Reference: *Biol. Bull.* **180:** 135–153. (February, 1991)

Sulfide-Driven Autotrophic Balance in the Bacterial Symbiont-Containing Hydrothermal Vent Tubeworm, *Riftia pachyptila* Jones

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Abstract. Hydrothermal vent tubeworms, *Riftia pachyptila* Jones, were maintained alive and studied on board ship using flow-through pressure aquaria. Simultaneous measurements of O_2, ΣCO_2, ΣH_2S fluxes showed that the intact symbioses reach maximum rates of uptake of ΣCO_2 (>2 μmole g^{-1} h^{-1}) at about 90 μM ΣH_2S. Measurements were made of hemolymph and coelomic fluid ΣCO_2, ΣH_2S, thiosulfate, pH, and hemoglobin concentrations in worms kept under various conditions of O_2 and ΣH_2S. Normal hemolymph pH appears to be about 7.5 and is not affected by ΣH_2S and ΣCO_2 concentrations within the ranges observed. We conclude that *Riftia* is specialized to provide sulfide to its symbionts with minimal interaction of sulfide with the animal metabolism. The uptake of sulfide is apparently by diffusion into the hemolymph, facilitated by the sulfide-binding properties of the hemoglobins. Both ΣCO_2 and P_{CO_2} are elevated in the hemolymph above their levels in the medium, although they are reduced under autotrophic conditions. Thus inorganic carbon is apparently concentrated from the medium into the hemolymph by an unknown mechanism.

Introduction

The giant hydrothermal vent tubeworm, *Riftia pachyptila* Jones, is perhaps the most distinctive of the animals living around the deep-sea hydrothermal vents. Like all vestimentiferan tubeworms, adults of this species lack a mouth and a gut (Jones, 1981, 1988; Jones and Gardiner, 1988; Southward, 1988). The adult worms appear to derive their nutritional needs from the large population of sulfur-oxidizing chemolithoautotrophic bacterial symbionts that live in cells within a specialized organ—the trophosome—in their trunk (Cavanaugh *et al.,* 1981; Felbeck, 1981). The trophosome is a highly vascularized organ lying between two coelomic cavities that contain a hemoglobin-rich fluid (Jones, 1988). This anatomy requires that the animal supply the needs of the symbionts through its circulatory system (Arp *et al.,* 1985; Felbeck and Childress, 1988). Because these symbionts are sulfide-oxidizing autotrophs (Felbeck, 1981; Belkin *et al.,* 1986; Fisher *et al.,* 1989), the worm must take up sulfide, oxygen, and carbon dioxide from the medium and transport them to the symbionts. These substances can be taken up from the water by the large obturacular plume, a highly vascularized organ that has a large surface area and brings the hemolymph very close to the surrounding water (Arp *et al.,* 1985; Jones, 1988). The hemolymph and the coelomic fluid both have abundant extracellular hemoglobins which are believed to play a key role in the transport of all three of these metabolites (Childress *et al.,* 1984; Arp *et al.,* 1985).

Two hemoglobins are found in the extracellular fluids of these worms. One has a molecular weight of about 1.7 $\times 10^6$ M_r and is found primarily in the hemolymph, while the second is smaller (0.4 $\times 10^6$ M_r) and is found in both the coelomic and vascular compartments (Terwilliger *et*

Received 10 September 1990; accepted 24 November 1990.

al., 1980; Arp and Childress, 1981; Terwilliger and Terwilliger, 1985; Arp *et al.*, 1987). Both hemoglobins bind oxygen and sulfide reversibly with a high affinity (Arp and Childress, 1981, 1983; Childress *et al.*, 1984; Arp *et al.*, 1987; Fisher *et al.*, 1988a). The sulfide binding does not affect the simultaneous binding of oxygen, and appears to occur at a site removed from the heme (Childress *et al.*, 1984; Arp *et al.*, 1987). When sulfide and oxygen are below saturation in the hemolymph, their normally rapid, spontaneous reaction is suppressed (Fisher and Childress, 1984). Further, the hemoglobin can protect the animal tissues from sulfide toxicity by binding the sulfide with a higher affinity than does the site of toxic effects, cytochrome-c-oxidase (Powell and Somero, 1983, 1986). The hemoglobin does, however, release sulfide to the symbionts while simultaneously protecting them from sulfide toxicity by holding free sulfide concentrations down (Fisher and Childress, 1984; Fisher *et al.*, 1988a, 1989). The hemoglobins also buffer the hemolymph for carbon dioxide transport (Childress *et al.*, 1984). Thus, the hemolymph apparently has the properties required to take oxygen, carbon dioxide, and sulfide from the medium and to transport them to the endosymbionts.

Most of the experiments described in this paper were performed to test the role of the hemolymph in gas uptake and transport in intact, living *Riftia pachyptila* individuals. In particular, we were concerned with demonstrating the continuous uptake and oxidation of sulfide by the intact organisms, evaluating the role of the hemoglobins in concentrating sulfide from the medium, looking for the possible roles of other forms of sulfur in the symbiosis, examining the impact of sulfide and symbiont autotrophy on internal CO_2 pools and pH, and observing the pattern of exchange of gases between the coelomic fluid and the hemolymph.

A consistent chemical terminology will be used throughout this paper. Sulfide and inorganic carbon refer to these substances without specifying the chemical species involved. ΣH_2S and ΣCO_2 refer to the amounts of these gases analyzed from acidified samples using the analytical methods described below. They are measures of the sum of the various chemical forms in which these substances are found. H_2S, HS^-, S^{2-}, S^0, CO_2, HCO_3^- and any other chemical formulae refer only to the chemical species symbolized. "Free" refers to that fraction of a substance in the body fluids that is not bound to the hemoglobins.

Materials and Methods

The tubeworms used in these studies were collected from depths of about 2600 m at sites on the Galapagos Rift (00°48.247′N, 86°13.478′W) and the East Pacific Rise (12°48′N, 108°57′W) by deep submersibles (*Alvin* at the Galapagos Rift site and *Nautile* at the East Pacific Rise site). Both submersibles pulled the worms off the rocks using their manipulators, placed them in thermally insulated containers, and brought them to the surface about 2 to 8 h after capture. Once at the surface the worms were quickly transferred to cold seawater (7°C) where undamaged worms were set apart for the whole animal experiments described here. The worms chosen were then carefully removed from their natural tubes and placed in straight plastic tubes of appropriate size so they could be fitted into the pressure vessels necessary for their maintenance. They were then quickly placed in pressure aquaria.

The worms were routinely maintained in flowing-water pressure aquaria (Quetin and Childress, 1980) at 200 atm pressure, 8°C, and more than 100 μM O_2. Water was pumped through these stainless steel pressure vessels at about 12 l/h. Previous studies (Childress *et al.*, 1984) and preliminary observations during this study indicate that although the worms live at a hydrostatic pressure of about 260 atm at these sites, they are able to survive and display apparently "normal" behavior at pressures as low as about 100 atm. The symbionts themselves do not show significant effects of pressure on carbon fixation rates within the pressure range used here (Fisher *et al.*, 1989). In the present study pressures as low as 120 atm were used in some experiments, but the experience cited above suggests that these lower pressures should have little effect on the results.

Studies involving the maintenance of the worms at known sulfide concentrations were carried out in flowing-water aquaria (120 atm, about 4 l/h) using transparent acrylic pressure vessels (Quetin and Childress, 1980), allowing the activity of the worms to be observed during the experiments. Anaerobic sulfide stock solution (5 or 10 mM sodium sulfide in seawater at ph 7.0 or 7.5) was added continuously at the intakes of the pressure pumps with low pressure metering pumps to achieve stable sulfide concentrations in the pressure vessels. The effluent water from the vessels was periodically sampled with a 0.5 ml glass syringe, and the gases were analyzed by gas chromatography (Childress *et al.*, 1984). The pH of the effluent water was measured with a double junction electrode and was between 7. and 8.1, depending on the experiment.

Metabolism measurements

Measurements of whole animal metabolism were made in a flowing water system similar to that used by Anderson *et al.*, (1987), but adapted for use at the high pressures required for the survival of the worms. The system pumped seawater through the respirometer chambers using HPLC pressure pumps with small acrylic pressure vessels as respirometer chambers. The water in this system was first passed through a series of filters (5.0 and 0.2 μm) and a UV sterilizer. It was then continuously mixed by

means of metering pumps with an antibiotic solution to achieve a final concentration of 150 mg penicillin-G per liter and with a sulfide solution (pH 7.5 in seawater) to achieve the desired sulfide concentration. It then went to a vertically oriented column measuring 1 × 0.1 m, with the seawater entering at the top and exiting near the bottom. The pH of the water in the column was maintained at 7.5 by a pH controller that pumped 1 *M* acid (HCL) or base (NaOH) into the column. Oxygen and N_2 bubbled via the bottom of the column mixed the water in the column while maintaining the desired O_2 concentration. The water was then pumped through the respirometer chambers to a gas chromatograph for analysis. Two respirometer streams were continuously used in these measurements, one with animals in the respirometer chamber and the other an identical system without animals, which served as a control for spontaneous oxidation of sulfide. Fluxes of the measured gases due to the animals were calculated from the differences in gas concentrations in the water exiting the experimental and control chambers. These experiments were carried out at 130 atm hydrostatic pressure.

Ammonium flux was measured for several worms while they were in the respirometer system described above. The ammonium concentrations in the effluents from the two chambers were measured by flow injection analysis (Willason and Johnson, 1986).

Dissection procedure

Worms were dissected so that samples of hemolymph, coelomic fluid, and trophosome could be obtained for further analysis. Worms to be sacrificed were quickly removed from the pressure aquaria and the plastic tubes and then stretched out in a dissecting tray. The body wall below the vestimentum was carefully slit for a few centimeters parallel to the main axis of the worm on the ventral side. A sample of coelomic fluid (1–5 ml) was quickly drawn, with a blunt needle, from the pool of this fluid in the coelomic space and placed on ice. Subsamples for the various analyses were quickly taken. The remaining coelomic fluid was then drained from the worm, and a 1-ml syringe with a 30-ga needle was used to remove hemolymph from the major dorsal vessel leading from the trophosome to the plume of the worm. Aliquots of this post-trophosome (pre-branchial) hemolymph sample were quickly taken for the various analyses. Samples of trophosome tissue were also frozen for later analysis of elemental sulfur. If the trophosome appeared "unhealthy" [the pinkish appearance correlated with lack of CO_2 fixation in trophosome preparations (Fisher *et al.*, 1989), occurred in 7 of the 50 animals used] for an individual worm, the data from that worm were excluded from further consideration. These unhealthy worms were always characterized by low (<7.0) hemolymph pH values.

Analytical methods

Gas chromatographic methods similar to those described by Childress *et al.* (1984) were used to analyze gases in body fluids and seawater. Briefly, water samples were acidified with phosphoric acid, and gases were stripped from them using a glass and teflon extractor, in-line with a thermal conductivity gas chromatograph. This system allowed the analysis of the O_2, CO_2, H_2S, N_2, CH_4, and CO concentrations in fluid samples of 0.2 to 1.0 ml. The limit of sensitivity for these gases was between 1 and 20 μM, depending on the gas and the sample size. Throughout this paper, the terms ΣH_2S and ΣCO_2 refer to the amounts measured using this analytical method without regard for the chemical species present at the very different pH values and conditions in the worms.

To measure pH, a sample of hemolymph or coelomic fluid was drawn from an animal with a syringe. The dead space of the syringe was filled with blood by drawing a small amount of sample into the syringe and then expelling the air and excess blood before drawing the sample for analysis. Without air exposure, the sample was immediately injected into a Radiometer glass capillary electrode (Radiometer America G298A) used in conjunction with a reference electrode (Radiometer K171) in a water jacketed chamber. Precision buffers (Radiometer S1500 & S1510) were used to calibrate the electrode.

The abundances of the two hemoglobins in the hemolymph were quantified by separating them by HPLC gel filtration and measuring the absorbance as they eluted from the column (Arp *et al.*, 1987). A TSK-50 column, 7.5 mm in diameter and 300 mm long, was used with a TSK guard column (7.5 mm by 75 mm). The eluent was a citric acid/phosphate buffer (1.63 g citric acid and 26.17 g KH_2PO_4/l) at pH 7.5, pumped at 0.3 ml/min at 5°C. The run time was about 40 min, and an undiluted 1-μl sample was used. The absorbance was measured at 415 nm as the eluent left the column.

Determinations of thiosulfate and other unbound thiols in the body fluids were made by HPLC analysis of samples derivatized by monobromobimane using the methods of Newton *et al.* (1981) and Fahey *et al.* (1983) as modified by Vetter *et al.* (1989). Derivatives were separated on a 15 cm C-18 reversed phase column and detected using a 235 nm filter for excitation and a 442 nm filter for detection of fluorescence. The eluent flow rate was 1.5 ml per min, using an increasing hydrophobic gradient of HPLC grade methanol and 2% acetic acid, starting at 10% methanol and increasing to 100% during the run.

Elemental sulfur in the extracts was quantified by gas chromatography according to the method of Richard *et al.* (1977) as modified by Fisher *et al.* (1988b). Pieces of tissue (0.5–2.0 g wet weight) were dried for 18 h in a 100°C drying oven, and then extracted for 24 h with cyclohexane

in a micro-Soxhlet apparatus. The extracts were "cleaned up" by passing them through a fluorosil column to remove lipids, and concentrated by evaporation. The injector temperature was 240°C, and the initial column temperature was 150°C, programmed to 220°C during the separation. A six foot (1.8 m) glass column with a 2 mm bore, packed with 5% SP2401 on 100/120 mesh Supelcoport, was used to separate sulfur. The sulfur was detected and quantified using a thermal conductivity detector. The detection limit for elemental sulfur was *ca.* 0.001% of the dry weight of the sample (depending somewhat on sample size). The identity of the separated sulfur was confirmed by the distinctive smell of sulfur vapor coming out of the gas chromatograph detector at the time of the putative sulfur peak.

Estimation of free ΣH_2S and H_2S

Because ΣH_2S, pH, and hemoglobin contents were measured, it was possible, using previously published data, to estimate the concentration of free (unbound) ΣH_2S as well as the various species of sulfide. Free ΣH_2S was estimated by using the Hill equation describing the relationship between fractional saturation and free sulfide measured at 6°C, pH 7.5 in a mixture of coelomic fluid and hemolymph (Fisher *et al.*, 1988a): ln [% saturation/(100 − % saturation)] = 0.737(ln free ΣH_2S μM) − 1.778.

To use this equation, the capacity of each fluid sample to bind sulfide was estimated by multiplying the small hemoglobin aggregate concentration by one sulfide/heme and the large aggregate concentration by three sulfides/heme. These estimates were derived from a multiple regression of the sulfide concentrations in nine coelomic fluid samples dialyzed at saturating sulfide concentrations against the concentrations of the two aggregates in those samples (data from Arp, 1987). This regression had an r^2 of 0.97 and gave coefficients of 0.90 ± 0.27 (95% C. I.) and 2.97 ± 0.86, respectively, for the two hemoglobins. From the estimated capacity for binding sulfide and the measured ΣH_2S in the fluid, the percent saturation was approximated, and the above equation was solved for free sulfide. This approximation of free sulfide was then used with the estimated sulfide binding capacity [% saturation = 100 (bound sulfide/binding capacity)] in the Hill equation to estimate the sulfide bound to the hemoglobin. This procedure was then carried through several iterations until the estimates converged on a single value for free sulfide. This value was then used to calculate the percentage saturation of the fluid.

The free H_2S in each sample was calculated from the free ΣH_2S using a pK_1 value (8°C, 35‰ and 120 atm) of 6.784 (Millero, 1986; Millero *et al.*, 1988) and the pH measured in that particular sample.

Estimation of P_{CO_2}

Because ΣCO_2, pH, and hemoglobin contents were measured, it was possible, using previously published data, to estimate the P_{CO_2} in the fluids. The data relating pH, ΣCO_2, and P_{CO_2} in *Riftia* coelomic fluid at 10°C (Childress *et al.*, 1984) were used as the basis of a family of curves that predict P_{CO_2} from pH and ΣCO_2. However, although the coelomic fluid and hemolymph are quite similar in ionic composition, the hemolymph often has much higher hemoglobin content. Because hemoglobin is the only protein in any concentration in the hemolymph (Arp *et al.*, 1987), we used the concentration of heme as an indicator of protein content in these fluids. An approximate correction factor for hemoglobin concentration was developed by equilibrating subsamples, brought to different concentrations in *Riftia* saline, of the same hemolymph sample with gases of known P_{CO_2} and then measuring the ΣCO_2 in these subsamples using the gas chromatographic method. These subsamples (0.909 and 3.554 mM heme) were equilibrated with 2.09 torr P_{CO_2} and a final pH of 7.70 (Arp *et al.*, 1987). These measurements indicated that the effect of heme concentration on ΣCO_2 in this range was 0.50 mmole ΣCO_2/mmole heme. This was added to the final equation used to calculate P_{CO_2} as a factor that changed the slope of the relationship between P_{CO_2} and ΣCO_2 at different pH values. The equation was: P_{CO_2} = (4.199 − 0.537 pH) + ΣCO_2[(e^(−1.845 pH + 13.396)] + 0.0667(heme − 0.79). P_{CO_2} in the medium was estimated from the medium pH and ΣCO_2 with the pK_{app} estimated from the equation given by Heisler (1984), and αCO_2 (0.06345) at 8°C (Skirrow, 1975).

Statistical methods

Statistical analyses were carried out using Statview SE+ and SuperANOVA (Abacus Concepts) and Fastat (Systat Inc.). The Kendall rank correlation was used to test for a relationship between two parameters without any assumptions about the form or linearity of the relationship. Testing for differences in the medians in paired data sets employed the Wilcoxon signed rank test. The Mann-Whitney U test was used to test for differences in medians between unpaired datasets. Simple and multiple linear regressions of raw and in transformed data were used to describe the relationships between parameters.

Results

Whole animal metabolism

Due to a variety of equipment problems, only one such experiment was successfully conducted. In this experi-

ment, two worms (8.7 and 5.0 g) were run in their natural tubes in one chamber for 68 h. This experiment was started at 13.5°C without sulfide. After 6 h, sulfide was added continuously and 14 h later the animals showed net ΣCO_2 uptake (autotrophy). For the next 20 h the effects of different sulfide concentrations on the fluxes of O_2, ΣH_2S and ΣCO_2 were measured while maintaining O_2 between 105 and 209 μM (Fig. 1B). After that time the temperature of the system was lowered to 8.4°C over 2 h, and a similar set of measurements repeated at O_2 concentrations between 72 and 211 μM during the next 24 h (Fig. 1A). The set of observations at 8.4°C started at 92 μM ΣH_2S, decreased in steps to 0.0 μM ΣH_2S, and then was then raised to 41–49 μM ΣH_2S for 6 h. As can be seen in Figure 1A, the lower ΣH_2S concentrations resulted in less uptake of ΣCO_2, and without added sulfide the ΣCO_2 balance was fully heterotrophic (+3.05 μmoles ΣCO_2 $g^{-1}h^{-1}$). For the first three hours after the reintroduction of sulfide, this balance remained heterotrophic (+1.93 μmoles ΣCO_2 $g^{-1}h^{-1}$, high point at 43 μM ΣH_2S in Fig. 1A), but autotrophy was reached in the next 3 h (−0.64 μmoles ΣCO_2 g^{-1} h^{-1}, at 49 μM ΣH_2S in Fig. 1A). The worms were then removed and their tubes replaced in the vessels. The tubes alone did not show significant ΣCO_2 flux (<0.1 μmole ΣCO_2 g^{-1} worm h^{-1}) in the presence of 130 μM ΣH_2S. When the worms were dissected after the experiment, S° was visible in their trophosomes.

These data demonstrate that these worms were dependent on ΣH_2S levels greater than about 50 μM to break even on carbon flux and more than 90 μM was required for maximum uptake of ΣCO_2. They also show that the lag-time for changes in ΣCO_2 flux when ΣH_2S was removed was short, suggesting that use of stored S° was not quantitatively very important. In contrast, when sulfide was introduced after an absence, the lag time was relatively long (3–14 h).

Both O_2 and ΣCO_2 flux were significantly dependent on the ΣH_2S flux (Fig. 1C). The slope of the line relating ΣCO_2 flux to ΣH_2S flux was 0.92 ± 0.18 (95% C. I.) indicating that 0.92 mole CO_2 was fixed for each mole H_2S consumed. The slope of the line relating O_2 flux to ΣH_2S flux (Fig. 1C) was 1.14 ± 0.17 (95% C. I.), indicating that 1.14 mole O_2 was consumed for each mole ΣH_2S consumed. The lines relating ΣCO_2 and O_2 fluxes to ΣH_2S flux both intercept the y-axis at virtually the fluxes found for the worms in the absence of sulfide. This indicates that sulfide does not interact with the metabolism of carbon or O_2 by the animal tissues. The R. Q. in the absence of sulfide is 0.83 suggesting a metabolism based on a mixture of the major substrates.

The autotrophic *Riftia* experiment described above failed to show net uptake of N_2, supporting other negative data that this species' symbionts do not fix N_2. A preliminary study has also been carried out on ammonia flux

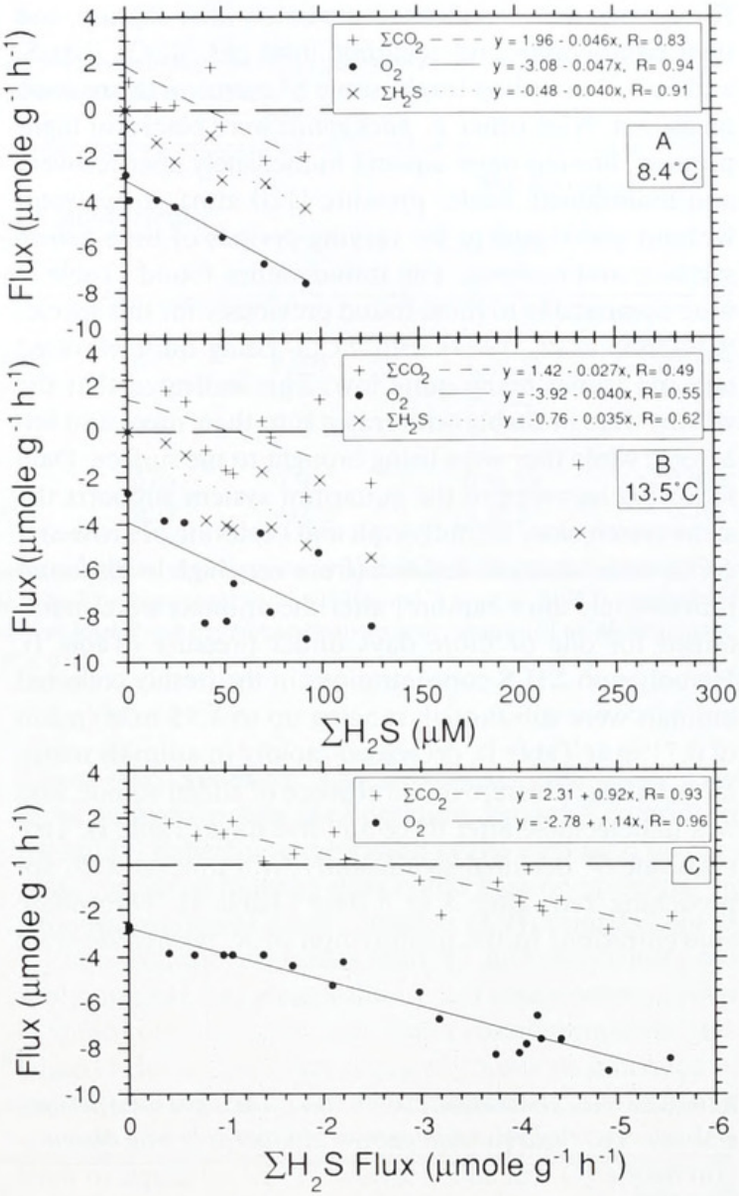

Figure 1. *Riftia pachyptila* metabolic fluxes in a flowing water, pressure respirometer system. Closed circles are oxygen fluxes, x symbols are sulfide fluxes, and crosses are CO_2 fluxes. (A) Fluxes presented as functions of the ambient sulfide concentrations measured at 8.4°C. (B) Fluxes presented as functions of the ambient sulfide concentrations measured at 13.5°C. (C) Fluxes at both temperatures combined, presented as functions of sulfide consumption rate as manipulated by controlling the sulfide concentration around the worms.

in *Riftia*. Three different animals (3.3, 6.1, and 17.2 g wet weight) in flowing water pressure respirometers in heterotrophic carbon balance showed appreciable rates of ammonia excretion (0.07, 0.19, and 0.27 μmol g^{-1} h^{-1}, respectively).

Hemolymph parameters after capture and maintenance without sulfide

In these experiments, several properties related to autotrophic metabolism in *Riftia pachyptila* were followed over time, after capture and recovery of the tubeworms.

Five worms were sacrificed immediately after capture, and their hemolymph and coelomic fluid pH, ΣCO_2, ΣH_2S, and $S_2O_3^{2-}$ as well as trophosome S° concentrations were measured. Nine other *R. pachyptila* were placed in high-pressure, flowing-water aquaria immediately after recovery and maintained, under pressure (120 atm), in seawater without added sulfide for varying periods of time before sacrifice and analysis. The initial values found (Table I) were comparable to those found previously for this species (Childress *et al.*, 1984) with ΣCO_2 being quite elevated and pH values being quite low. This indicated that the worms were probably withdrawn into their tubes and anaerobic while they were being brought to the surface. Data following recovery in the aquarium system supports the same conclusion. Hemolymph and coelomic pH rose and ΣCO_2 concentration declined (from very high levels found immediately after capture) after the animals were maintained for one or more days under pressure (Table I). Hemolymph ΣH_2S concentrations in the freshly collected animals were substantial, ranging up to 1.75 m*M* (mean of 0.71 m*M* Table I), decreased rapidly in animals maintained under pressure in the absence of added sulfide, and was undetectable after three and five days (Table I). Trophosome S° declined significantly with time as well, approaching zero after 3 to 5 days (Table I). Thiosulfate concentrations in the hemolymph of *R. pachyptila* were always very low (less than 36 μM, average = 24 μM) and did not decline during the five days in captivity (Table I).

To examine the hypothesis that the pattern of high ΣCO_2 and low pH found in the hemolymph of freshly recovered worms resulted from oxygen deprivation, we maintained two individuals for 24 h in the flowing water aquarium system at 14 μM O_2 and 15 μM ΣH_2S. Prior to this experiment these worms had been kept in the aquarium system for 2 days with no sulfide and more than 100 μM O_2. The hemolymph pH was depressed (6.48 and 6.82), supporting the suggestion that depressed pH values after recovery are the result of anaerobic metabolism (Childress *et al.*, 1984). The ΣCO_2 values were low (3.357 and 3.280 *mM*), but at the low pH values these represent high P_{CO_2} values (13.9 and 8.0 torr). The failure of these worms to accumulate the higher ΣCO_2 concentrations found in freshly recovered worms (Table I) probably resulted from their plumes remaining extended and thus continuing to exchange CO_2 with the medium during the experiment. In contrast, during recovery from the bottom, worms were constrained in a box and could not extend their plumes to exchange gases. This is consistent with observations that *Riftia pachyptila* individuals release substantial amounts of ΣCO_2 to the medium under hypoxic conditions (Childress *et al.*, 1984). The hemolymph ΣH_2S contents were substantial (5.497 and 5.013 m*M*)

Table I

Riftia pachyptila *hemolymph, coelomic fluid, and trophosome parameters immediately after capture and after maintenance in the absence of sulfide in flowing water, pressure (120 atm) aquaria*

Days after capture	n	Tissue	pH	ΣCO_2 (mmoles/l)	ΣH_2S (mmoles/l)	$S_2O_3^{2-}$ (mmoles/l)	S° (%wet wt.)
0	5	Hemolymph	7.07 ± 0.07	10.37 ± 1.05	0.714 ± 0.332	0.024 ± 0.004	
	5	Coelomic	7.14 ± 0.78	11.56 ± 2.81	0.089 ± 0.087	0.013 ± 0.017	
	5	Trophosome					2.76 ± 1.38
1	1	Hemolymph	7.39	7.78	0.066	0.000	
	1	Coelomic	7.48	8.67	0.000	0.000	
	1	Trophosome					1.94
3	2	Hemolymph	7.38, 7.47	5.48, 9.45	0.000, 0.000	0.000, 0.013	
	2	Coelomic	7.42, 7.39	6.39, 9.17	0.000, 0.000	0.000, 0.000	
	2	Trophosome					1.75, 0.03
5	5	Hemolymph	7.49 ± 0.40	5.91 ± 0.25	0.000 ± 0.000	0.014 ± 0.013	
	5	Coelomic	7.59 ± 0.12	5.91 ± 0.56	0.000 ± 0.000	0.003 ± 0.006	
	5	Trophosome					0.092 ± 0.21
Test of change over time in captivity (Kendall rank correlation *tau*, P =)							
		Hemolymph	0.67, 0.0014	−0.77, 0.0005	−0.70, 0.0009	−0.29, 0.19	
		Coelomic	0.71, 0.0007	−0.77, 0.0005	−0.55, 0.0085	−0.20, 0.35	
		Trophosome					−0.54, 0.0097

"n" indicates the number of worms and samples at each time period and the parameter values are shown as mean ± standard error of the mean. ΣCO_2 and ΣH_2S indicate the total concentration of all forms of these substances, released by acidification of the samples in the process of analysis. The Kendall rank correlation tests the significance of changes over time in captivity (underlined *tau* values indicate $P < 0.05$) and are listed beneath each parameter tested.

Table II

Coelomic fluid hemoglobin concentrations as functions of hemolymph hemoglobin concentrations in Riftia pachyptila *after maintenance (24 h) in high-pressure (120 atm), flowing-water aquaria at various fixed ΣH_2S concentrations ≥0.0 and ≤800 μM.*
Data on freshly collected worms from Arp et al. *(1987)*

Parameter	n	[coelomic] = a + b[hemolymph]				Wilcoxon signed-rank	
		b ± 95% CI	a	r^2	*P*	Coel:Hemo +, =, −	*P*
All Worms							
Heme (m*M*)	34	0.304 ± 0.204	0.873	0.47	0.005	1, 0, 33	<0.0001
Hemoglobin FI (m*M*)	34			0.04	0.145	0, 0, 34	<0.0001
Hemoglobin FII (m*M*)	34			0.02	0.187	18, 0, 16	0.228
Data from Arp *et al.* (1987)							
Hemoglobin FI (m*M*)	18			0.05	0.70	0, 0, 18	0.0002
Hemoglobin FII (m*M*)	18	0.413 ± 0.276	0.633	0.39	0.0059	11, 0, 7	0.013

"n" indicates the number of worms and samples and the regression coefficients are shown ±95% confidence intervals (CI). FI is the large hemoglobin aggregate (1,700,000 M_r) and FII is the smaller aggregate (400,000 M_r) described by Terwilliger *et al.* (1980) and Arp *et al.* (1987). Underlined regression coefficients and Wilcoxon distributions are significant at the level of at least $P < 0.05$. Not all analyses were completed on all specimens. Regressions are given only when they are significant at the level of at least $P < 0.05$.

and apparently in equilibrium (0.52 and 0.57 fractional sulfide saturation) with the external ΣH_2S (Fig. 5A, B). This is consistent with uptake being due solely to the binding of sulfide by the hemoglobins.

Functioning of tubeworms exposed to various concentrations of oxygen and sulfide

To test the existing hypotheses concerning carbon dioxide and sulfide transport in the hemolymph (Rau and Hedges, 1979; Arp and Childress, 1983; Childress *et al.*, 1984; Arp *et al.*, 1985; Felbeck, 1985; Fisher *et al.*, 1989; Fisher *et al.*, 1990) and to examine responses of this species to different external sulfide concentrations, a series of experiments were conducted in which individual tubeworms were maintained under different conditions before dissection and analysis. In these experiments, *R. pachyptila* individuals were maintained in the high-pressure flowing-seawater aquaria in the absence of sulfide for two days after capture. This allowed the worms to recover from capture and to metabolize most of their internal stores of inorganic sulfur compounds. These worms were then exposed continuously to constant concentrations of sulfide (0–805 μ*M* ΣH_2S) for 24 to 36 h while in the high-pressure (120 atm) flowing-seawater aquaria at 8°C. Oxygen concentrations in the seawater during these experiments were between 0 and 276 μ*M*. After the sulfide exposure, the worms were dissected and samples taken. The hemolymph and coelomic fluid samples were analyzed for pH, ΣCO_2, ΣH_2S, $S_2O_3^{2-}$, and the two hemoglobin fractions. Trophosome samples were analyzed for S°. Upon dissection, seven of the worms were found to have substantial amounts of trophosome that appeared to be in poor condition (see Materials and Methods), and these were dropped from further consideration, leaving 43 worms in the study. Extensive exploration of the data with scatterplots suggested that the data could best be presented in two groups; one of these consisted of 28 animals kept at O_2 concentrations greater than 42 μ*M* and whose hemolymph pH was greater than 7.2. These worms showed evidence of autotrophy and blood circulation (to be discussed below). The other group of 15 worms consisted of individuals kept at O_2 concentrations of 42 μ*M* or less (8 worms) and individuals whose hemolymph pH was less than or equal to 7.2 (11 worms). The low O_2 apparently limited sulfide oxidation while the low pH values apparently indicated anaerobic metabolism in the worms due to behavioral (remaining contracted in the tubes) or undetected physiological constraints. In the figures and tables to be presented, the numbers for each analysis are often less than the total number of individuals, because not all analyses were successfully executed on all specimens.

The heme contents of the hemolymph and coelomic fluid samples of the worms were significantly correlated, but the hemolymph samples had much higher heme concentrations than did the coelomic fluid ones (Table II). There was no significant correlation between the concentrations of the large hemoglobin (FI) in the two compartments, but the concentration in the hemolymph was always much higher (Table II, Fig. 2). In contrast, there was no significant difference in the concentrations of the small hemoglobin (F II) between the two compartments (Table II). However, because there was no significant correlation between the concentrations in the two compartments, it is apparent that they are not confluent. Arp *et al.* (1987) suggested that these two compartments may be

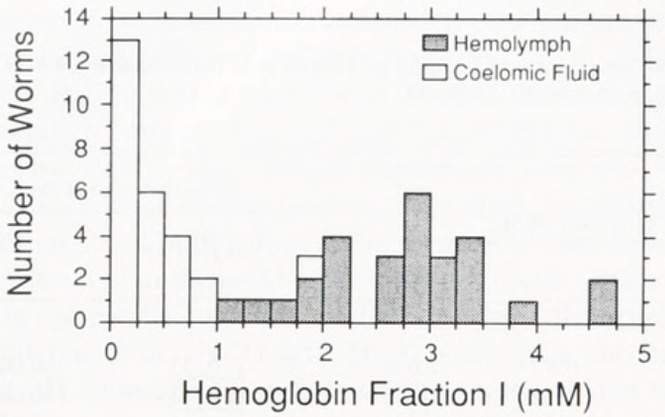

Figure 2. Frequency distributions of the concentrations of the large hemoglobin [FI, 1.7×10^6 M_r, (Terwilliger *et al.*, 1980; Arp *et al.*, 1987)] in coelomic fluid and hemolymph of *Riftia pachyptila* kept for 24 h at different external sulfide and O_2 concentrations.

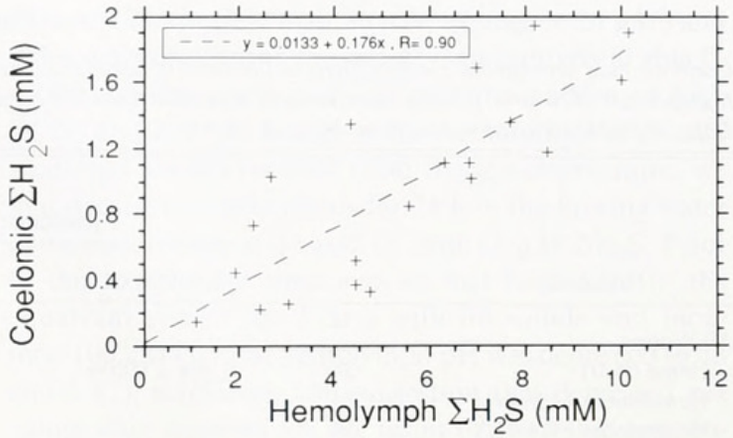

Figure 3. Coelomic fluid ΣH_2S as a function of hemolymph ΣH_2S in *Rifta pachyptila* kept for 24 h at different external sulfide concentrations and >42 μM O_2.

confluent at the size of the smaller hemoglobin, because the concentrations in the two compartments were significantly correlated. However, re-analysis showed that while the concentrations were significantly correlated in their data, the coelomic fluid had higher concentrations precluding confluence for this size molecule (Table II).

Although the two compartments do not exchange hemoglobin molecules, it is apparent that small dissolved molecules are readily exchanged, because the higher O_2, higher pH group had highly significant correlations between the values of all of the measured parameters between the two compartments (Table III). In addition, there were significant differences in the values of all of these parameters, except thiosulfate, between the compartments, apparently resulting from their interactions with the hemoglobins. ΣH_2S was in much higher concentrations in the hemolymph (Fig. 3) than in the coelomic fluid because of the much higher concentrations of hemoglobin FI, which binds 3 moles of sulfide per mole of heme, although this binding is not to the heme group itself (Arp *et al.*, 1987), *versus* 1 mole of sulfide per mole of heme for F II (Fig. 2). These correlations and distributions indicate that these worms were circulating their blood effectively. In contrast, the low O_2, low pH group had no significant correlations between the two compartments for these parameters, and the values were sig-

Table III

Riftia pachyptila *coelomic fluid parameters as a function of the same parameters in hemolymph after maintenance (24 h) of the worms in flowing water, pressure (120 atm) aquaria at various fixed ΣH_2S concentrations between 0.0 and 600 μM with external O_2 concentrations > 42 μM and hemolymph pH > 7.2*

		[coelomic] = a + b[hemolymph]				Wilcoxon signed-rank	
Parameter	n	b ± 95% CI	a	r^2	*P*	Coel:Hemo +, =, −	*P*
pH	26	0.841 ± 0.394	1.223	0.45	0.0002	16, 2, 8	0.042
ΣCO_2 (mM)	27	0.990 ± 0.105	0.520	0.94	<0.0001	25, 0, 2	<0.0001
P_{CO_2} (torr)	20	0.744 ± 0.171	0.638	0.82	<0.0001	6, 0, 14	0.04
ΣH_2S (mM)	27	0.176 ± 0.034	0.013	0.81	<0.0001	0, 7, 20	<0.0001
% Hb sulfide saturation	20	0.759 ± 0.123	−0.019	0.90	<0.0001	0, 7, 13	0.0015
Free ΣH_2S (mM)	19	0.140 ± 0.031	0.002	0.84	<0.0001	0, 6, 13	0.0015
Free H_2S (mM)	19	0.133 ± 0.024	0.0003	0.89	<0.0001	1, 6, 12	0.0024
$S_2O_3^{2-}$ (mM)	22	1.54 ± 0.25	−0.021	0.89	<0.0001	7, 3, 12	0.41

"n" indicates the number of worms and samples and the regression coefficients are shown ±95% confidence intervals (CI). ΣCO_2 and ΣH_2S indicate the total concentration of all forms of these substances, bound and free, released by acidification of the samples in the process of the analyses. Free ΣH_2S is an estimate of the free sulfide of all molecular species. Free H_2S is an estimate of the free (*i.e.*, unbound) concentration of this molecular species. Underlined Wilcoxon distributions are significant at the level of at least $P < 0.05$. These data include observations for all parameters for seven animals that were not exposed to sulfide during the experiment and had internal sulfide concentrations of zero. Not all analyses were completed on all individuals studied.

nificantly different for only three of the parameters (Table IV). Such lack of equilibration indicates a lack of opportunity for exchange between the two fluids, suggesting that circulation was impaired in this group of worms.

Because the hemolymph and coelomic fluid parameters were always parallel and closely correlated, and because the low O_2, low pH worms do not, for the most part, show signs of autotrophy and effective circulation, the hemolymph parameters from the higher O_2, higher pH worms will be emphasized in considering the responses of the internal parameters to external sulfide (Table V). The low O_2, low pH group (Table VI) will be considered primarily in contrast to the other group.

In the higher O_2 group, hemolymph and coelomic ΣH_2S were correlated with external ΣH_2S. They were at least one order of magnitude higher than the external concentration in all cases (Table V, VII, Fig. 4A), clearly demonstrating the ability of this worm to concentrate sulfide from its environment. However, their hemoglobin was maintained well below sulfide saturation at all external sulfide concentrations tested (Fig. 5A) showing 50% saturation at an external ΣH_2S of 122 μM as compared to an *in vitro* affinity of 50% saturation at 11.2 μM ΣH_2S (Fisher *et al.*, 1988a). The hemolymph free ΣH_2S and free H_2S also increased with external ΣH_2S, but remained about an order of magnitude lower than the external concentrations (Table V, Fig. 5B, C). Thus, although the ΣH_2S concentration in the hemolymph was much higher than outside the worm, there was a significant gradient from the outside to the inside for the free chemical species. The latter gradient could only be maintained by the consumption of sulfide within the worm, presumably by the symbionts.

In contrast, the low O_2, low pH group shows only three barely significant correlations between external ΣH_2S and any of the hemolymph sulfide parameters (Table VI). Further, the hemolymph sulfide saturation is close to that expected *in vitro*, and 50% saturation is close to the *in vitro* value (3.3 and 11.2 μM ΣH_2S, respectively, Fig. 5A). Thus, the ability of these worms to concentrate ΣH_2S in their hemolymph appears to be explained entirely by the binding of sulfide by the hemoglobins. In addition, the hemolymph free ΣH_2S and free H_2S were essentially in equilibrium distributions with the corresponding external parameters (Fig. 5B, C) indicating that no gradient for passive uptake exists in these worms. Symbiont sulfide oxidation had apparently essentially ceased under these conditions so the hemoglobins could no longer function to depress free sulfide concentrations.

It is also apparent from the data that sulfide is the only sulfur compound of importance in the hemolymph. Although thiosulfate was found in both groups, and increases significantly in the presence of external sulfide (Table V), it is typically less than hemolymph ΣH_2S by more than one order of magnitude (Fig. 4).

The hemolymph ΣCO_2 and P_{CO_2} values are both reduced at higher external ΣH_2S concentrations in the higher O_2 group (Table V, VII, Fig. 6A, B), suggesting the removal of inorganic carbon by the autotrophic symbionts. There were no significant relations between these parameters for the low O_2, low pH group (Table V, Fig. 6A, B). In addition, the internal P_{CO_2} was higher than the external under virtually all conditions (Fig. 6B, Table V, VI) precluding uptake by passive diffusion into the hemolymph.

Table IV

Coelomic fluid parameters as a function of the same parameters in hemolymph of Riftia pachyptila *after maintenance (24 h) in high-pressure (120 atm), flowing-water aquaria at various fixed sulfide concentrations between 0.13 and 800* μM *with O_2 concentrations ≤ 42* μM *or hemolymph pH ≤ 7.2*

		[coelomic] = a + b[hemolymph]				Wilcoxon signed-rank	
Parameter	n	b ± 95% CI	a	r^2	*P*	Coel:Vasc +, =, −	*P*
pH	15			0.19	0.105	11, 0, 4	0.125
ΣCO_2 (mM)	14			0.45	0.054	10, 0, 3	0.022
P_{CO_2} (torr)	13			0.05	0.47	0, 0, 13	0.0015
ΣH_2S (mM)	14			0.00	0.96	2, 0, 12	0.0019
% Hb sulfide saturation	13			0.03	0.56	5, 0, 8	0.34
Free ΣH_2S (mM)	13			0.04	0.49	5, 0, 8	0.60
Free [H_2S] (mM)	13			0.03	0.55	4, 0, 9	0.34
$S_2O_3^{2-}$ (mM)	7			0.04	0.691	5, 0, 2	0.13

"n" indicates the number of worms and samples and the regression coefficients are shown ±95% confidence intervals (CI). ΣCO_2 and ΣH_2S indicate the total concentration of all forms of these substances, released by acidification of the samples in the process of the analyses. Free ΣH_2S is an estimate of the unbound sulfide of all molecular species. Free [H_2S] is an estimate of the concentration of this molecular species. Underlined Wilcoxon distributions are significant at the $P < 0.05$ level. No regressions were significant at the $P < 0.05$ level and therefore none are listed.

Table V

Riftia pachyptila hemolymph parameters and S° in trophosome as functions of external conditions after maintenance (24 h) in high-pressure (120 atm), flowing-water aquaria at various fixed ΣH_2S concentrations greater than 0.0 and less than 600 μM (external O_2 concentrations > 42 μM and hemolymph pH > 7.2). Kendall correlations, but not the regressions or Wilcoxon tests, include seven individuals at 0.0 ΣH_2S

X Variable	Kendall correlation			[hemolymph parameter] = aX^b					Wilcoxon signed-rank	
Hemolymph Parameter	n	*tau*	*P* =	n	b ± 95% CI	a	r^2	*P*	External:Hemo +, =, −	*P*
X = External ΣH_2S										
pH	26	−0.165	0.24	19			0.05	0.38		
ΣCO_2 (m*M*)	27	−0.629	<0.0001	20	−0.224 ± 0.124	1.565	0.44	0.0014		
P_{CO_2} (torr)	26	−0.529	<0.0001	19	−0.170 ± 0.150	1.525	0.25	0.031		
ΣH_2S (m*M*)	27	0.826	<0.0001	20	0.448 ± 0.121	12.53	0.76	<0.0001	0, 0, 20	<0.0001
Sulfide saturation	19	0.772	<0.0001	18	0.417 ± 0.138	1.057	0.72	<0.0001		
Free ΣH_2S (m*M*)	25	0.861	<0.0001	18	1.05 ± 0.41	0.105	0.65	<0.0001	17, 0, 1	0.0002
Free H_2S	25	0.832	<0.0001	18	1.086 ± 0.438	0.0219	0.63	<0.0001		
$S_2O_3^{2-}$ (m*M*)	22	0.487	0.0015	15			0.11	0.13		
S° (% wet wt.)	24	0.504	0.0006	14			0.017	0.66		
X = External H_2S										
Free H_2S	25	0.694	<0.0001	18	1.053 ± 0.593	0.179	0.47	0.016	17, 0, 1	0.0004
X = External P_{CO_2}										
P_{CO_2} (m*M*)	25	−0.361	0.011	18			0.007	0.74	1, 0, 17	0.0002
X = External pH										
pH	26	0.123	0.38	19			0.018	0.68	14, 0, 5	0.0079

"n" indicates the number of worms and samples and the regression coefficients are shown ±95% confidence intervals (CI). ΣCO_2 and ΣH_2S indicate the total concentration of all forms of these substances, released by acidification of the samples in the process of the analyses. H_2S indicates only this chemical species itself. "Free" indicates an estimate of quantity present in a fluid but not bound to the hemoglobin. Only regressions that were significant at the $P < 0.05$ level are shown.

Hemolymph pH appeared to be unaffected by external or internal sulfide or external pH (Table V, VI, VII, Fig. 6C), although it is somewhat variable. This lack of interaction suggests that pH is not apt to be a significant factor in the uptake and distribution of sulfide or inorganic carbon from the environment into the hemolymph. The strong effect of low O_2 on hemolymph pH is probably due to the accumulation of acidic endproducts of anaerobic metabolism.

These various parameters followed the same trends in the coelomic fluid. However, the multiple regression analyses consistently showed that the strongest predictor of a chemical parameter in the coelomic fluid is not an external parameter but the corresponding parameter in the hemolymph. This indicates that the route of transport to the coelomic fluid is via the hemolymph and not the body wall.

Discussion

Autotrophy

Riftia pachyptila, like all vestimentiferan tubeworms, lacks a mouth and a gut as an adult, and as a result, its nutrition has been a matter of considerable investigation and speculation. While net ΣCO_2 uptake (autotrophy) by the intact symbioses between sulfur-oxidizing, autotrophic bacteria and marine invertebrates living around deep-sea hydrothermal vents has been widely assumed (Cavanaugh *et al.,* 1981; Felbeck, 1981, 1985; Felbeck *et al.,* 1981; Cavanaugh, 1985; Southward, 1987), the data presented here are the first actually to demonstrate this in a vestimentiferan tubeworm or any hydrothermal vent animal. Actual autotrophic balance for the tubeworm symbiosis of course depends on the reasonable assumptions that much of the fixed carbon is available to the animal tissues, and the production of mucus and loss of small organic molecules are not large compared to the rate of carbon fixation.

The only previous demonstration of autotrophic balance in an animal/bacterial symbiosis was for the shallow-living, gutless protobranch bivalve, *Solemya reidi* (Anderson *et al.,* 1987). In that case, the clams showed a maximum net ΣCO_2 uptake of 0.89 μmole g^{-1} h^{-1} which equals about 0.24% of the clam's total organic carbon per day. In contrast, *Riftia pachyptila* apparently has a considerably higher maximum rate of net ΣCO_2 uptake (2.74 μmole g^{-1} h^{-1} maximum in this study). This high rate, combined with the relatively low carbon content of 5.5%

Table VI

Hemolymph parameters and S° in trophosome as functions of external sulfide in Riftia pachyptila *after maintenance (24 h) in high-pressure (120 atm), flowing-water aquaria at various fixed ΣH_2S concentrations between 0.013 and 800 μM (external O_2 concentrations ≤ 42 μM or hemolymph pH ≤ 7.2)*

X Variable	Kendall correlation			[hemolymph parameter] = aX^b					Wilcoxon signed-rank	
Hemolymph Parameter	n	tau	*P* =	n	b ± 95% CI	a	r^2	*P*	External:Hemo +, =, −	*P*
X = External ΣH_2S										
pH	15	−0.154	0.42	15			0.00	0.97		
ΣCO_2 (m*M*)	15	−0.174	0.37	15			0.10	0.26		
P_{CO_2} (torr)	14	−0.223	0.91	14			0.02	0.61		
ΣH_2S (m*M*)	15	0.385	0.045	15			0.08	0.30	0, 0, 15	0.0007
Sulfide saturation	14	0.425	0.034	14	0.098 ± 0.088	0.885	0.33	0.033		
Free ΣH_2S (m*M*)	14	0.438	0.029	14	0.646 ± 0.523	0.270	0.38	0.020	8, 0, 6	0.55
Free H_2S	14	0.402	0.040	14	0.669 ± 0.562	0.100	0.36	0.023		
$S_2O_3^{2-}$ (m*M*)	7	−0.410	0.20	7			0.56	0.053		
S° (% wet wt.)	11	−0.185	0.94	11			0.028	0.62		
X = External H_2S										
Free H_2S	14	0.291	0.148	14			0.20	0.106	4, 0, 10	0.177
X = External P_{CO_2}										
P_{CO_2} (m*M*)	14	−0.205	0.31	14			0.005	0.82	0, 0, 14	0.001
X = External pH										
pH	15	−0.216	0.26	15			0.021	0.87	13, 0, 2	0.002

"n" indicates the number of worms and samples and the regression coefficients are shown ±95% confidence intervals (CI). ΣCO_2 and ΣH_2S indicate the total concentration of all forms of these substances, released by acidification of the samples in the process of the analyses. H_2S indicates only this chemical species itself. "Free" indicates an estimate of quantity present in a fluid but not bound to the hemoglobin.

of wet weight of this species (Fisher *et al.*, 1988b), results in a much higher estimate of 1.4% of the tubeworm's total organic carbon per day. This suggests a high potential growth rate in this species, which is supported by some evidence of growth in length *in situ* (Roux *et al.*, 1989). Very rapid growth has been hypothesized to be important in this species' apparent domination of young vent sites (Childress, 1988; Fustec *et al.*, 1988; Hessler *et al.*, 1988).

The maximum rate of carbon fixation (2.74 μmole ΣCO_2 g $worm^{-1}h^{-1}$) for the intact symbiosis corresponds to a rate of 17.9 μmole ΣCO_2 g $trophosome^{-1}h^{-1}$, assuming that trophosome accounts for 15.3% of the wet weight of the intact symbiosis (Childress *et al.*, 1984). This rate is in good agreement with the maximum rates of fixation of $H^{14}CO_3^-$ (13 to 28 μmol ΣCO_2 g $trophosome^{-1}h^{-1}$) observed in preparations of trophosome tissue from *Riftia pachyptila* containing viable endosymbiotic bacteria using sulfide as a substrate (Fisher *et al.*, 1989).

Substrates used by the symbiosis

Studies of the isolated symbionts of *Riftia pachyptila* have indicated that these symbionts use only sulfide and not thiosulfate as a source of externally derived reducing power (Belkin *et al.*, 1986; Fisher *et al.*, 1989; Wilmot and Vetter, 1990). The data presented in this paper support the view that vestimentiferan tubeworms are specialized to supply only sulfide to their symbionts (Childress *et al.*, 1984), because sulfide is concentrated from the medium and is quickly used by the symbionts (Fig. 4, 5, Table I). In contrast, thiosulfate, an endproduct of animal sulfide oxidation (Vetter *et al.*, 1987; O'Brien and Vetter, 1990), is always at a very low concentration in the hemolymph (Fig. 4) and is not quickly used by the symbiosis (Table I). The lack of interaction (either inhibition or utilization as substrate) of sulfide with the animal metabolism is also shown by the fact that the regressions of O_2 and ΣCO_2 fluxes *versus* ΣH_2S flux pass essentially through the values of O_2 and CO_2 flux measured in the absence of ΣH_2S (Fig. 1C). The low levels of sulfide oxidase activity reported from the body wall of *R. pachyptila* (Powell and Somero, 1986) are apparently of little significance in the overall metabolism of this species because so little thiosulfate is found in the body fluids. Thus, the animal metabolism has little interaction with sulfide, delivering it intact to the symbionts.

This arrangement is quite different from that of symbiont-containing bivalves, which appear to oxidize sulfide to thiosulfate and to supply this to the symbionts. *Solemya reidi* mitochondria can produce ATP from the oxidation of sulfide to thiosulfate (Powell and Somero, 1985; O'Brien and Vetter, 1990), which can then be supplied

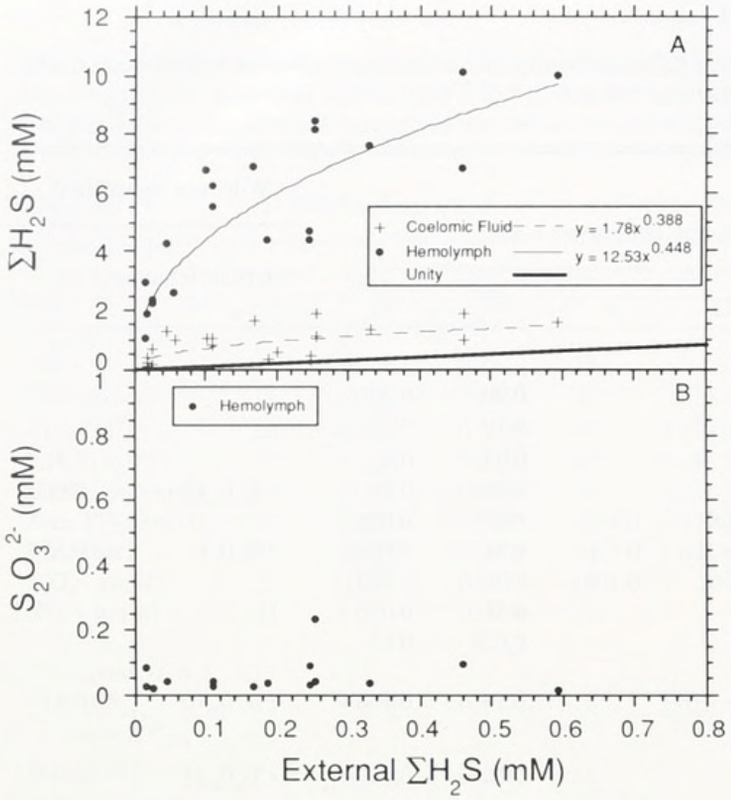

Figure 4. ΣH_2S and thiosulfate concentrations in hemolymph and coelomic fluid in *Riftia pachyptila* kept for 24 h at different external sulfide concentrations and >42 μM O_2. (A) The broad solid line represents equal concentrations of ΣH_2S outside the worm and in its fluids. The narrow solid line and the closed circles apply to the hemolymph while the dashed line and crosses apply to the coelomic fluid. (B) The closed circles represent the concentrations of thiosulfate in the hemolymph in these same worms.

to the symbionts to support their metabolism (Anderson *et al.,* 1987; Vetter *et al.,* 1989). In this species, the oxidation of sulfide apparently has substantial effects on the animal metabolism, reducing the animal carbon oxidation, which indicates that the sulfide oxidation is of metabolic significance to the animal tissues (Anderson *et al.,* 1987). Similarly, the symbionts of *Bathymodiolus thermophilus,* the vent mussel, and *Calyptogena magnifica,* the vent clam, both appear to be able to use thiosulfate to drive carbon-fixation (Belkin *et al.,* 1986; Childress *et al.,* 1991), and the animals involved accumulate substantial thiosulfate in their body fluids (Fisher *et al.,* 1988c; Childress *et al.,* 1991). Whether these other animals can obtain energy from the oxidation of sulfide remains to be tested.

Riftia pachyptila symbionts are dependent on the immediate availability of sulfide to drive significant rates of autotrophy. While the symbionts do store S° at concentrations up to 3200 μg atoms/g fresh weight (Fisher *et al.,* 1988b) and can oxidize the stored S° in the absence of external ΣH_2S (Table I), the metabolism experiment reported here demonstrated that both O_2 and autotrophic ΣCO_2 fluxes were dependent upon an external supply of ΣH_2S. The stored S° did not support a detectable rate of ΣCO_2 uptake for even a few hours. This is the same situation found in the bivalve *Solemya reidi* (Anderson *et al.,* 1987). Thus, it appears that while the substantial S° stores often found associated with sulfur-oxidizing symbionts (Vetter, 1985; Somero *et al.,* 1989) can be used by the symbionts, these rates are only a small fraction of the rates of oxidation of sulfide or thiosulfate. This may well be the case for free-living sulfur-oxidizing bacteria as well (Nelson *et al.,* 1986). S° sulfur stores may be of significance for the survival of the symbionts during times of sulfide deprivation, but apparently do not represent a significant store to support the symbiosis.

Respiratory fluxes in response to sulfide

Oxygen and ΣCO_2 fluxes in *Riftia pachyptila* are dependent upon the sulfide flux. Net ΣCO_2 uptake requires the presence of both O_2 and sulfide. About 90 μM ΣH_2S was necessary to reach the maximum ΣCO_2 uptake rate (Fig. 1), and external O_2 concentrations greater than 42 μM appeared to be necessary for the use of sulfide by the symbionts (Fig. 5). These concentrations are similar to those that stimulate maximal autotrophy in *S. reidi* (Anderson *et al.,* 1987). However, autotrophic ΣCO_2 uptake by the *R. pachyptila* symbionts in the intact symbiosis did not appear to be inhibited by external sulfide concentrations up to 600 μM (Fig. 5B), unlike that of the symbionts of *S. reidi,* which are inhibited in the intact symbiosis at external ΣH_2S concentrations of about 250 μM (Anderson *et al.,* 1987).

These environmental requirements of *R. pachyptila* appear to match closely the environmental conditions where the species is found. Where *Riftia* is in abundance, the flow of vent water is high, ΣH_2S can approach 350 μM in the vent water and O_2 in the ambient water is around 110 μM (Fisher *et al.,* 1988b; Johnson *et al.,* 1988b). *In situ* measurements of sulfide and O_2 distributions around the tubeworms have suggested that they take up sulfide from concentrations above about 60 μM and O_2 from concentrations above 70 μM, with maximal uptake rates from concentrations around 100 μM in both cases (Johnson *et al.,* 1988b). The worms gain access to both substrates at high concentrations because the water around them is not well mixed, and they are therefore exposed to conditions that fluctuate between vent water (15°C, 350 μM ΣH_2S, 0 μM O_2) and ambient water (2°C, 0 μM ΣH_2S, 110 μM O_2) on time scales of fractions of a second and longer (Johnson *et al.,* 1988a). This species then appears to be specialized for high rates of autotrophic function, and, as a result, it requires the high concentration and supply of sulfide associated with rapid venting. These stringent habitat requirements make *Riftia pachyptila* vulnerable to either natural reduction in vent flow over time or diversion of vent flow by mussels. *R. pachyptila*

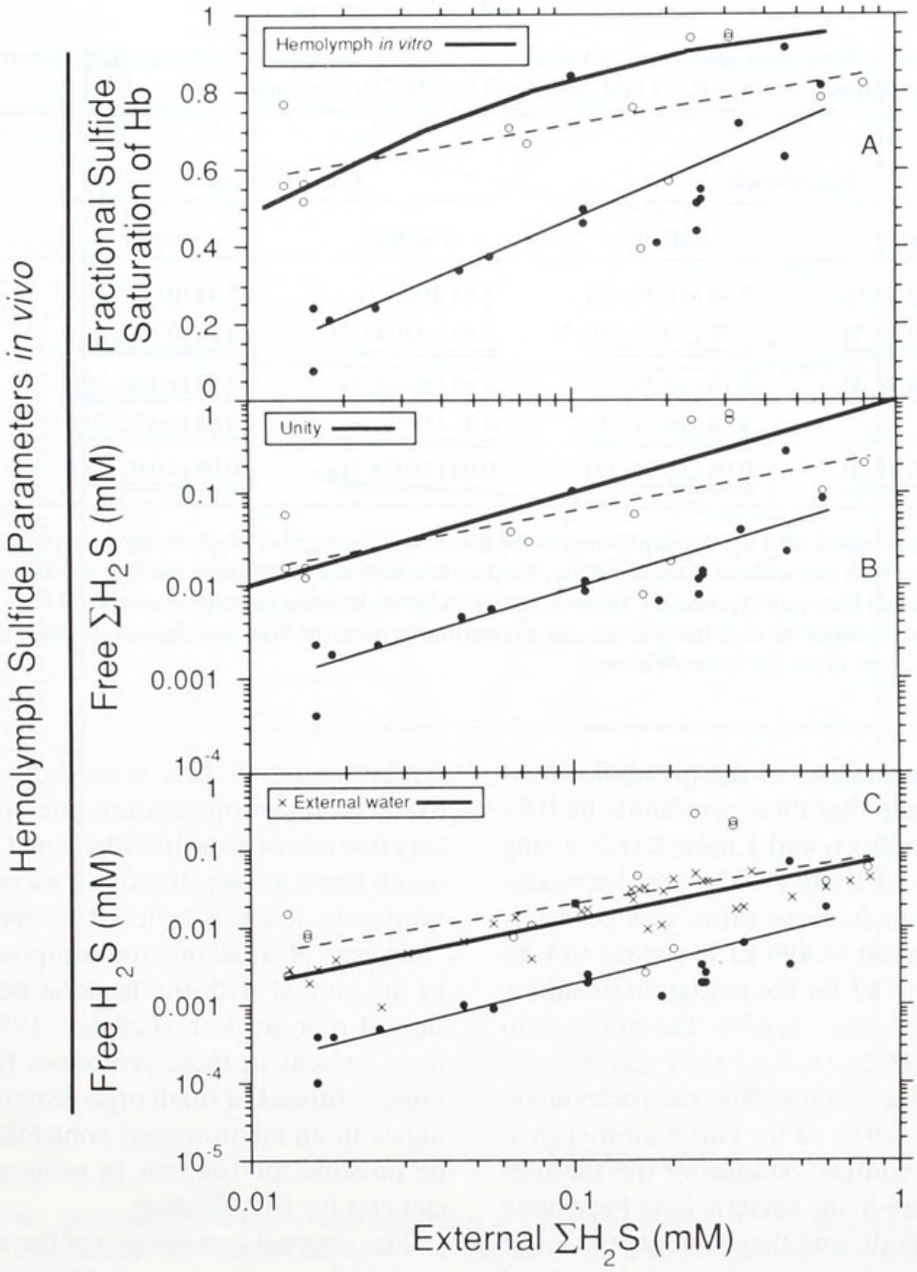

Figure 5. *Riftia pachyptila* hemolymph sulfide parameters as a function of external ΣH_2S concentrations in worms kept for 24 h at fixed ΣH_2S concentrations. Closed circles and narrow solid lines represent values from worms that were kept at >42 μM O_2 and had hemolymph pH values > 7.2. Open circles and dashed lines represent values from worms that were kept at ≤42 μM O_2 or had hemolymph pH values ≤7.2. Regression equations for the plotted lines may be found in Tables V and VI. (A) The fractional saturation of the hemoglobins *in vivo* with sulfide as estimated from the hemoglobin concentrations, hemolymph ΣH_2S and *in vitro* sulfide binding properties. The broad solid line is the saturation *versus* ΣH_2S relationship determined *in vitro* (Fisher *et al.*, 1988a). (B) The relationship between free (unbound) ΣH_2S and external ΣH_2S in hemolymph. The broad solid line represents equal concentrations in the hemolymph and outside. (C) The relationship between free (unbound) H_2S and external ΣH_2S. The x symbols and broad solid line represent the external H_2S concentrations in these same experiments.

numbers might therefore decline at a vent site long before venting ceased, as has been observed at the Galapagos Rift Rose Garden site (Hessler *et al.*, 1988).

Molar ΣCO_2:O_2:ΣH_2S ratios

The maximal measured uptake rates of O_2, ΣH_2S, and ΣCO_2 by *Riftia pachyptila* are high (Fig. 1); they are about twice those of *S. reidi* for O_2 and ΣH_2S and three times that of *S. reidi* for ΣCO_2 (Anderson *et al.*, 1987). The relationships between the O_2 and ΣCO_2 fluxes and the ΣH_2S flux provide quantitative estimates of the dependences of the former fluxes upon the latter (Fig. 1C). As noted earlier, these relationships suggest that there is little direct interaction of the animal metabolism and the ΣH_2S flux, and thus they apparently reflect the symbiont me-

Table VII

Comparisons of hemolymph and coelomic fluid parameters in Riftia pachyptila *after maintenance (24 h) in high pressure (120 atm), flowing-water aquaria either in the absence or presence (0.016 to 0.593 m*M*, mean = 0.19 m*M *ΣH_2S) of sulfide*

	Hemolymph		Coelomic fluid		Wilcoxon Coel:Hemo +, =, −	
Parameter	0 sulfide	sulfide	0 sulfide	sulfide	0 sulfide	sulfide
pH	7.47 (±0.32,7)	7.40 (±0.26, 19)	7.54 (0.05, 7)	7.44 (0.03, 20)	5, 0, 2	14, 0, 5
ΣCO_2 (m*M*)	5.76 (±0.66, 7)	2.78 (±0.25, 20)	6.44 (±0.049, 7)	2.78 (0.26, 21)	6, 0, 1	19, 0, 1
P_{CO_2} (torr)	5.81 (±0.78, 7)	3.16 (±0.28, 18)	4.95 (±0.00, 7)	2.92 (±0.00, 19)	1, 0, 6	1, 0, 17
ΣH_2S (m*M*)	0.0 (7)	5.38 (±0.61, 20)	0.0 (7)	0.95 (±0.12, 21)	0, 7, 0	0, 0, 20
$S_2O_3^{2-}$ (m*M*)	0.01 (±0.01, 7)	0.06 (±0.01, 15)	0.00 (±0.00, 7)	0.06 (±0.02, 17)	0, 3, 4	7, 0, 8

Parameter values are shown as means with the standard errors of the means and the number of observations in parentheses. For the hemolymph and coelomic fluid comparisons with and without external sulfide, the data sets were compared using the Mann-Whitney U test. Single underlined pairs of means are from groups that have null hypothesis P values < 0.05. Double underlining indicates P values < 0.005. The relative concentrations of each substance were compared between the coelomic and vascular compartments using the Wilcoxon signed-rank test. Single and double underlining have the same meanings for this test as for the Mann-Whitney.

tabolism. The regression analyses of the metabolism experiment (Fig. 1C) indicate that these symbionts fix 0.92 mole ΣCO_2 using 1.14 mole O_2 and 1 mole ΣH_2S. Using the calculation methods of Kelly (1982), the thermodynamic efficiencies implicit in these ratios can be determined. Given a requirement of 496 kJ to reduce CO_2 to hexose, and a $\Delta G = -716$ kJ for the oxidation of sulfide to sulfate, the resulting efficiency is 63%. The molar ratio observed for *S. reidi,* 0.38 ΣCO_2:0.92 O_2:1 ΣH_2S, gives an efficiency of 40% if one assumes that the bacteria are using thiosulfate ($\Delta G = -936$ kJ for two S atoms) (Anderson *et al.,* 1987). In contrast, studies of the thermodynamic efficiencies of free-living bacteria have been done using very different methods, and they have shown lower efficiencies. The studies of free-living bacteria have used the y_{max} or "true growth yields" to estimate fixation independent of maintenance metabolism (Kelly, 1982). *Thermothrix thiopara* has the highest ratios yet determined for aerobic sulfur-oxidizers (0.58 ΣCO_2:1 thiosulfate at 72°C), corresponding to a thermodynamic efficiency of 29% (Mason *et al.,* 1987). While the ratios and efficiencies for the *S. reidi* and *R. pachyptila* symbionts seem unusually high, this may be a result of the symbiotic lifestyle.

Measurements of the y_{max} in free-living bacteria are generally made in a chemostat that maintains constant, optimal conditions for the growth of the bacteria. The production of bacterial biomass is then measured. This situation is very different from that in a symbiosis in that microbial growth involves the synthesis of a variety of complex compounds, not primarily the production of small organic molecules as is typical of animal/algal symbioses and is probably typical of most animal/bacterial symbioses as well. In *S. reidi,* the symbionts "leak" newly fixed carbon within seconds and are apparently held at a very low rate of growth (Fisher and Childress, 1986). While much less is known about this aspect of the *R. pachyptila* symbiosis, it too is believed to operate primarily by the "leakage" of small organic compounds from the bacteria to the animal with the bacteria being held in a state of slowed reproduction (Felbeck, 1985). Under the conditions present in these symbioses (low microbial growth rates, synthesis of small organic compounds, and maintenance in an environment controlled by the host) it may be possible for bacteria to achieve unusually high efficiencies for CO_2 fixation.

The internal consistency of the molar ratios for *Riftia pachyptila* can also be evaluated from the O_2:ΣH_2S ratios. The ratio of 1.14:1 falls well short of the expected 2:1 if all of the sulfide is oxidized to sulfate in the absence of other reductive processes. However, because carbon fixation is a reductive process, the reducing equivalents used in carbon fixation must also be taken into account. Following the reasoning of Kelly (1982), each CO_2 fixed to the level of CH_2O *via* the Calvin-Benson cycle requires $4e^-$ and $4H^+$. For our ratio of 0.92 ΣCO_2:1 ΣH_2S, CO_2 fixation requires $0.92 \times 4(H) = 3.68$ of the 8(H) available from complete oxidation of sulfide. Thus, $8 - 3.68 = 4.32(H)$ remain for the reduction of O_2, and the predicted O_2 uptake would be $4.32/8 \times 2 = 1.08$ O_2, compared with our value of 1.14. This agreement supports the validity of the observed ratios.

In contrast, the ratios determined for *S. reidi* showed a considerable discrepancy (0.92 O_2:1ΣH_2S observed *versus* 1.62:1 calculated as above) with insufficient O_2 consumption seemingly to account for the observed fixation

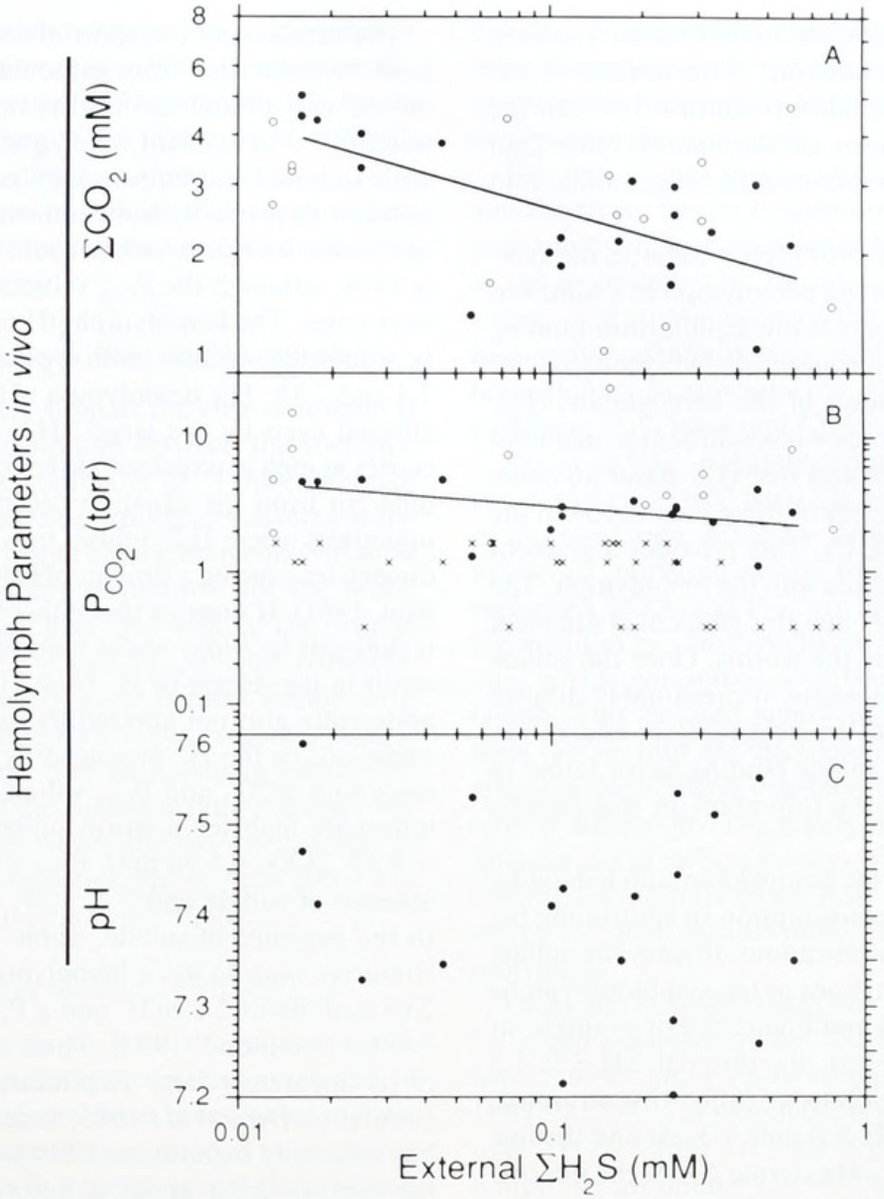

Figure 6. *Riftia pachyptila* hemolymph parameters as a function of external ΣH_2S in worms kept for 24 h at a given ΣH_2S. Closed circles and solid lines represent values from worms which were kept at >42 μM O_2 and had hemolymph pH values >7.2. Open circles represent values from worms which were kept at ≤42 μM O_2 or had hemolymph pH values ≤7.2. Regression equations for the plotted lines may be found in Table V. Where no line is plotted, the relationship was not significant (Tables V and VI). (A) Hemolymph ΣCO_2 as a function of external ΣH_2S. (B) P_{CO_2} as a function of external ΣH_2S in hemolymph and in the external water (x symbols). (C) Hemolymph pH as a function of external ΣH_2S.

of carbon and oxidation of sulfide (Anderson *et al.*, 1987). This discrepancy was attributed to the interactions between the animal sulfide and carbon metabolism. Therefore, the agreement observed for *R. pachyptila* may be yet another indicator of the degree to which the animal metabolism is isolated from the sulfur metabolism of the symbiosis.

Uptake and transport processes

The data presented here provide much new information on the processes for the uptake and transport of sulfide and carbon dioxide that are operative in these worms. The central role of the sulfide-binding hemoglobins in sulfide uptake, transport, and toxicity control (Arp and Childress, 1983; Powell and Somero, 1983; Childress *et al.*, 1984; Powell and Somero, 1986; Fisher *et al.*, 1988a, 1989) is fully supported by these data. In particular, the worms can bind sulfide reversibly *in vivo* (Table I); they can concentrate ΣH_2S from the medium by a factor of 1 to 2 orders of magnitude (Fig. 4A); and when the worms are not in autotrophic balance (low O_2, low pH group), the hemolymph hemoglobins approach equilibrium sulfide binding (half saturation near 11 μM ΣH_2S) at all sulfide concentrations tested (Fig. 5A). In addition, free ΣH_2S and free H_2S in the hemolymph of these worms is in equi-

librium with the same parameters in the external medium (Fig. 5B, C). Because the endpoint, in the absence of autotrophy, appears to be sulfide equilibrium between the hemolymph and the medium, no mechanism other than diffusion appears to be functioning to bring sulfide into the hemolymph.

When the worms are in autotrophic balance, the symbionts remove sulfide from the hemolymph at a sufficient rate to keep the hemoglobins below equilibrium binding of sulfide, resulting in an apparent *in vivo* affinity of 122 μM ΣH_2S for half saturation of the hemoglobins (Fig. 5A). This uptake by the symbionts is sufficient to maintain the hemolymph free ΣH_2S and free H_2S about an order of magnitude below the values of those parameters in the external medium (Fig. 5B, C). This provides a gradient to drive the diffusion of sulfide into the hemolymph. The available evidence indicates that this gradient is sufficient for the uptake of sulfide by the worms. Once the sulfide is transported to the trophosome, it presumably diffuses from the hemolymph into the bacteriocytes and subsequently to the bacteria. A sulfide binding factor found in the trophosome may also be important in this process (Childress *et al.*, 1984).

The cooperative role of the hemoglobin sulfide-binding and the symbiont sulfide consumption in controlling hemolymph free ΣH_2S concentrations to prevent sulfide toxicity to either the host tissues or the symbionts can be appreciated from Figure 4 and Figure 5. For example, at an external ΣH_2S of 100 μM, the internal ΣH_2S is 4.5 mM, however, the hemoglobins are only 47% saturated, and, as a result, the free ΣH_2S is only 9.3 μM and the free H_2S is 1.8 μM. Above 300 μM external ΣH_2S, the internal free sulfide rises rapidly due to the increasing saturation of the hemoglobins. Because 50% inhibition of *R. pachyptila* cytochrome c oxidase activity occurs at about 25 μM ΣH_2S *in vitro* at pH 7.0 (Powell and Somero, 1986), the observed hemolymph free ΣH_2S concentrations indicate a significant degree of protection for this critical enzyme at the usual external ΣH_2S concentrations found in this species' environment, with 50% inhibition being reached at about 250 μM external ΣH_2S concentration (Fig. 5B). While the sensitivity of the *R. pachyptila* symbionts to sulfide *in vitro* has not been precisely defined, the onset of inhibition of carbon fixation at pH 7.5 appears to occur at about 300 μM free ΣH_2S (Fisher *et al.*, 1989). In autotrophic worms, such concentrations would not be reached until the hemoglobin was more than 90% saturated, which would not be expected until external ΣH_2S concentrations reached more than 900 μM ΣH_2S (Fig. 5). Thus, the proposed protective role of the hemoglobin sulfide binding activity, both for the tubeworm tissues and the symbionts, is supported by these observations of *in vivo* sulfide concentrations.

The uptake and transport of inorganic carbon appear to be very different from those for sulfide. The environmental pH around the worms ranges from 7.0 in vent water to 7.9 in ambient water, and the ΣCO_2 is about the same in both (K. Johnson, pers. comm.). While O_2 must be taken up primarily from ambient water and ΣH_2S from vent water, inorganic carbon could be taken up from either or both, although the P_{CO_2} values would be higher in the vent water. The hemolymph pH of the worms appears to be somewhat variable, with typical values being between 7.4 and 7.55. The hemolymph pH does not appear to be affected even by the large ΣH_2S concentrations that it carries at high external sulfide concentrations. This is very different from the situation described for nonsymbiotic organisms where H_2S diffuses into the organism and then dissociates causing a drop in pH (Jaques, 1936; Groenendaal, 1981). It suggests that either the uptake mechanism is different in *Riftia* or the binding mechanism does not result in the release of H^+ from H_2S. Hemolymph pH is apparently also not affected by the ΣCO_2 concentration variations or the H^+ produced by sulfide oxidation. Hemolymph ΣCO_2 and P_{CO_2} values in *R. pachyptila* were unusually high for a worm under oxic conditions (pH = 7.47, ΣCO_2 = 5.76 mM, P_{CO_2} = 5.81 torr at 8°C in the absence of sulfide and 7.4, 2.78, and 3.16, respectively, in the presence of sulfide, Table VII). For comparison, *Arenicola marina* has a hemolymph pH of about 7.53, a ΣCO_2 of about 2.5 mM, and a P_{CO_2} of about 1.1 torr at 7.5°C (Toulmond, 1977). Thus, it appears that, in spite of its apparently large respiratory surface and effective circulation (Arp *et al.*, 1985; Jones, 1981), *R. pachyptila* has unusually high internal ΣCO_2 and P_{CO_2} levels. When the worms were in apparent autotrophic balance, the hemolymph ΣCO_2 and P_{CO_2} were decreased significantly as a function of external ΣH_2S, with minimal values approaching 1 mM and 1 torr, respectively, but were still above the environmental values (Table VII, Fig. 6A, B). While we believe that the observed distributions represent conditions in the worms under net autotrophy, it is possible that the observed distribution could result from the experimental worms not being in net autotrophic balance. The decrease in ΣCO_2 under autotrophic conditions is most likely due to the demand of the symbionts, because they have been shown to readily fix inorganic carbon (Belkin *et al.*, 1986; Fisher *et al.*, 1989). The implication of the observed distributions is that these tubeworms concentrate ΣCO_2 to relatively high P_{CO_2} values in the hemolymph and then depend on diffusion through the bacteriocytes to supply the symbionts.

This hypothesis is supported by the unusual $\delta^{13}C$ values of *Riftia pachyptila* of between −9 and −15.6‰ (Rau, 1981; Fisher *et al.*, 1988b, 1990). These workers have suggested that these low values of isotope discrimination result from carbon fixation in this species operating under

conditions approaching carbon limitation, as can happen in marine plankton (Degens *et al.*, 1968). Because the K_m for the carbon fixation by the *Riftia* symbionts is between 400 and 700 μM ΣCO_2 at pH 7.5 (Fisher *et al.*, 1988d), the hemolymph carbon dioxide values (as low as 1100 μM ΣCO_2 in the presence of sulfide) might well be low enough to limit carbon isotope discrimination under conditions of active autotrophy.

Relationship between coelomic fluid and hemolymph

The data presented here support the view that there is free exchange of small molecules between the coelomic fluid and hemolymph (Childress *et al.*, 1984), although this exchange does not extend to molecules as large as the hemoglobins. The much higher hemoglobin concentration in the hemolymph is clearly responsible for the much higher ΣH_2S in that fluid, and may well be responsible for differences in pH, ΣCO_2, and P_{CO_2} as well. However, the cause of the consistently lower percent sulfide saturation, free ΣH_2S, and free H_2S in the coelomic fluid is not apparent at this time. The new data reported here support the concept that the coelomic fluid is a reservoir of O_2, ΣCO_2, and ΣH_2S which the worms can use to buffer the effects of brief fluctuations in vent flow (Arp and Childress, 1981; Childress *et al.*, 1984).

Model of the functioning of the intact symbiosis

Riftia pachyptila appears to have the greatest autotrophic potential and as a result the fastest growth rate of any of the sulfur-oxidizing symbioses investigated to date. It, and probably all vestimentiferans, appears to be unique among the studied species in that the animal is specialized to minimize the interaction of the animal metabolism with sulfide and to provide only sulfide to symbionts that are only capable of using sulfide. Central to the ability of the vestimentiferan symbioses to use sulfide are the hemoglobins, which reversibly bind both sulfide and oxygen to different sites simultaneously. These hemoglobins enable the worms to concentrate sulfide from the medium and by almost two orders of magnitude. Yet, because of the high affinity of the hemoglobins for sulfide as well as the consumption of sulfide by the symbionts, which holds the hemoglobins well below sulfide saturation, the worms can maintain their hemolymph free ΣH_2S concentrations an order of magnitude lower than external ΣH_2S concentrations. The high capacitance of the hemolymph for sulfide is essential for the transport of sufficient quantities of sulfide to the symbionts *via* the circulatory system. The low free sulfide concentrations are essential for preventing the inhibition of animal metabolism or symbiont carbon fixation by sulfide. Diffusion of sulfide across the plume into the hemolymph appears sufficient to explain the movement of sulfide into the worms. Because the symbionts can take sulfide from the hemoglobins, diffusion from the hemolymph into the bacteriocytes in the highly vascularized trophosome may well be sufficient to supply the needs of the symbionts.

The uptake and supply of O_2 to both the symbionts and the worm tissues is apparently accounted for by the high affinity of the hemoglobins for oxygen and the ability of the symbionts and the tissues to use O_2 at low P_{O_2} values. The combination of the high O_2 affinity and the high sulfide affinity is responsible for the ability of these hemoglobins to suppress the spontaneous oxidation of sulfide by O_2 (Fisher and Childress, 1984).

About half the inorganic carbon fixed by the symbionts is potentially derived from the heterotrophic metabolism of the symbiosis, while the remaining half requires the uptake of inorganic carbon from the medium. The hemolymph ΣCO_2 and P_{CO_2} are apparently elevated above the medium by some mechanism, other than a pH-based one, which concentrates carbon dioxide in the hemolymph. This elevated hemolymph inorganic carbon can then diffuse into the bacteriocytes and to the bacteria, although the available evidence indicates that, at maximal rates of autotrophy, this supply may approach values limiting the rate of carbon fixation. The supply of fixed carbon from the symbionts to the host is presumably predominantly *via* small organic molecules transported in the hemolymph.

Using the available data, one can evaluate this model by creating a hypothetical 100-g worm that has 5 ml of hemolymph and 15 g of trophosome (Childress *et al.*, 1984). At 200 μM external ΣH_2S, one would expect 5.9 mM ΣH_2S, 5 mM O_2, and 2 mM ΣCO_2 in the hemolymph. At an uptake rate of 5 μmole ΣH_2S $g^{-1}h^{-1}$, the O_2 uptake rate would be 8 μmole $g^{-1}h^{-1}$ and the net ΣCO_2 uptake would be 2 μmole g^{-1} h^{-1}. If one assumes that the hemolymph makes one circuit per minute, one can calculate that 27% of the ΣH_2S, 53% of the O_2, and 33% of the ΣCO_2 must be exchanged on each circuit. These numbers are not unreasonable, while at the same time the similarity of the percentages provides some confidence that the values used for the hemolymph concentrations are approximately correct.

Acknowledgments

This work was supported by NSF grants OCE-8609202 and OCE-9012076 to J.J.C. and OCE-8610514 to J.J.C. and C.R.F. and funding from IFREMER to A.-M. Alayse. Some travel support was provided by NATO grant D.880423 to H. Felbeck. We would like to thank R. Van Buskirk, V. Vanderveer, and D. Gage for technical assistance during the cruises. Thanks are also due to the captains and crews of the *RV Melville, RV Thomas Thompson,* and *N/O Nadir,* as well as the sub crews and pilots

of the submersibles *Alvin* and *Nautile,* without whom this work would not have been possible. This manuscript has benefited from discussions with and comments by A. Anderson, H. Felbeck, R. Trench, and J. Feigenbaum.

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