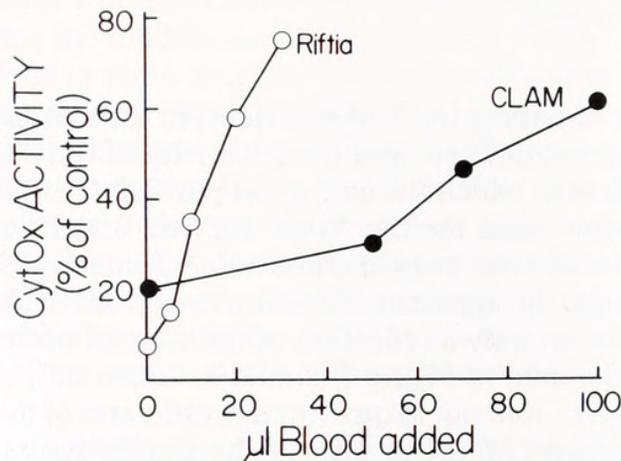


FIGURE 3. Protection by blood of CytOx activity from sulfide inhibition. The line labeled *Riftia* was *Riftia* plume enzyme and blood, and the line labeled clam was *Calypptogena* heart enzyme and blood. The *Riftia* enzyme had been twice acid-precipitated (Powell and Somero, 1983); the clam enzyme preparation was a crude homogenate. The controls were the activities of the enzyme preparations without sulfide added. Sulfide concentration of *Riftia* assays was 5 μM, and of *Calypptogena magnifica* assays was 500 μM.

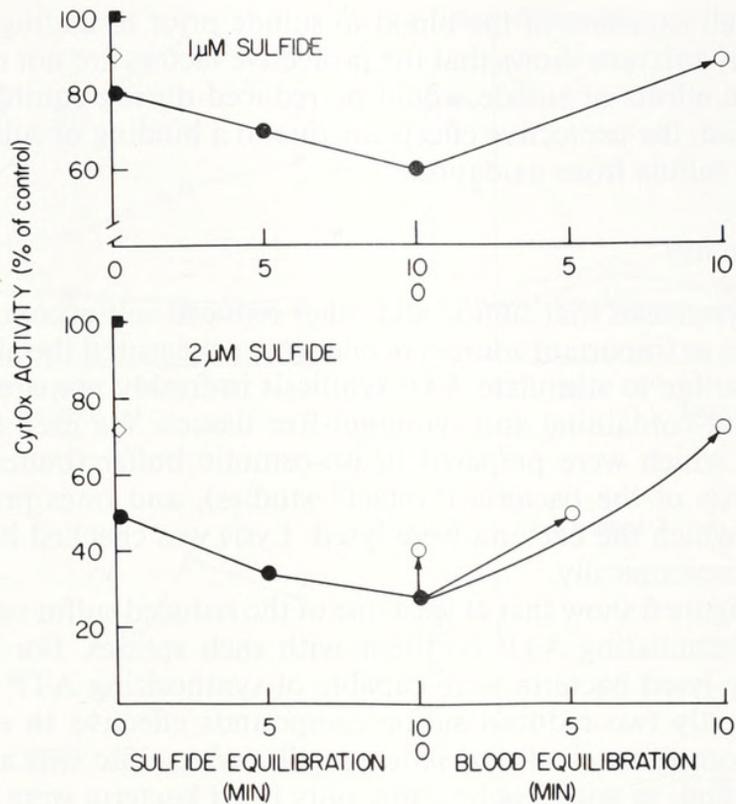


FIGURE 4. Time-dependent effects of sulfide on *Riftia pachyptila* plume CytOx activity, and time-dependent reversal of inhibition by blood. Sulfide was added to CytOx preparations, and activity was measured at the times indicated. The filled circles represent time of pre-incubation of enzyme preparations with sulfide prior to initiation of assay. The open diamond symbols represent addition of 10 μl of blood at t = 0, and immediate assay. Open circles represent addition of blood after 10 min pre-incubation of enzyme preparations with sulfide; assays were then initiated 0, 5, and 10 min after blood addition. Filled squares represent CytOx activity of enzyme preparations without sulfide added.

of sulfide reduced or eliminated the ability of the blood to protect CytOx activity, indicating a saturation of the sulfide-binding capacity of the blood at higher sulfide concentrations. The finding that the protective effects of blood could be reduced or

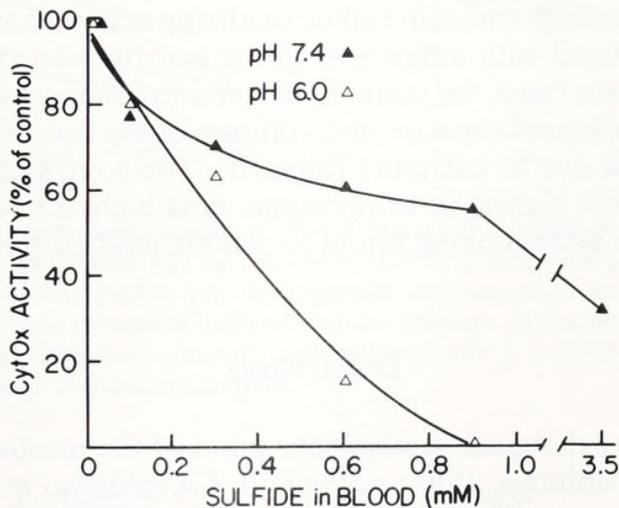


FIGURE 5. Effects of pH and blood sulfide concentration on protection by blood of CytOx activity. Ten μl of blood containing known amounts of sulfide were added to CytOx assays inhibited by 2 μM sulfide (pH 6.0), and 10 μM sulfide (pH 7.4). Sulfide-free blood (10 μl) provided 100% protection (equal to control activity without sulfide added) under the assay conditions used.

eliminated through exposure of the blood to sulfide prior to adding a blood sample to the CytOx assay mixture shows that the protective factors are not oxidizing sulfide, in which case the effects of sulfide would be reduced during equilibration of blood and sulfide. Instead, the protective effects are due to a binding of sulfide that leads to protection of the sulfide from oxidation.

ATP synthesis studies

To test the hypothesis that sulfide and other reduced sulfur compounds are used in these symbioses as important sources of energy, we measured the abilities of sulfide, thiosulfate, and sulfite to stimulate ATP synthesis in freshly prepared tissue homogenates of symbiont-containing and symbiont-free tissues. We used two types of homogenates: ones which were prepared in iso-osmotic buffer (buffered seawater) to avoid osmotic lysis of the bacteria ("intact" studies), and ones prepared in dilute HEPES buffer, in which the bacteria were lysed. Lysis was checked by examining the homogenates microscopically.

The data in Figure 6 show that at least one of the reduced sulfur compounds tested was effective in stimulating ATP synthesis with each species. For homogenates of trophosome, only lysed bacteria were capable of synthesizing ATP, and sulfite and sulfide were the only two reduced sulfur compounds effective in stimulating ATP production. In homogenates of *Calyptogena* gill, only sulfite was able to stimulate ATP production and, as with trophosome, only lysed bacteria were effective in synthesizing ATP under our experimental conditions. Homogenates of gill of *Bathymodiolus* differed in three respects from those of the other species. First, only intact bacteria exhibited ATP production in response to reduced sulfur compounds. Second, thiosulfate was the most effective reduced sulfur compound in driving ATP synthesis. Third, the amount of ATP produced was much lower in *Bathymodiolus* preparations than in the other species.

Stimulation of ATP synthesis by reduced sulfur compounds was not observed for any of the symbiont-free tissues tested (*Riftia* body wall, foot and mantle of *Calyptogena* and *Bathymodiolus*; data not shown).

Bacterial contents of tissues

To determine if the differences in sulfide oxidizing activities and ATP synthesizing capacities were correlated with differences in the contribution of the bacterial endosymbionts to total tissue mass, we counted bacteria in homogenates of the symbiont-containing tissues, measured the sizes and volumes of the bacteria, and computed the percent of tissue mass due to bacteria (Table III). The contribution of bacterial cells to total tissue mass was highest in trophosome, next highest in clam gill, and lowest in the mussel gill, the same ranking found for sulfide oxidizing and ATP synthesizing activities.

DISCUSSION

These studies were designed to elucidate some of the mechanisms by which the three dominant vent animals, *Riftia pachyptila*, *Calyptogena magnifica*, and *Bathymodiolus thermophilus*, succeed in exploiting the energy of the sulfide molecule while avoiding its toxic effects on aerobic respiration. Our results indicate that the three symbioses share certain mechanisms in common for metabolizing and detoxifying sulfide, yet differ in some aspects of their sulfide relationships.

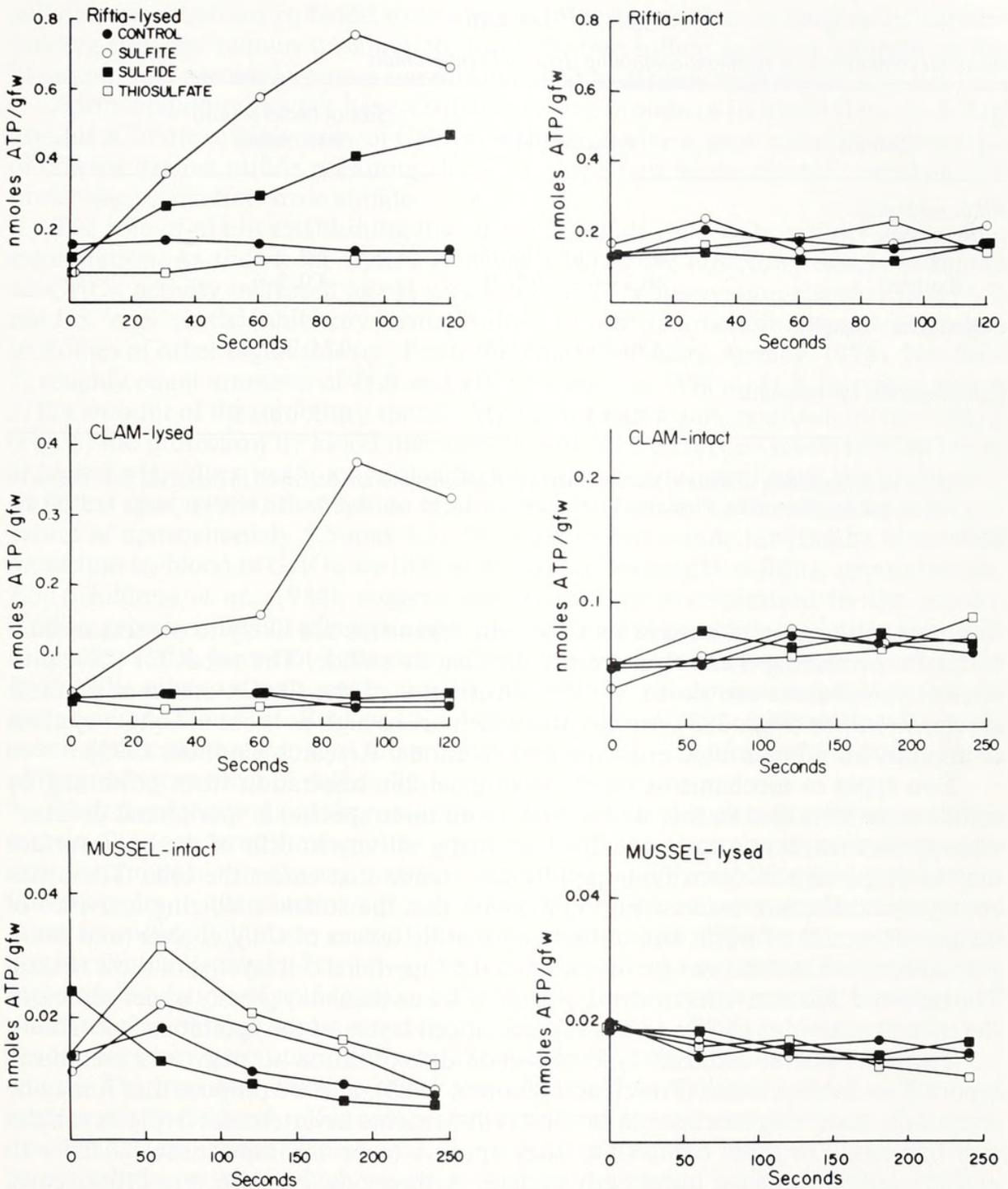


FIGURE 6. ATP concentrations (nmoles ATP/gFW tissue) in homogenates incubated with reduced sulfur compounds [sulfide (■), sulfite (○), or thiosulfate (□)] at 1 mM final concentration or no sulfur compound [control (●)]. Lysed samples were homogenized and assayed in low osmolarity buffer; intact samples were homogenized and assayed in buffered artificial seawater. Intactness of bacteria was judged by phase-contrast microscopy. Each frame presents data gathered with a different tissue homogenate. Each symbol represents the average of duplicate samples.

Protection of aerobic respiration from poisoning by sulfide

The occurrence of CytOx activity in all three species (Table II) and the observation that *Riftia* (Childress *et al.*, 1984) and *Calyptogena* (Arp *et al.*, 1984) consume oxygen at rates comparable to those reported for shallow-living species, at comparable mea-

TABLE III

Bacterial composition of symbiont-containing tissues of vent animals

Sample	# Bacteria/gFW	Size of bacteria (μm) (avg, range)	% Bacteria
<i>Riftia pachyptila</i> trophosome (top)	$4.37 \pm 1.05 \times 10^9$ (3)	4.0, 2-6	15
(bottom)	$10.3 \pm 5.6 \times 10^9$ (3)	4.0, 2-6	35
<i>Calyptogena magnifica</i> gill	$3.30-3.64 \times 10^{11}$ (2)	0.75, 0.5-1.0	7.7
<i>Bathymodiolus thermophilus</i> gill	$1.70-1.81 \times 10^{11}$ (2)	0.75, 0.5-1.0	4.0

Bacterial composition of tissues. Measurements and calculations are described in Materials and Methods.

For *R. pachyptila*, mean \pm standard deviation (n) are given, and for the bivalves range and (n) are given.

surement temperatures, suggest that the vent organisms are likely to possess mechanisms for protecting themselves from poisoning by sulfide. The needs for these protective mechanisms are shown by the sensitivities of the CytOx enzymes of these species to sulfide (Figs. 2, 3), sensitivities which are as high as those of CytOx systems of animals from low-sulfide environments (National Research Council, 1979).

Two types of mechanisms for protecting aerobic respiration from poisoning by sulfide were identified in this study. First, in all three species, a "peripheral defense" strategy that employs a zone of sulfide oxidizing activity in cells of the body surface may be important in detoxifying rapidly any sulfide that enters the cells. The histochemical localization studies (Fig. 1) indicate that the sulfide oxidizing activities of body wall muscle of *Riftia* and of foot and mantle tissues of *Calyptogena* (and *Bathymodiolus*; data not shown) are restricted to the superficial cell layer(s) of these tissues. The reported SOx activities in terms of gFW of tissue thus may grossly under-represent the specific activities of SOx in the superficial cell layers of the symbiont-free tissues.

This "peripheral defense" type of sulfide detoxification strategy was previously reported in *Solemya reidi* (Powell and Somero, 1985), and we propose that it may be a generally occurring mechanism in soft-bodied marine invertebrates living in sulfide-rich habitats. For these organisms, there appears to be no impermeable barrier to exclude sulfide from the outer body surface. Arthropods, e.g., the vent crustaceans, appear to lack this "peripheral defense" mechanism, and instead detoxify any sulfide that has entered their circulation by oxidizing it in the hepatopancreas (Vetter *et al.*, 1986). Arthropods may be highly impermeable to sulfide, except at areas where the exoskeleton is very thin, so most of the body surface may not be threatened by sulfide entry and poisoning of respiration.

A second mechanism for preventing poisoning of respiration by sulfide exists in *Riftia* and *Calyptogena*. High molecular weight factors in the bloods of the tube worm (Arp and Childress, 1983; Fisher and Childress, 1984) and the clam (Arp *et al.*, 1984) bind sulfide tightly, and appear capable of extracting sulfide from the environment and transporting it via the circulation to the bacterial symbionts. These binding factors, which are proteins in *Riftia* (Arp and Childress, 1983; Childress *et al.*, 1984) and probably also in *Calyptogena* (Arp *et al.*, 1984) appear to be important in holding free

sulfide concentrations in blood to low values. We suggest that as long as the sulfide-binding proteins remain unsaturated, too little free sulfide exists in solution in the blood of these species to pose a significant threat to aerobic respiration.

Bathymodiolus does not have a sulfide-binding protein in its blood (Drs. A. J. Arp and J. J. Childress, University of California, Santa Barbara, pers. comm.), so this type of defense against sulfide poisoning does not play a part in the mussel's strategies for protecting respiration from sulfide.

The role of pH in establishing the effects of sulfide on CytOx activity bears close examination. As shown for CytOx of *Riftia* (Fig. 2), the inhibitory effects of sulfide on CytOx activity increased as pH was reduced. This observation suggests that H_2S , not HS^- or S^{2-} , is the inhibitory form of sulfide. A similar conclusion has been reached in studies of other organisms (*cf.*, Environmental Protection Agency, 1976). Near pH 7, roughly equal amounts of H_2S and HS^- are present. When pH is increased above 7, the amount of the inhibitory species, H_2S , falls rapidly and, as shown by our results (Fig. 5) the protection by blood increases as well. The enhanced protection by blood at higher pH values in the physiological pH range is consistent with the findings of Childress *et al.* (1984) that sulfide binding by blood of *Riftia* increased between pH values of approximately 5.5 and 7.5. The finding that sulfide binding by blood and protection by blood of CytOx are high at the average blood pH of *Riftia*, approximately 7.5, (Childress *et al.*, 1984), suggests that protection of respiration by the sulfide-binding protein is highly effective under physiological pH conditions. Intracellular pH values for *Riftia* have not been determined, but it seems reasonable to assume that *Riftia*, like other animals, maintains its intracellular pH approximately 0.4–0.5 pH units acidic to blood (*cf.*, Reeves, 1977). Thus, any free sulfide entering the cells would pose a significant threat to CytOx activity since at pH 7.0–7.1 substantial concentrations of the inhibitory form of sulfide, H_2S , would be present.

Several questions about the mechanisms used to defend against poisoning by sulfide remain. One concerns the means by which sulfide passes from the ambient vent water into the animals' circulatory systems without poisoning mitochondrial respiration. For example, in the plume of *Riftia*, which is thought to serve as the major entry route for sulfide (Childress *et al.*, 1984), large amounts of sulfide must cross a highly aerobic (Table II) tissue. The low sulfide oxidase activities in plume (Table I) suggest that little oxidation of sulfide takes place during the transport process. Oxidation of sulfide during passage across a transport surface into the circulatory system, where it is complexed in a non-toxic form by the sulfide-binding proteins, is undesirable, of course. The maximal energy yield from sulfide will be attained only if sulfide, not some partially oxidized sulfur compound, is delivered to the site of sulfur metabolism, *e.g.*, the bacteria in the trophosome. We suggest that sulfide transport in plume may be pericellular, a route which would avoid the problems of sulfide inhibition of respiration and the partial oxidation of this energy resource.

A second unanswered question about the interactions of aerobic respiration with sulfide concerns the mussel, *Bathymodiolus*, which appears to lack sulfide binding proteins in its blood. The activities of CytOx present in tissues of the mussel may be protected entirely by sulfide oxidase enzymes serving a detoxification role. However, these sulfide oxidizing enzymes could reduce the amount of sulfide reaching the bacterial symbionts (see below).

Reduced sulfur compounds as energy sources

Discussions of the symbioses between sulfide biome invertebrates and their bacterial symbionts have emphasized the potential contributions that reduced sulfur compounds

like sulfide might make to the energy needs of these organisms (*cf.*, Felbeck and Somero, 1982; Jannasch and Mottl, 1985). It has been emphasized that carbon dioxide fixation via the Calvin-Benson cycle could be driven by the energy released in bacterial sulfide oxidation, and that the reduced carbon compounds synthesized in the bacterial symbionts could be translocated to the host to satisfy some fraction of its nutritional requirements.

In the clam, *Solemya velum*, Cavanaugh (1983) demonstrated that sulfide and thiosulfate were effective in stimulating carbon dioxide fixation. Belkin *et al.* (1986) showed that sulfide, but not thiosulfate, stimulated the fixation of carbon dioxide in homogenates of *Riftia* trophosome; thiosulfate, but not sulfide, was effective in stimulating carbon dioxide fixation in homogenates of gill from *Bathymodiolus*. To our knowledge, these are the only studies that have demonstrated directly the roles of reduced sulfur compounds in driving endergonic processes in these symbioses.

To gain additional understanding of the efficacy of reduced sulfur compounds in supplying energy for these symbioses, we reasoned that it would be especially important to determine how these compounds affected the ATP synthesis of the organisms. Because ATP is the dominant "energy currency" of the cell, changes in ATP concentrations in response to exposure to different reduced sulfur compounds could be a very sensitive indicator of the importance of these compounds to the generation of biologically useful forms of energy in the symbioses. Prior to our studies the capacities of these symbioses to trap the energy released during the oxidation of reduced sulfur compounds in the form of ATP had not been investigated. Neither had there been study of the relative abilities of different reduced sulfur compounds, *e.g.*, sulfide, sulfite, and thiosulfate, to supply energy in these symbioses.

The results of our experiments suggest that the symbiont-containing tissues of *Riftia*, *Calyptogena*, and *Bathymodiolus* can exploit the energy of reduced sulfur compounds. Because we used unfractionated homogenates in our studies, *i.e.*, animal tissue and bacteria were present, our results do not demonstrate unambiguously that the symbionts were solely or primarily responsible for the ATP production driven by reduced sulfur compounds. The correlation between ATP production and bacterial contribution to tissue mass is consistent with a dominant role for the symbionts in this process (Table III); however, a role for animal-localized ATP synthesis mechanisms cannot be excluded (see below).

The reduced sulfur compounds most effective in driving ATP synthesis differed among the three symbioses (Fig. 6). Also, the total amount of ATP that was produced by oxidation of reduced sulfur compounds differed among the three symbioses studied (Fig. 6). In *Riftia* trophosome homogenates, sulfide and sulfite were most effective in stimulating ATP production, while in *Calyptogena* only sulfite was effective, and in *Bathymodiolus*, thiosulfate was most effective (Fig. 6). In *Riftia* and *Calyptogena*, only homogenates containing lysed bacteria exhibited the capacity to exploit reduced sulfur compounds, while in *Bathymodiolus* intact bacteria had to be present for ATP synthesis to occur. *Riftia* trophosome had the highest ATP synthesizing capacity, followed by the gills of *Calyptogena* and *Bathymodiolus*.

The finding that sulfide was effective only in the case of *Riftia*, where the highest activities of SO_x were found, and where SO_x was clearly localized in the bacterial symbionts (Fig. 1), suggests that the tube worm may be the only one of these three symbioses to supply sulfide directly to its bacterial symbionts.

In the two bivalves, where sulfide was not effective as an energy source for driving ATP production under our experimental conditions, the abilities of sulfite and thiosulfate to stimulate ATP synthesis suggest that only oxidation products of sulfide may be delivered by the animal to its symbionts. The sulfide oxidizing activities found in

the gills of the two vent bivalves could not be localized histochemically due to the very small sizes of the bacteria (Table III). Therefore, it is not clear whether the measured SO_x activities are animal or bacterial. However, the finding that in gills of *Solemya reidi* all of the SO_x activity is found in the animal compartment (Powell and Somero, 1985) is an interesting precedent in this context. Perhaps in the vent bivalves as well the initial step(s) in sulfide oxidation occur in the animal tissue.

Belkin *et al.* (1986) found sulfide to be the most effective energy source for driving net CO₂ fixation in trophosome of *Riftia*, and thiosulfate to be the most effective energy source in *Bathymodiulus*. Our findings on the relative abilities of reduced sulfur compounds to drive ATP synthesis agree with their results, and we suggest oxidation of sulfide by the animal tissues as the source of thiosulfate for the symbionts of *Bathymodiulus*.

If in the bivalves the initial step(s) in sulfide oxidation occur in the animal compartment of the symbiosis, not in the bacteria, then the possibility exists that mitochondrial ATP production might be driven by the energy released in sulfide oxidation. This is the case for mitochondria of *Solemya reidi* (Powell and Somero, 1986). Because the homogenization procedures we used in the studies of the three vent symbioses would have ruptured mitochondria, we cannot exclude the possibility that mitochondria in both symbiont-containing and symbiont-free tissues are able to exploit the energy released in mitochondrial sulfide oxidation to drive ATP synthesis. We plan to examine this possibility during future studies of the vent animals.

In conclusion, our studies show that, despite many similarities, the three symbioses studied also exhibit important differences, *e.g.*, in preferred reduced sulfur compounds for driving ATP synthesis, in the absolute capacity for ATP synthesis in the symbiont-containing tissues, and in mechanisms for transporting and detoxifying sulfide. The three symbioses may also differ in the relative roles played by the bacterial symbionts in the nutritional needs of the animal. This is suggested by both anatomical and biochemical results. *Riftia* entirely lacks a digestive system (Jones, 1981), and the unusual carbon isotope ratios of its tissues, ratios which are the same in symbiont-containing and symbiont-free tissues (Rau, 1981), suggest a strong reliance on the reduced carbon compounds translocated from bacterial to animal cells. *Calyptogena* has a greatly reduced digestive system (Boss and Turner, 1980), and has more bacterial endosymbionts per mass of gill than *Bathymodiulus*. The activities of SO_x and ATP synthesis are higher in the clam than in the mussel as well. *Calyptogena* is found exclusively at sites of active venting (see Arp *et al.*, 1984), whereas *Bathymodiulus* is found in active venting sites and at sites peripheral to the main venting regions (see Hessler and Smithey, 1983). *Bathymodiulus* has a well-developed digestive system, and a ciliary-mucus net feeding capacity (Kenk and Wilson, 1985). Thus, where *Riftia* and *Calyptogena* may depend absolutely on a symbiotic source of reduced carbon compounds, *Bathymodiulus* may be able to exploit a greater variety of microhabitats in the vent ecosystem.

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