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NET UPTAKE OF CO_2 DRIVEN BY SULPHIDE AND THIOSULPHATE OXIDATION IN THE BACTERIAL SYMBIONT-CONTAINING CLAM *SOLEMYA REIDI*

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SUMMARY

Solemya reidi Bernard is a gutless clam that lives in burrows in reducing sediments, and harbours intracellular sulphur-oxidizing bacteria in its gills. Clams were incubated in various concentrations of sulphide and thiosulphate for up to 65 h in a flow-through respirometer. Fluxes were determined by continuous sampling of the respiratory medium with analysis of CO_2, O_2 and sulphide by gas chromatography and analysis of thiosulphate, sulphite (and sulphide) by HPLC using monobromobimane-derivatized discrete samples. Net CO_2 uptake was shown to occur with exposure to 50–100 μmol l^{-1} sulphide and greater than 225 μmol l^{-1} thiosulphate; sulphide oxidation and thiosulphate uptake were also demonstrated. $^{45}CaCO_3$ deposition in the shells of *S. reidi* was found to be insignificant compared to the net CO_2 flux measured in the presence of low levels of sulphide.

In experiments conducted under various O_2 conditions, O_2 limitation, produced by a combination of low [O_2] and low water flow, was shown to inhibit sulphide oxidation and to prevent CO_2 uptake. However, if O_2 supply was not limited by low flow rates, in the presence of low [O_2] (25–40 μmol l^{-1}) *S. reidi* showed rates of O_2 and sulphide consumption and CO_2 uptake near the maximum levels determined under high [O_2] conditions, indicating the potential for net CO_2 uptake in the low [O_2] conditions presumed to exist in the animal's burrows.

Thiosulphate levels in the blood of *S. reidi* were analysed and shown to increase rapidly during incubation in sulphide. These levels reached an apparent steady state (approx. 300 μmol l^{-1}) in recently captured clams after 1 h of incubation. However, both O_2 limitation and time in captivity (>43 days after capture) caused a marked increase in the blood thiosulphate levels, which exceeded 2·5 mmol l^{-1} after 16 h of exposure to sulphide. These results indicate that blood thiosulphate is transported to the bacteria and further oxidized, and that sulphide and thiosulphate oxidation are oxygen-dependent. In analyses of gill tissues for elemental sulphur, we found a wide range in the levels of sulphur stores. Calculations indicated these to be a small fraction of the total flux of sulphur maintained during continuous sulphide oxidation.

Estimates of CO_2:O_2:sulphide ratios suggest CO_2 fixation efficiencies similar to those of chemolithoautotrophic bacteria. Assuming translocation and oxidation of symbiont-fixed organic compounds, the net uptake of CO_2 by *S. reidi* in the presence

Key words: *Solemya reidi*, chemoautotrophy, sulphide oxidation, symbiosis, thiosulphate oxidation.

of reduced sulphur compounds suggests that this intact symbiosis may be able to meet its organic carbon needs through autotrophy.

INTRODUCTION

Solemya reidi is a protobranch bivalve that lacks both a mouth and a gut (Bernard, 1980; Reid & Bernard, 1980). This burrowing marine clam inhabits highly reducing sediments such as pulpmill effluent sites (Reid, 1980) and sewage outfalls (Felbeck, 1983), where sulphide levels up to 22 mmol l^{-1} have been measured (Childress & Lowell, 1982). *S. reidi* is one of numerous bacteria–invertebrate symbioses that have been described from reducing marine habitats (Southward *et al.* 1981; Cavanaugh, 1983; Felbeck, Childress & Somero, 1983; Felbeck, Liebezeit, Dawson & Giere, 1983; Fisher & Hand, 1984; Schweimanns & Felbeck, 1985) since the discovery of the symbiosis between the hydrothermal vent tubeworm *Riftia pachyptila* and its intracellular bacteria (Cavanaugh *et al.* 1981; Felbeck, 1981). Available evidence indicates that the symbionts of many of these associations are chemolithoautotrophic bacteria that oxidize reduced sulphur compounds (Felbeck, 1981; Felbeck, Childress & Somero, 1981; Giere, 1981; Southward *et al.* 1981; Southward, 1982; Cavanaugh, 1983, 1985*a*; Dando *et al.* 1985). Such bacteria can use the energy released by their oxidation of sulphur compounds to generate ATP and reducing power [NAD(P)H], fix CO_2 *via* the Calvin–Benson cycle, and reduce nitrate (Felbeck & Somero, 1982). Several investigators have proposed (Cavanaugh *et al.* 1981; Southward *et al.* 1981; Felbeck, 1981, 1983; Spiro, Greenwood, Southward & Dando, 1986), and some have empirically demonstrated (Fisher & Childress, 1986), that the organic products of symbiont CO_2 fixation may provide a significant source of nutrition for their hosts. The enhancement of $^{14}CO_2$ fixation in the presence of reduced sulphur compounds has been reported for a few of these symbioses (Dando *et al.* 1985; Belkin, Nelson & Jannasch, 1986), including *S. velum* (Cavanaugh, 1983), a congener of *S. reidi*.

Studies of *S. reidi* have shown that it: (1) houses large numbers of intracellular bacterial symbionts within its gills (Felbeck, 1983); (2) has high activities of enzymes involved in sulphur metabolism, CO_2 fixation (*via* the Calvin–Benson cycle) and nitrate reduction in its symbiont-containing gills (Felbeck *et al.* 1981); (3) contains bacteria in its gills that fix CO_2 and shows translocation of a portion of the resulting organic carbon products to host tissues (Fisher & Childress, 1986); and (4) can take up dissolved organic compounds across its body surfaces (Felbeck, 1983). *S. reidi* is able to oxidize sulphide (Powell & Somero, 1985), and the initial step of this enzyme-mediated sulphide oxidation occurs in the animal tissue, not in the bacteria (Powell & Somero, 1985). In addition, this sulphide oxidation in the clam tissues is coupled with the mitochondrial production of ATP (Powell & Somero, 1986).

Given the evidence of sulphide oxidation, CO_2 fixation and carbon translocation, we asked two questions. What is the quantitative significance of symbiont-fixed carbon to the carbon flux of the intact symbiosis, and how are the separate components of animal and bacterial activities coupled?

In the present study, our working hypothesis was that if symbiont-fixed carbon makes a significant contribution to the animal's carbon needs, then the net CO_2 flux of the intact association would reflect this CO_2 fixation in the presence of a usable energy source such as sulphide or another reduced sulphur compound. Moreover, in the presence of such an energy source, a net negative flux, or uptake, of CO_2 from the experimental medium would indicate the potential for autotrophy to satisfy some or all of the organic carbon needs of the symbiosis. It is important to note that this approach is quite different from experiments by others using ^{14}C-labelled bicarbonate (Felbeck, 1983; Cavanaugh, 1983; Fisher & Childress, 1986). Though these studies are useful for elucidating the pathway of CO_2 fixation (Felbeck, 1983) and demonstrating the site of CO_2 fixation and subsequent translocation of fixed products (Fisher & Childress, 1986), they cannot be used to measure the net carbon flux of the association, as ^{14}C techniques estimate only total fixation rates and cannot simultaneously measure respiratory CO_2 loss. In this study, we have instead taken the approach of measuring the net CO_2 flux of the intact symbiosis to assess directly the quantitative importance of carbon autotrophy in the intact association in relation to fluxes of other metabolites.

To test the hypothesis, live animals were placed in a flow-through respirometer and the fluxes of O_2, CO_2 and sulphide or thiosulphate were continuously monitored for 48 h both in the presence and in the absence of varying concentrations of the reduced sulphur compounds. In addition, the dependencies of respiratory O_2 and CO_2 fluxes and sulphide oxidation flux on dissolved oxygen were determined. To ascertain the quantitative contribution of deposition of shell carbonate to the net CO_2 flux determined in the respiration studies, rates of calcium deposition were determined using radiolabelled $^{45}CaCl_2$ both in the presence and in the absence of sulphide.

To develop a scheme for the complete oxidation of sulphide (and potential sites of energy production) by the separate animal and bacterial components of the symbiosis, the blood of clams incubated in continuously flowing sulphide was assayed for reduced sulphur compounds. Results from these experiments and those determined with flow-through respirometry suggest a model for the coupling between sulphur oxidation and CO_2 fixation in the animal and bacterial components of the intact association.

MATERIALS AND METHODS

Animal collection and maintenance

Solemya reidi were collected on five separate cruises in 1985 and 1986 by Van Veen grab sampling at the Hyperion sludge outfall in Santa Monica Bay, California from a depth of approximately 90 m. They were transported to Santa Barbara in chilled sea water and maintained at 7·5 °C in a 200-l tank filled with sludge collected at the site, with sea water flowing slowly over the sludge. The O_2 content of the water in the clam burrows in the holding tank was determined intermittently by gas chromatography (Childress, Arp & Fisher, 1984).

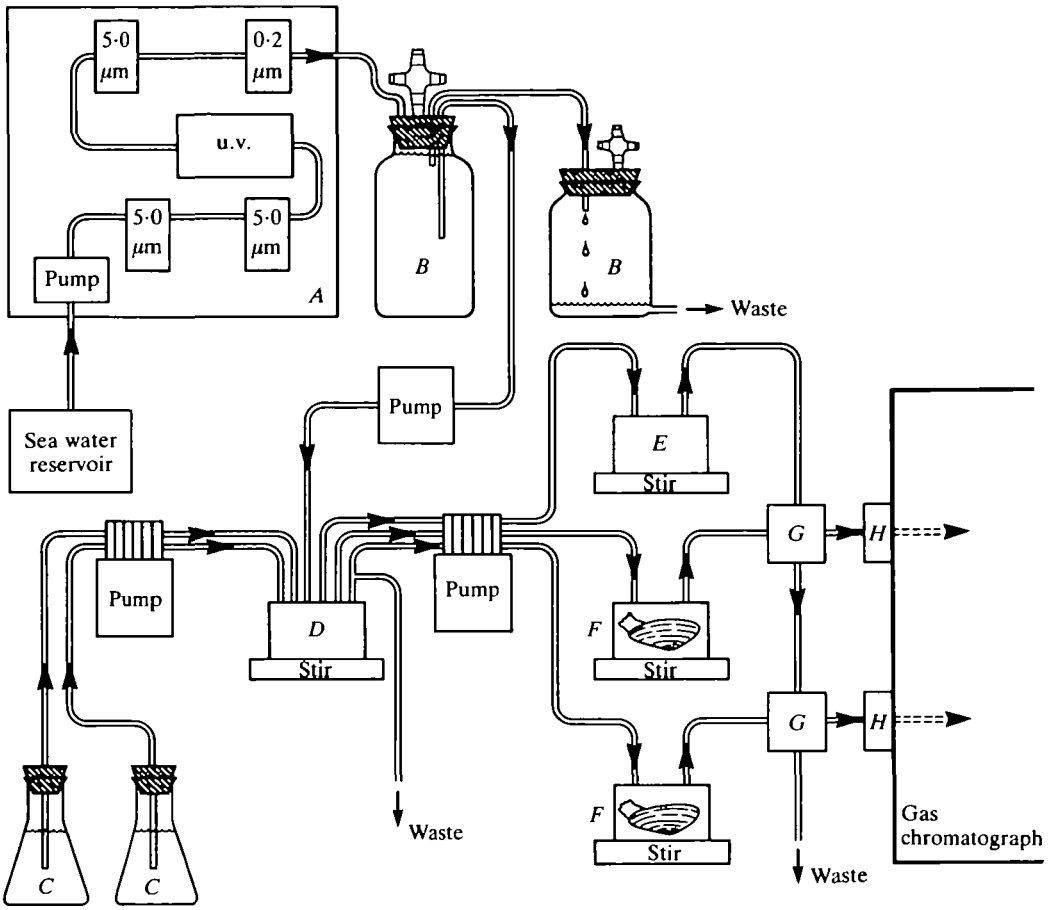

Fig. 1. Schematic diagram of flow-through respirometer, including: (*A*) sea water filtration and sterilization system, (*B*) sterile, filtered sea water holding vessel and drip overflow container, both fitted with bacterial air vents, (*C*) antibiotic and reduced sulphur-compound solution containers, (*D*) mixing chamber, (*E*) control chamber and (*F*) two animal respiration chambers, (*G*) automated switching valves for chamber effluent, and (*H*) gas chromatograph with two injection chambers for simultaneous analysis of two samples. Stir, magnetic stirrer; pump, peristaltic or piston metering pump.

Flow-through respirometry

A flow-through, multiple-chamber, automated respirometer was developed to determine the fluxes of respiratory gases under various conditions (Fig. 1). The source sea water from offshore Santa Barbara was pumped through two 5·0-μm filters, ultraviolet sterilized (purifier Model RM-2, REFCO Purification Systems, Inc.), and finally filtered through 5·0- and 0·2-μm filters. This sterilized sea water (SSW) was continuously pumped into an autoclaved 20-l Nalgene jug fitted with an overflow drip system and an air vent sealed with a membrane filter. The SSW was then pumped to a chamber where it was mixed with solutions of antibiotics and

sodium sulphide or sodium thiosulphate, which were pumped from static reservoirs. Flow rates in all the lines were monitored using rotameters.

The mixed solution was delivered by peristaltic pump at the same flow rate to each of three respiration chambers; two contained animals and one served as a control. Flow rates used in these studies ranged from 2 to 7 ml min^{-1}.

The effluent from the respiration and control chambers flowed through two automated four-port switching valves which directed either the experimental or control chamber effluent to the sample injection valves on a gas chromatograph. The Hewlett-Packard 5880A gas chromatograph (GC) was plumbed for the extraction of gases from liquid samples (Childress *et al.* 1984) and, using its own microprocessor, controlled the operation of the switching and sample injection valves. The GC was fitted with two sets of injection valves, in-line extractors, columns and thermal conductivity detectors so that two samples could be analysed simultaneously. Samples were injected and analysed approximately every 25 min over the course of the experiments, which generally lasted 48 h. The gases were stripped from the liquid samples by a combination of heat, vigorous stirring and treatment with 50% phosphoric acid (Childress *et al.* 1984). The GC integrators were calibrated to analyse CO_2, H_2S, O_2 and N_2 in $\mu mol\,l^{-1}$. The range of analytical variation in replicated samples was less than $\pm 5\%$ for oxygen, sulphide and nitrogen and less than $\pm 2{\cdot}5\%$ for CO_2. The pH of each chamber was monitored periodically throughout the experiment using a double-junction electrode. The entire flow-through apparatus (chambers, tubing, flowmeters, etc.) was cleaned with a dilute hypochlorite solution after each run and rinsed with SSW until no trace of chlorine could be detected (<0·1 p.p.m.).

The respirometer, control and mixing chambers were constructed from 140 ml Pyrex containers, which were water-jacketed and fitted with 0·6 cm thick Plexiglas lids with O-ring seals. Threaded nylon fittings in the lids provided for the entry and exit of the flowing sea water through 0·3 cm nominal o.d. polypropylene tubing. The chambers were cooled to 7·5 °C by a recirculating water bath.

Antibiotic and reduced sulphur solutions were mixed in SSW, 0·2 μm filtered, and kept in autoclaved, sealed flasks. The air inlets of the rubber flask stoppers were sealed with membrane filter air vents. Antibiotics used were Penicillin G and Streptomycin sulphate (Sigma Chemicals) and were both delivered at a final concentration of 50 mg l^{-1}. To minimize spontaneous oxidation, sodium sulphide solutions were mixed in deoxygenated SSW and titrated with HCl to pH 6·5 (Chen & Morris, 1972) and a nitrogen gas atmosphere was maintained in the sealed flask. All forms of sulphide present in solution (H_2S, HS^- and S^{-2}) are referred to as 'sulphide'. At the pH range of our experiments, 7·82–7·98, about 90% of the sulphide is in the HS^- form.

Determinations of thiosulphate and sulphite levels in the flow-through chambers were made by high-pressure liquid chromatography (HPLC) analysis of monobromobimane (mBB) derivatized samples using the methods of Newton, Dorian & Fahey (1981) and Fahey, Dorian, Newton & Utley (1983) as modified by Vetter, Wells, Kurtsman & Somero (1987). A Gilson HPLC and model 121 fluorometer

were used for the determinations. Derivatives were separated on a 15 cm Supelco C-18 reversed phase column and detected using a 235 nm filter for excitation and a 442 nm filter for detection. The eluent flow rate was 1·5 ml min^{-1}, and an increasing hydrophobic gradient of HPLC grade methanol and 2% acetic acid was used.

Respiration procedure

The experiments were conducted with animals that had been in captivity for no more than 15 days. The duration of each respiration procedure and number of different experiments necessitated five separate collections (August and November 1985; February, May and October 1986) to complete the study. Wet tissue masses of the clams ranged from 6·3 to 10·3 g and were determined after dissection of the clams from their shells at the end of each experiment.

Before a respiration experiment, the clams were removed from the holding tank and placed in partially deoxygenated (approximately half-saturated) SSW for 3–4 h so that their mantle cavities would be free of mud. Both the respiration and control chambers were covered with a black cloth throughout the experiment to mimic light conditions in their natural habitat. For the majority of the sulphide studies, each clam was exposed to 2–3 concentrations of sulphide during each experiment following initial measurements of respiration in sulphide-free SSW (SFSW). For most of the studies, the levels of sulphide were delivered in order of increasing concentration. It was necessary to maintain each sulphide concentration for at least 12 h to reach steady state and to determine rates of exchange. Each clam was exposed to only two or three of the experimental sulphide levels, as the potential for bacterial contamination of the system increased with time. In the studies using thiosulphate rather than sulphide, the responses of six animals were determined during exposure to one of three experimental thiosulphate concentrations following initial exposure to SSW.

To examine responses over longer periods, two respiration experiments were conducted in which two clams were exposed to either SFSW alone or 100 μmol l^{-1} sulphide for 36–65 h. To examine whether the responses measured during the respiration experiments with sulphide were dependent upon sulphide concentration or order of exposure to the various sulphide levels, six studies were conducted in which the clams were exposed first to high levels, then to low levels, and again to high levels of sulphide, as opposed to the usual regime noted above.

For the sulphide incubations, the dependence of sulphide consumption rates on oxygen concentration was examined by using various concentrations of sulphide (50–250 μmol l^{-1}) and oxygen (35–130 μmol l^{-1}). Varying flow rates to the respiration and control chambers were also employed. To partially deoxygenate the SW for these runs, the unfiltered SW was passed through a 1·5 m × 5 cm column with an airstone fitted into the bottom stopper. Nitrogen gas was bubbled continuously into the column and the deoxygenated SW was then pumped through the filtration/ultraviolet sterilization apparatus and on to the SSW holding vessel.

When steady state had been established (gas concentrations stable over time in each of the chambers), rates of flux (μmol g wet $mass^{-1}$ h^{-1}) were calculated from the

differences in gas concentrations between the effluents of the respiration and control chambers. The effluent from the chamber being analysed was sampled 3–4 times each for 1·5 h. Any chromatograms in which problems were detected (due to bubbles in the sample as shown by high N_2 values) were excluded. In this manner, an average concentration of each gas was determined every 1·5 h for alternating (control and animal) chambers on each of the two independent channels of the gas chromatograph. The average control chamber concentrations which temporally bracketed those of an animal chamber were averaged, and the differences between the animal and control chamber concentrations taken. Control chamber concentrations changed very little over time once steady state had been reached for each sulphide level. Using this concentration difference, plus the flow rate and clam's wet tissue mass, a rate of flux for each gas was calculated for each 1·5-h segment of measurements from an animal chamber. By averaging these rates, a response for each clam (μmol g^{-1} wet $mass^{-1}$ h^{-1}) at each sulphide concentration was determined for each 12-h exposure. Linear regression analyses of rate changes showed that no significant change occurred during this interval.

In preliminary studies, made with sea water lacking filtration/ultraviolet sterilization and with non-sterile antibiotic and sulphide solutions, we were unable to prevent significant levels of bacterial contamination. In addition to adopting sterilization procedures for the present studies, two different control methods were used to confirm that bacterial contamination was not significant. At the end of each respiration experiment the clams were removed and the experimental chambers resealed and re-equilibrated, or the shells of the freshly dissected experimental clams were replaced in the experimental chambers before resealing and re-equilibrating. For the majority of the respiration experiments, within a short time (2–4 h) after resealing the chambers the gas concentrations in the experimental chambers were observed to equal those in the control chamber. Thus, bacterial contamination was considered to be insignificant and the respiration data reliable.

Determination of products of sulphide oxidation in the blood

Because the oxidation of sulphide is known to occur in the animal tissues of *S. reidi* (Powell & Somero, 1985), we determined the identity and concentrations of some of the sulphur species in the circulating blood which might be supplied to the bacteria for further oxidation. As both oxygen availability (see Results) and number of days post-capture (A. E. Anderson, in preparation) affect the rates of sulphide consumption, we conducted four experiments that included two variables: oxygen availability and days post-capture of clams. Concentrations of thiosulphate, sulphite and sulphide in the blood of both laboratory-maintained and sulphide-incubated clams were analysed.

S. reidi were incubated in 100 μmol l^{-1} sulphide at a pH of 7·8–7·9 at 5·5 °C in a 2-l sidearm flask set up as a flow-through chamber. In one experiment, 10 recently captured clams (in captivity 14 days) were exposed to 100 μmol l^{-1} sulphide at high flow rates (i.e. no O_2 limitation). In a paired experiment, 10 clams that had been maintained in the laboratory mud tank for 180 days were exposed to sulphide under

the same conditions. In the third experiment, six recently captured clams (18 days post-capture) were exposed to 100 μmol l^{-1} sulphide under conditions of O_2 limitation. Finally, 12 clams, 43 days after capture, were exposed to 100 μmol l^{-1} sulphide under conditions of O_2 limitation.

At selected times during the incubations, a clam was removed, weighed and the blood collected by syringe after quickly rinsing and draining the mantle cavity. The blood was then derivatized with mBB, heated for 15 min at 60°C (after addition of concentrated acetonitrile) to precipitate the protein, and analysed by HPLC using the methods noted above. In addition, if the sample size was >1 ml, a subsample of blood was analysed for oxygen and sulphide content by gas chromatography. Sulphide and oxygen concentrations of the incubation medium were analysed by gas chromatography throughout each experiment.

Gill ciliary activity in the presence of sulphide

The activity of gill cilia was monitored in various concentrations of sulphide for visual evidence of the inhibition of aerobic metabolism. Freshly excised gills of *S. reidi* were placed in a respirometer maintained at 7·5°C by a recirculating water bath. A dissecting microscope was used to monitor ciliary movement of carmine particles in 50, 100, 250 and 500 μmol l^{-1} sulphide (O_2 present) and in 2·5 mmol l^{-1} sulphide (no O_2 present). The incubation water was replaced every 15 min to maintain the sulphide concentration. Activity was observed and recorded for 30–45 min at each concentration. Activity in sulphide-free sea water was observed following exposure to sulphide to confirm that the excised gill was alive. A gill was used for no more than 2 h after dissection.

Determination of elemental sulphur

Elemental sulphur analysis was carried out following the methods of Richard, Vick & Junk (1977) as modified by Fisher, Childress, Oremland & Bidigare (1987). Pieces of gill tissue (0·1–4·0 g wet mass) were dried at 60°C and extracted with cyclohexane in a micro-soxhlet apparatus. The extracts were passed through a fluorosil column and concentrated by evaporation. Sulphur in the extract was quantified by gas chromatography using a thermal conductivity detector and a 1·8 m glass column with a 2 mm bore, packed with 5% SP2401 on 100/120 mesh Supelcoport. Detection limits for this procedure were about 0·003% dry mass.

Calcium deposition experiments

Using radiolabelled calcium, the rates of deposition of calcium were determined both in the presence and in the absence of sulphide (100 μmol l^{-1}) to assess the contribution of shell $CaCO_3$ deposition to the flux of CO_2 determined in the respirometry studies. For each of the incubations, 20 live clams (4–6 g wet mass) were put into a 2-l vessel at 7·5°C. To test for non-biological exchange of calcium

with the shell, four whole frozen/thawed clams (whole clams that had been killed by placing in a −80°C freezer, gaping and resembling a live clam when thawed) and the shells of four freshly killed clams were used. The clams to be incubated in the presence of 100 μmol l^{-1} sulphide were pre-incubated in sulphide for 5 h prior to addition of the labelled calcium. As soon as all the clams had opened their valves, 20 μCi of $^{45}CaCl_2$ (2 mCi in 0·09 ml, 8·37 mCi per mg calcium) was added to the incubation chamber containing the clams. Initial and final activities of $^{45}Ca^{2+}$ in the experimental chamber were determined by placing 100 μl samples of the incubation medium in glass scintillation vials with 10 ml of Hydrofluor (National Diagnostics) and counting as noted below. Antibiotics at the same concentration used in the respiration studies were used in these incubations. The incubation medium was periodically analysed for O_2 and sulphide content by gas chromatography during the experiment, and sulphide was added as needed to maintain a concentration of approximately 100 μmol l^{-1}. Oxygen concentrations ranged from 100 to 185 μmol l^{-1}.

At times of 2, 3, 4 and 5 h, three live clams, one empty shell and one frozen/thawed clam were removed from the labelled SSW medium and quickly weighed. Each live clam was cut open, its foot and gill were excised, and all parts put into hot ethanol (60°C) to terminate deposition activity. The empty control shell and the frozen/thawed clam were treated similarly. Mantle tissue was subsequently removed from all shells, the shells were dried, and weights determined. 2 mol l^{-1} HCl was used to dissolve the calcified portion of the whole shell, and the remaining proteinaceous shell portion was dried and weighed.

The dissolved shell solutions were neutralized with NaOH and duplicate 800-μl samples were placed in glass scintillation vials with 10 ml of Hydrofluor. The samples were counted in a liquid scintillation counter and corrections made for background and counting efficiency. $CaCO_3$ deposition rates (mg Ca^{2+} mg dry calcified shell^{-1} h^{-1}) were calculated based on the specific activity of the incubation medium (which did not vary more than 6 % over the course of 5 h), the measured amounts of $^{45}Ca^{2+}$ in the dissolved shells, a calcium concentration in sea water of 0·412 g l^{-1} (0·01 mol l^{-1}) and a half-life of 247 days for $^{45}Ca^{2+}$. The rates were then transformed to μmol CO_2 g tissue^{-1} h^{-1}, assuming an approximately 1:1 molar ratio of calcium to carbonate deposited (Wheeler, Blackwelder & Wilbur, 1975), so that comparisons could be made with the CO_2 flux determined in the respiration experiments.

Statistics

The data were initially examined *via* multiple regressions and two-way analyses of variance (ANOVAs) (STATPRO statistics software package, Penton Software Inc.) to determine the appropriate statistical methods for our final analyses, which are presented in the Results. The significance of correlation coefficients was tested according to Sokal & Rohlf (1969), and a significance level of $P < 0{\cdot}05$ is assumed unless otherwise noted.

RESULTS

Effects of sulphide and thiosulphate on respiratory gas flux

In a preliminary study, it was found that there were significant decreases in the rates of CO_2 uptake, O_2 consumption and sulphide oxidation with increasing time in captivity of clams kept in our holding tank. Significant declines in these rates were also measured in clams that had been kept in oxygenated SSW for 36–48 h before the respiration experiments, when compared to the rates of clams kept in half-saturated SSW for 3–4 h (A. E. Anderson, in preparation). Because of these findings, the data presented in this study were collected from clams that had been kept in SSW for only 3–4 h before each experiment (see Materials and Methods) and had been in captivity no more than 15 days (unless otherwise noted). The preliminary studies indicate that a decline of 15–35 % in the rates is to be expected in clams held in captivity for 15 days compared to those analysed within 2 days of capture. This 15-day duration was selected as a compromise between the time required to do experiments and the change in animal condition.

The rates of CO_2, O_2 and sulphide flux determined from the flow-through studies are shown in Fig. 2 and summarized in Table 1. In SFSW the CO_2 production was approximately equal to the O_2 consumption (RQ = 1·0). In the presence of either 50–80 or 100 μmol l^{-1} sulphide, there was a net uptake of CO_2 from the respiratory medium, with the higher uptake rates occurring at 100 μmol l^{-1}. This uptake occurred quickly and was quantifiable as soon as the system reached steady state (within 3–4 h after the addition of sulphide). At sulphide concentrations greater than 100 μmol l^{-1}, there was a net production of CO_2, though at lower rates than without sulphide. O_2 consumption rates increased over those found in SFSW with exposure to increasing sulphide concentrations up to 100 μmol l^{-1}, at which concentration the

Table 1. *Responses of individual* Solemya reidi *to various sulphide concentrations and oxygen conditions*

[Oxygen] (μmol l^{-1})	[Sulphide] (μmol l^{-1})	Flux (μmol g wet mass^{-1} h^{-1})			N
		CO_2	O_2	Sulphide	
75–150	0	+2·17 ± 0·25	−1·97 ± 0·15	—	
	50–80	−0·22 ± 0·15	−2·99 ± 0·29	−1·48 ± 0·13	4
	100	−0·89 ± 0·18	−4·27 ± 0·24	−3·07 ± 0·22	10
	250	+0·35 ± 0·14	−3·23 ± 0·22	−2·79 ± 0·25	8
	500	+1·84 ± 0·28	−2·61 ± 0·44	−2·11 ± 0·43	4
0–20*	0	+0·36 ± 0·04	−0·53 ± 0·06	—	4
	100	+0·45 ± 0·16	−0·36 ± 0·003	−0·21 ± 0·01	2
24–44†	0	+1·13 ± 0·12	−0·76 ± 0·015	—	2
	100	−0·44 ± 0·16	−2·53 ± 0·09	−2·25 ± 0·38	2

Values are means ± S.E.M.

Each rate represents the mean response of (N) animals determined over >12 h exposure to specified chamber conditions.

Positive values = production, negative values = consumption.

* O_2 limitation imposed by low flow rates of incubation medium.

† No O_2 limitation.

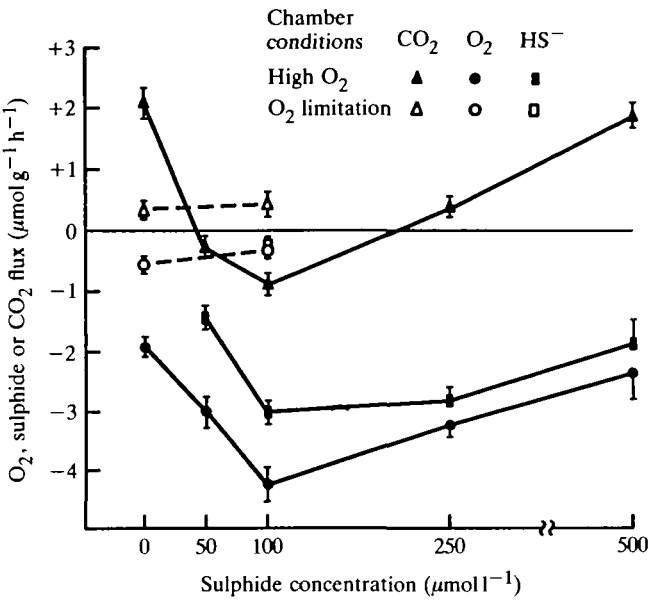

Fig. 2. Mean CO_2, O_2 and sulphide fluxes of *Solemya reidi* at varying sulphide concentrations and oxygen conditions. Positive flux rates indicate production; negative rates indicate uptake. O_2 limitation at ambient $[O_2]$ of 0–20 μmol l^{-1}. Each symbol represents an average ± S.E. from (N) animals (see Table 1). Temperature, 7·5°C.

average O_2 consumption rate was more than twice that with no added sulphide (4·27 *vs* 1·97 μmol g^{-1} h^{-1}). At sulphide concentrations greater than 100 μmol l^{-1}, O_2 consumption declined and was only slightly greater at 500 μmol l^{-1} sulphide (2·61 μmol g^{-1} h^{-1}) than in SFSW (1·97 μmol g^{-1} h^{-1}). Sulphide consumption rates were also highest at 100 μmol l^{-1} sulphide and declined at higher concentrations. Therefore, it appears that the maximum autotrophic balance with respect to CO_2 is achieved near 100 μmol l^{-1} sulphide, with a decline in CO_2 uptake both above and below this level.

Visual examinations of the chambers revealed that the clams remained open and apparently ventilating in SFSW and in 50–100 μmol l^{-1} sulphide, but that their valves were frequently partially closed when incubated in 250 or 500 μmol l^{-1} sulphide.

In incubations with 100 μmol l^{-1} thiosulphate, CO_2 production decreased and net CO_2 uptake occurred in thiosulphate concentrations greater than or equal to 250 μmol l^{-1}. In two of three experiments, net CO_2 uptake (0·30–0·34 μmol g^{-1} h^{-1}) did occur within 3–4 h of thiosulphate addition as it did after the addition of sulphide; in the third, CO_2 levels fluctuated and did not show substantial net uptake until after 13 h (final uptake rate 0·7 μmol g^{-1} h^{-1}). However, the addition of thiosulphate did cause an immediate increase in O_2 consumption rates in all experiments, and greater increases in O_2 consumption were found in 250–350 μmol l^{-1} than in 100 μmol l^{-1} thiosulphate. As there was substantial

variation between individuals in the measured rates of O_2 consumption in the small sample of animals used for these determinations, means of the rates would obscure the patterns observed and are not presented here. The increases in O_2 consumption ranged from 0·2 to 1·78 μmol g^{-1} h^{-1}. Thiosulphate consumption rates were determined for four experiments and showed marked variation between individual clams (range = 0·52–1·45 μmol g^{-1} h^{-1}). Blood thiosulphate levels of 114 and 143 μmol l^{-1} were found in two clams incubated in 350 μmol l^{-1} thiosulphate, additional evidence of uptake of this compound (the average blood thiosulphate level of clams incubated in SSW for 3–4 h was 12·5 μmol l^{-1}). Upon dissection of the clams at the end of the experiment, the gill tissues of clams exposed to 250 μmol l^{-1} (and greater) thiosulphate appeared creamy-yellow, an appearance which correlates with stores of elemental sulphur in the gills of other symbiont-containing bivalves (Vetter, 1985; Dando *et al.* 1985). The data indicate that *S. reidi* is able to take up thiosulphate and use it or one of its reaction products to drive CO_2 fixation.

There was no significant correlation between the mass-specific rates and the wet masses of the clams used over the relatively small size range of clams tested (6–10 g) in any of the respiration studies.

65-h exposure to 100 μmol l^{-1} sulphide

During a 65-h experiment in 100 μmol l^{-1} sulphide (Fig. 3), net uptake of CO_2 occurred quickly after exposure to sulphide. Though CO_2 flux varied somewhat during this experiment, net CO_2 uptake was maintained throughout and thus is not a transient phenomenon.

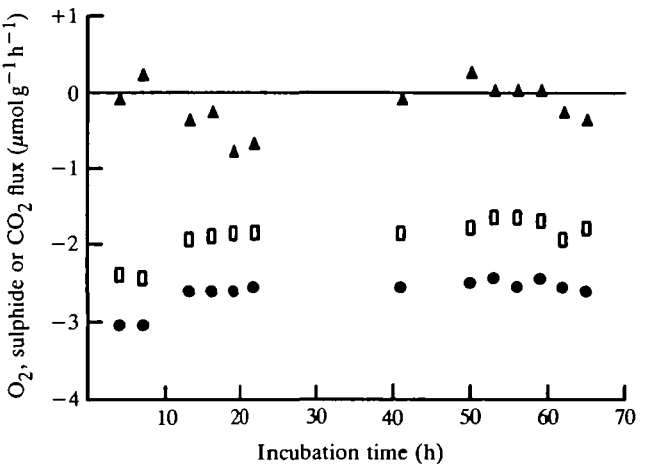

Fig. 3. CO_2 (▲), O_2 (●) and sulphide (□) fluxes of one individual *Solemya reidi* over time during a 65-h incubation in 100 μmol l^{-1} sulphide at 7·5 °C. Each point represents the mean of 2–3 gas analyses of respiration chamber medium taken over alternate 1·5-h intervals (see Materials and Methods).

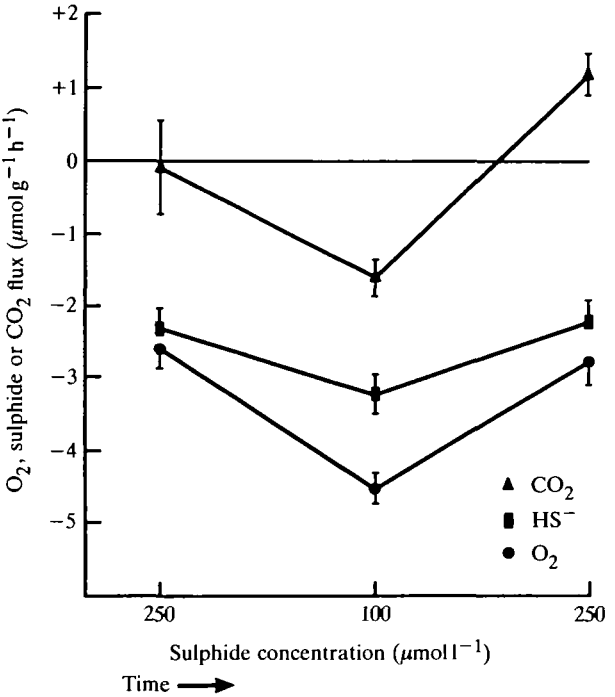

Fig. 4. Order of exposure *vs* concentration dependence of responses of *Solemya reidi* to various sulphide concentrations. Order of exposure to changing sulphide levels as presented on abscissa; 250, 100, 250 μmol l^{-1}. Each point is the mean response for one animal determined over a 12-h exposure to each sulphide concentration (±S.E.). Temperature, 7·5 °C.

Order of exposure versus *concentration dependence of rates of flux*

To examine whether the rates were dependent on time or concentration, the order in which the clams were exposed to the different levels of sulphide was changed. We found that the order of exposure to varying levels of sulphide did not affect the responses: exchange rates of CO_2, O_2 and sulphide appear to be concentration-dependent. In separate instances where exposure to 100 μmol l^{-1} followed 250 or 500 μmol l^{-1} sulphide, O_2 and sulphide consumption rates increased and the CO_2 production characteristic of the higher sulphide levels was replaced by net CO_2 uptake (after 250 μmol l^{-1} sulphide) or steadily declining rates of CO_2 production (after 500 μmol l^{-1} sulphide) after a period of 12 h in 100 μmol l^{-1} sulphide (Fig. 4).

Effects of oxygen concentration on respiratory gas flux

In the presence of limited oxygen (35–50 μmol l^{-1} delivered at low flow rates to the chambers), the flux rates were greatly decreased (Fig. 2; Table 1). In SFSW, the average O_2 consumption rate (0·53 μmol g^{-1} h^{-1}) was very low at the lowest flow rate, where O_2 levels in the clam chambers were depleted to 0–17 μmol l^{-1} ($N = 4$). An increase in O_2 consumption rates was found with increasing flow rates and showed a significant correlation ($r = 0{\cdot}586$, d.f. = 37, $P < 0{\cdot}01$). At the highest flow

rates, where O_2 levels of 24–44 μmol l^{-1} ($N = 4$) (data not shown) were maintained in the clam chambers, O_2 consumption rates nearly equalled the rates found in the high O_2 experiments, indicating little inhibition by this O_2 concentration.

In the low-O_2 studies with added sulphide, only O_2 (not sulphide) was limiting. At 100 μmol l^{-1} sulphide and at chamber O_2 levels of 0–20 μmol l^{-1} (O_2-limiting flow rates), sulphide was consumed, but at rates an order of magnitude lower than in the presence of unlimited oxygen (0·21 *vs* 3·07 μmol g^{-1} h^{-1}). Net CO_2 uptake did not occur under these O_2-limited conditions. However, during two of the 100 μmol l^{-1} sulphide experiments, the flow rate of this low-O_2 medium to the chambers was increased (to 24–44 μmol l^{-1} ambient chamber O_2) so that O_2 supply was not limiting, allowing the clams to increase their O_2 and sulphide consumption rates. In these experiments, CO_2 uptake rates nearly equalled those found in high-O_2 conditions (Table 1). Therefore, O_2 is necessary for the consumption of sulphide. Furthermore, limiting O_2 and thereby decreasing sulphide oxidation inhibits the uptake of CO_2, presumably by limiting the energy and reducing power available for CO_2 fixation. In addition, O_2 and sulphide consumption and CO_2 uptake rates are apparently not limited by ambient O_2 concentration as low as 20–37 μmol l^{-1}. Though the O_2 content of the burrow water in the clams' natural environment is unknown, that in the burrows of the laboratory-maintained clams ranged from 30 to 60 μmol l^{-1} ($N = 4$).

Oxygen to sulphide ratios

The strong correlation between oxygen and sulphide consumption rates is evident in Fig. 5A,B. Using the method of Zerbe, Archer, Banchero & Lechner (1982) to compare lines with unequal slopes, the predicted y-values of the two lines (for 50–100 and 250–500 μmol l^{-1} sulphide) were found to be significantly different ($P < 0·05$) over the range of $x = 0·85–3·47$, within which all but three of the points occurred. The lower intercept for the 250–500 μmol l^{-1} sulphide data (Fig. 5A) indicates a decline in the background (non-sulphide related) O_2 consumption. The cluster of points near the origin (Fig. 5B) represents the rates at the lowest oxygen concentrations and slowest flow rates (i.e. greatest O_2 limitation) and the remaining points are from experiments in which increased flow rates or slightly higher O_2 concentrations were used, increasing the O_2 supply. The rates of both O_2 and sulphide consumption are dependent upon the availability of oxygen (Fig. 5B). The lower intercept in Fig. 5B as compared to Fig. 5A reflects the depression in O_2 consumption rates imposed by O_2 limitation.

Correlation of sulphide oxidation with CO_2 uptake

A direct relationship was found between sulphide oxidation rates and CO_2 uptake rates, indicating that CO_2 fixation is dependent on the energy made available through sulphide oxidation. Though a significant ($P < 0·05$) relationship occurred at all sulphide concentrations, the best fit was obtained by using only the data from 50–100 μmol l^{-1} sulphide experiments, in which net CO_2 uptake occurred. The

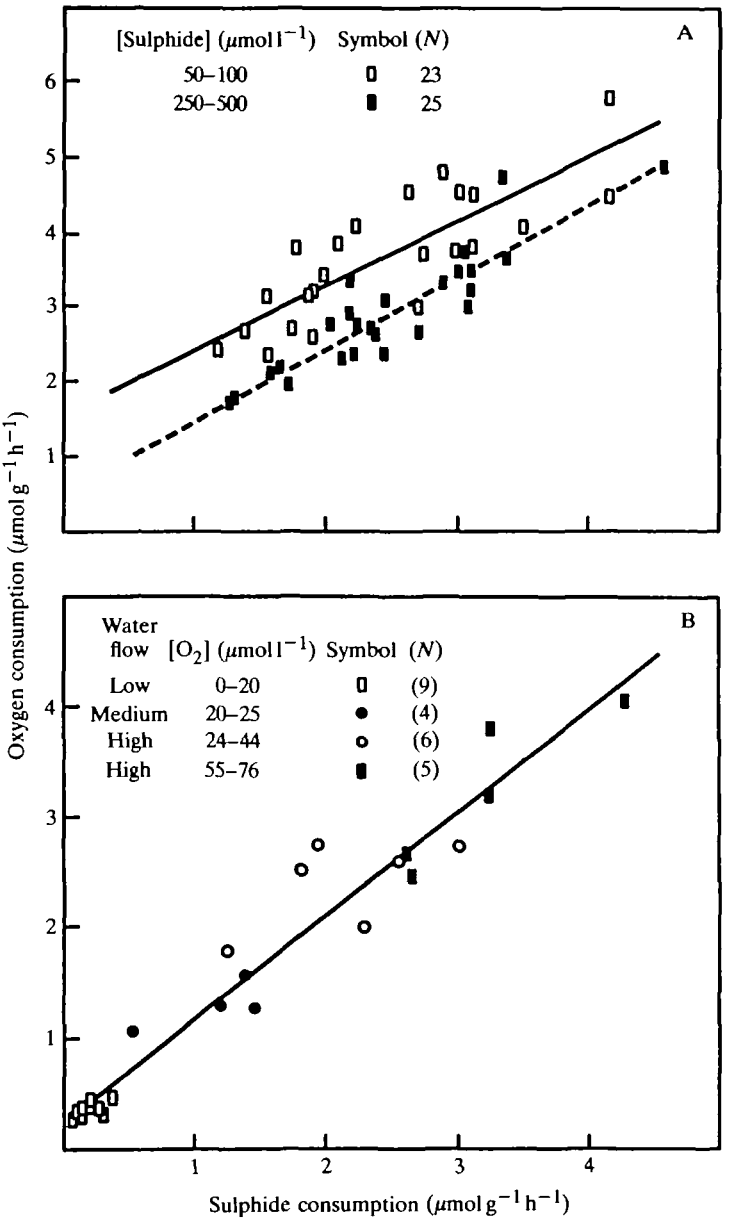

Fig. 5. Oxygen consumption of *Solemya reidi* plotted as a function of sulphide consumption. (A) Incubations in all sulphide levels with no oxygen limitation. For 50–100 μmol l^{-1} sulphide, $y = 1{\cdot}56(\pm 0{\cdot}71) + 0{\cdot}86(\pm 0{\cdot}28)x$ ($r = 0{\cdot}81$, $P < 0{\cdot}01$). For 250–500 μmol l^{-1} sulphide, $y = 0{\cdot}51(\pm 0{\cdot}51) + 0{\cdot}97(\pm 0{\cdot}20)x$ ($r = 0{\cdot}91$, $P < 0{\cdot}01$). (B) Low oxygen concentration studies, varying flow rates. $[O_2] = O_2$ concentration in animal chamber at steady state. $y = 0{\cdot}238(\pm 0{\cdot}19) + 0{\cdot}93(\pm 0{\cdot}10)x$ ($r = 0{\cdot}97$, $P < 0{\cdot}001$).

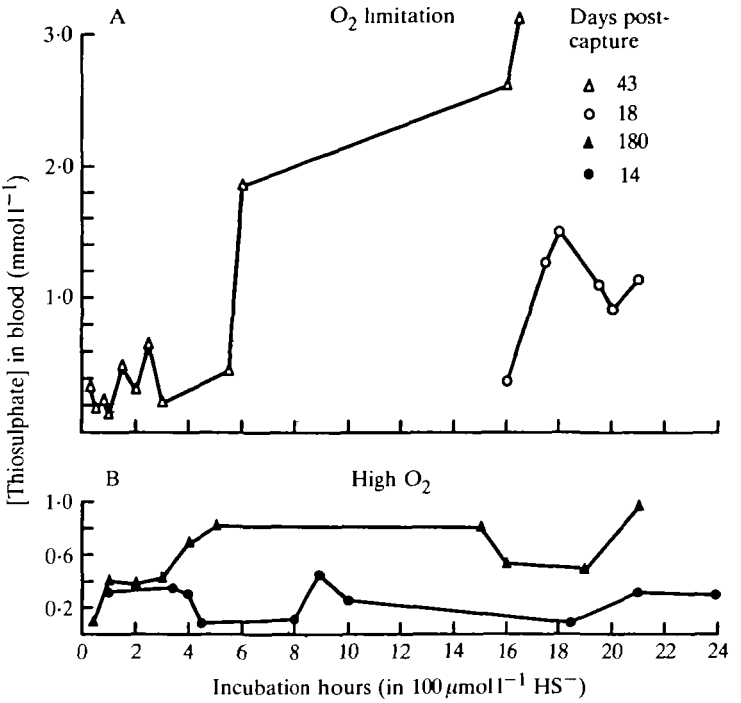

Fig. 6. Thiosulphate levels in blood of *Solemya reidi vs* incubation time (h) in 100–125 μmol l^{-1} sulphide. Each point represents assay of blood of one animal. Temperature, 5·0°C. (A) Oxygen limitation; (B) no oxygen limitation. At 45 days post-capture or greater, symbiont population appears to be reduced (see Results).

linear regression of sulphide consumption (x) *vs* CO_2 uptake (y) at 50–100 μmol l^{-1} sulphide yielded the equation $y = 0{\cdot}52 - 0{\cdot}38(\pm 0{\cdot}31)x$ ($r = 0{\cdot}485$, $N = 23$, $P < 0{\cdot}05$).

Sulphide oxidation products in blood

Thiosulphate and sulphite are present in the blood of freshly captured clams (R. Vetter, Scripps Institution of Oceanography, personal communication), while sulphide is not (J. J. Childress, personal observation). The blood of nine clams maintained in our sludge tank for 15–180 days contained thiosulphate levels that ranged from 3 to 105 μmol l^{-1}. Sulphite detected in the blood of five of these clams varied greatly and ranged from 28 to 320 μmol l^{-1}. No sulphide was detected in any of these samples.

Fig. 6A, shows plots of incubation time (h) *vs* thiosulphate concentration in the blood of *S. reidi* individuals incubated in a continuous flow of sea water containing 100–125 μmol l^{-1} sulphide. The data are from four separate experiments (see Materials and Methods). Though there was marked variation among individual clams and treatments, [thiosulphate] increased in the blood with exposure to sulphide in all treatments. In the non-O_2-limited experiments (Fig. 6B), initial thiosulphate concentrations in the blood of the 14-day post-capture clams taken from the holding tank averaged 12·5 μmol l^{-1} ($N = 4$) after 3–4 h in sea water and reached

300 μmol l^{-1} within 1 h in sulphide (Fig. 6B). Values over the following 23 h remained between 100 and 450 μmol l^{-1}, with no trend of further increases in concentration. Thiosulphate levels in the blood of the 180-day post-capture clams continued to rise for the first 5 h of the incubation to a concentration of 820 μmol l^{-1} and remained in this range for the following 16 h. No sulphide was detected in the blood of any of the clams in these non-O_2-limited incubations. [The results of earlier experiments indicated that clams held in captivity for >1 month showed activities of ribulosebisphosphate carboxylase (RuBPCase) that were substantially lower than those in freshly captured clams (A. E. Anderson, unpublished data) and decreased rates of CO_2 uptake (A. E. Anderson, in preparation). Thus, we assumed a symbiont population of lower viability or decreased size in the 43–180 day post-capture *vs* 14–18 day post-capture clams used in these experiments.]

In the O_2-limited experiments (Fig. 6A), great variation was found in the thiosulphate levels in the blood of 18-day post-capture clams, and concentrations were generally greater than 1·0 mmol l^{-1} after 17·5 h of incubation. For the 43-day post-capture clams exposed to O_2 limitation, final concentrations up to 3·1 mmol l^{-1} were reached after 16·5 h of incubation. Neither the 43-day nor the 18-day clams in the limited O_2 experiments showed the trend of stabilized thiosulphate concentrations seen in the high-O_2 study. In the O_2-limited studies, sulphide was detected in all of the blood samples, with concentrations ranging from 12 to 39 μmol l^{-1} in the 18-day clams and up to 105 μmol l^{-1} in the 43-day clams. Sulphite concentrations varied widely under all experimental conditions (0–175 μmol l^{-1}) and did not show a trend.

Both insufficient oxygen and a decreased or less-active symbiont population appear to reduce the further oxidation of thiosulphate. The rapid appearance of thiosulphate in the presence of added sulphide is additional evidence for the importance of this sulphur species in the sulphide oxidation pathway of the clam and the probability that it is the major energy-yielding substrate for the symbionts. The maintenance of an apparent steady state of about 300 μmol l^{-1} thiosulphate in the blood of the 14-day, high-O_2 animals in the presence of sulphide represents, with an average of 1·5 ml blood per clam ($N = 10$), less than 3·5 % of the sulphide oxidized per hour. Thus, blood thiosulphate does not represent a significant store of oxidizable substrate but probably a 'pool' that is rapidly turned over. Sulphide was present in the blood only under the conditions of limited O_2, i.e. O_2 levels apparently insufficient for the oxidation of all the sulphide entering the clams.

Gill ciliary activity in various levels of sulphide

In incubations of excised gills in sulphide, the movement of carmine particles indicated the maintenance of ciliary activity after more than 30 min in 100 μmol l^{-1} sulphide. In 250 μmol l^{-1} sulphide, this activity declined after 30 min; ciliary movement was greatly decreased after 20 min in 500 μmol l^{-1} sulphide. In 2·5 mmol l^{-1} sulphide (deoxygenated), moderate ciliary activity was detected for

10–15 min (after which it ceased), providing evidence for the inhibitory effects of sulphide.

Analyses of elemental sulphur in gills

The gills of both freshly caught and experimental clams exposed to sulphide or thiosulphate were frequently creamy yellow, in contrast to those of clams kept in the holding tank for long periods, which were reddish-brown. The yellow colour was particularly noticeable after incubations in 250 and 500 μmol l^{-1} sulphide and >250 μmol l^{-1} thiosulphate. Analyses of the gills of 13 experimental clams (100–500 μmol l^{-1} sulphide and 250 μmol l^{-1} or greater thiosulphate incubation conditions) revealed elemental sulphur contents that averaged 0·29 % (S.D. = 0·41) of dry mass (DM) and varied from less than 0·003 % (detection limit of analysis) to 1·33 % DM. Elemental sulphur in the gills of two freshly captured clams averaged 0·04 % DM. The gill from one animal incubated in 350 μmol l^{-1} thiosulphate contained 0·11 % DM elemental sulphur, while in that from a parallel experiment none was detected.

Elemental sulphur in the gills of three clams exposed to 100 μmol l^{-1} sulphide with no oxygen limitation ranged from undetectable ($N = 2$) to 0·22 % DM ($N = 1$). At the average sulphide oxidation rate found in 100 μmol l^{-1} sulphide (Table 1), 123 μmol of sulphide would be oxidized by a 10-g clam in 12 h. Elemental sulphur stores in the gill of 0·22 % DM would represent about 13·7 % of the sulphide oxidized in 12 h, whereas stores at the detection limit of the analysis method would represent 0·19 % of that oxidized over 12 h. These data suggest that, under the optimal conditions found for CO_2 fixation (100 μmol l^{-1} sulphide and unlimited oxygen), the quantities of elemental sulphur found in the gills would not represent a significant amount of the total sulphur flux that supports net CO_2 uptake. However, the range of amounts of elemental sulphur found in the gills from other than optimal conditions indicates the possibility of significant storage of sulphur, available for further oxidation when sulphide flux into the clam decreases and sufficient oxygen is available. As only the gills were analysed, it is possible that significant amounts of sulphur could be stored in other sulphide-oxidizing tissues, such as the outer layers of the foot (Powell & Somero, 1985) and perhaps the mantle tissue.

Calcium deposition studies

These studies were carried out to determine the quantitative contribution of calcification to CO_2 uptake. The average rates of deposition (Table 2), expressed as μg Ca^{2+} mg dry calcified shell^{-1} h^{-1}, were 0·021 (S.D. = 0·0004, $N = 6$) with sulphide and 0·020 (S.D. = 0·0001, $N = 12$) without sulphide. These rates were calculated for the linear portion of the slope (first 3 h with sulphide, 5 h without). The slope of the regression of μg Ca^{2+} deposited (y) *vs* incubation time (h) (x) was 0·0178 without and 0·0177 with sulphide. A comparison of the slopes of the two regression lin yielded no significant difference ($F_s = 0·0007$).

Table 2. *$^{45}Ca^{2+}$ deposition by individual* Solemya reidi *in the absence and presence of sulphide*

Sample	$^{45}Ca^{2+}$ deposition rate (μg Ca^{2+} mg dry shell^{-1} h^{-1})*			
	No sulphide†	(*N*)	100 μmol l^{-1} sulphide‡	(*N*)
Live clams	0·020 ± 0·001	(12)	0·021 ± 0·001	(6)
Empty shells	0·032 ± 0·030	(4)	0·049 ± 0·01	(2)
Frozen/thawed clams§	0·011 ± 0·006	(4)	0·024 ± 0·008	(2)

	CO_2 deposition rate (μmol g wet mass^{-1} h^{-1})¶	
Live clams	0·0625	0·0645

Values are means ± S.D.
N = number of animals or shells.
* Calcified portion of shell.
† Rates determined from 2, 3, 4 and 5 h incubations.
‡ Rates determined from 2 and 3 h incubations only (see Results).
§ See Materials and Methods for details.
¶ Transformations of average $^{45}Ca^{2+}$ deposition rates of live clams to deposition rate of CO_2 to estimate component of total CO_2 flux due to $CaCO_3$ deposition in shell.

The exchange rates (μg Ca^{2+} mg dry calcified shell^{-1} h^{-1}) determined for both the empty shells (0·032–0·049) and frozen/thawed clams (0·011–0·024) were much greater than the rates of deposition seen in the live animals (Table 2). Many of the frozen/thawed clams, used as a control in an attempt to resemble as closely as possible a live clam, fell apart over the 5 h, and thus exposed the inner calcified portion of the shell to the medium. These high exchange rates for the controls are undoubtedly due to the unusual shell morphology of *S. reidi*. As described by Beedham & Owen (1965), the outer surface of the shell of *S. reidi* is covered by a periostracum which extends well beyond the small central calcareous portion. Thus, in the live animal the calcareous portion of the shell is not exposed to the external environment and is also completely covered internally by the mantle tissues, which adhere to the perimeter of the periostracum. Thus, we do not consider that our data on exchange rates with empty shells and dead clams indicated background, non-biological exchange, and therefore these values were not subtracted from the rates found in the live clams, as is the usual procedure in bivalve calcification studies (Wilbur & Jodrey, 1952). Our deposition rates are, therefore, an overestimate, since they include an undetermined exchange factor.

Transformations of the $^{45}Ca^{2+}$ deposition rates to μmol CO_2 g wet tissue mass^{-1} h^{-1} were made [assuming 1 μmol CO_2 (μmol $^{45}Ca^{2+}$)$^{-1}$], and the mean value for the incubation with sulphide was 0·0645 and without sulphide 0·0625 (Table 2). These rates are not significantly different and can only be at most 7·6 % of the mean net CO_2 uptake rates determined in the respiration studies at 100 μmol l^{-1} sulphide (Table 1), and potentially make no contribution to the CO_2 uptake if the calcification rates measured are due entirely to exchange.

DISCUSSION

Autotrophy

The most significant result of this study was the demonstration of sulphur-driven autotrophy in this symbiont-containing bivalve. We define autotrophy [with regard to carbon, as it is commonly defined (Schlegel, 1975)] as the maintenance of a negative CO_2 balance: i.e. the uptake of CO_2 by the intact association is greater than the CO_2 respired. With the qualified assumption that the animal oxidizes the translocated symbiont-fixed organic products, our demonstration of net CO_2 uptake in the presence of reduced sulphur compounds suggests that autotrophy under these conditions does occur. This is the first time that net CO_2 uptake for any bacteria/invertebrate symbiosis has been demonstrated to be coupled with the consumption of reduced sulphur compounds.

The loss of organic carbon, such as in mucus or dissolved amino acids, was not measured in these studies. In studies on the net flux of usable organic carbon compounds, both net loss and net gain have been reported. If there is a net loss of such compounds from *S. reidi*, it is reasonable to assume that this loss would be no greater in the presence of sulphide than in its absence not, therefore, altering the evidence supporting autotrophy, as we have described it here.

The magnitude of this autotrophy can be appreciated by comparing it to the organic carbon content of the symbiosis. Assuming organic carbon to be approximately 10% of the wet tissue mass of *Solemya reidi*, the maximum observed net CO_2 uptake of $0{\cdot}89\,\mu mol\,g^{-1}\,h^{-1}$ (less 7·6% due to shell carbonate deposition) would equal 2·4% of the total organic carbon per day. This represents a high growth rate for a clam the size of these *S. reidi*.

Our experimental approach to examining the potential significance of carbon autotrophy by direct measurement of CO_2 flux is a novel one. Investigations of this sort for the sulphur-oxidizing chemoautotrophic bacteria have been made using growth measurements to estimate carbon fixed. Most commonly, these studies have used thiosulphate as the substrate and could not employ simultaneous direct measurement of oxygen and sulphur consumption. A notable exception is the recent work by Nelson, Jorgensen & Revsbach (1986) on *Beggiatoa*, grown in gradient tubes where both oxygen and sulphide consumption were measured during growth.

Respiratory flux in response to reduced sulphur compounds

Oxygen and CO_2 fluxes in *S. reidi* respond to the presence of sulphide and thiosulphate, with net CO_2 uptake observed in the presence of each compound. Maximum CO_2 uptake occurred at $100\,\mu mol\,l^{-1}$ sulphide, and at $250\,\mu mol\,l^{-1}$ or greater thiosulphate.

Oxygen and sulphide consumption and CO_2 flux were found to be sensitive to oxygen limitation and dependent upon sulphide concentration. Markedly decreased rates of sulphide consumption were induced by limited O_2, and CO_2 uptake did not occur under these conditions. However, despite the presence of low O_2 concentrations, near maximal rates of O_2 and sulphide consumption and CO_2 fixation

occurred if O_2 limitation was not imposed. Thus, in their natural habitat, where the O_2 concentration in their burrows is likely to be low (30–60 μmol l^{-1} in burrows in the laboratory holding tank) but that in the overlying water is not, autotrophy is possible.

We found that the absolute rates of O_2 and sulphide consumption, as well as CO_2 fixation increased between 50 and 100 μmol l^{-1} sulphide, but declined at concentrations of 250 μmol l^{-1} or more. This suggests that endogenous aerobic metabolism is maintained at 50–100 μmol l^{-1} sulphide but that it declines at higher concentrations. This is indicated in Fig. 5A by the lower intercept, which suggests reduced background aerobic respiration, for the 250–500 μmol l^{-1} sulphide regression as compared with the 50–100 μmol l^{-1} sulphide regression. Additional evidence of decreased aerobic metabolism above 100 μmol l^{-1} sulphide was provided by observations of ciliary activity of excised gills of *S. reidi* in SFSW compared with activity in various sulphide levels. This activity appeared unchanged in exposures to 100 μmol l^{-1} sulphide, but decreased in sulphide levels of 250 μmol l^{-1} or greater. In a similar experiment, Dando, Southward & Southward (1986) found that gill ciliary activity in the bacterial symbiont-containing *Lucinoma borealis* declined in greater than 100 μmol l^{-1} sulphide and ceased in 1·0 mmol l^{-1} sulphide. It is conceivable that above 100 μmol l^{-1} sulphide, molecular sulphide is able to diffuse into the tissues and blood, bypassing the detoxification step, as was shown to occur at 100 μmol l^{-1} sulphide under limiting O_2 conditions by analysis of clam blood. Thus, it is possible that the cause of the lower rates at higher sulphide concentrations is sulphide inhibition of the cytochrome *c* oxidase system, resulting in the observed decline in both oxygen consumption and sulphide oxidation [linked through cytochrome *c* (Powell & Somero, 1986)] capabilities of the host and possibly symbionts.

The cytochrome *c* oxidase in *S. reidi* occurs at high levels and is sensitive to sulphide inhibition (Hand & Somero, 1983). In incubations of isolated mitochondria from animal tissue of *S. reidi*, Powell & Somero (1986) found that, with added sulphide, the maximum rates of oxygen consumption occurred in a 20 μmol l^{-1} sulphide medium. O_2 consumption was inhibited at sulphide concentrations of greater than 20 μmol l^{-1}, an inhibition that was readily reversed at lower concentrations. At their experimental pH of 7·4, the concentration of H_2S, thought to be the inhibitory sulphide species (Broderius, Smith & Lind, 1977), would have been about 5·7 μmol l^{-1} ($pK_a = 7·0$). At the average pH value of 7·85 of our incubations, the concentration of H_2S during 50 and 100 μmol l^{-1} sulphide incubations would have been about 6·2 and 12·4 μmol l^{-1}, respectively, at steady state. However, at 250 and 500 μmol l^{-1} sulphide, the H_2S levels would have been about 30·9 and 61·8 μmol l^{-1}, respectively. Allowing for the probability that the intracellular concentration is lower than the medium concentration, the inhibition that is suggested by our whole animal studies to occur at [sulphide] of greater than 100 μmol l^{-1} is similar to that found in the mitochondria experiments of Powell & Somero (1986). As in the mitochondria experiments, the reversible nature of this inhibition was demonstrated in our study by the responses observed when the clams were transferred from 250 to 100 μmol l^{-1} sulphide (Fig. 4).

Finally, the absence of CO_2 fixation at greater than $100\,\mu mol\,l^{-1}$ sulphide, or at $100\,\mu mol\,l^{-1}$ sulphide with O_2 limitation (under which conditions sulphide was detected in the blood at substantial levels), might be explained by sulphide inhibition of bacterial cytochrome *c* oxidase (and resultant inhibition of bacterial CO_2 fixation) or insufficient oxygen available for thiosulphate oxidation. If bacterial (as well as animal) cytochrome *c* oxidase is inhibited by the presence of sulphide, this would prevent the generation of NAD(P)H, needed for CO_2 fixation reactions, by reverse movement of electrons through the cytochromes (Kelly, 1982). Insufficient available oxygen could prevent bacterial oxidation of thiosulphate and subsequent CO_2 fixation, as suggested by the increased thiosulphate levels in the blood of clams incubated in sulphide under O_2 limitation (Fig. 6A). Thus, the occurrence of any of these mechanisms could be expected to stop CO_2 fixation, as we observed in our study.

The evidence indicates that thiosulphate is the main product of sulphide oxidation in the animal tissues, and that it is transported to the bacteria where it is further oxidized. Levels of blood thiosulphate were found to be dependent both upon the availability of oxygen and upon the activity, viability or size of the symbiont population. Steady-state levels of thiosulphate were maintained for 24 h in the most freshly captured clams incubated in $100\,\mu mol\,l^{-1}$ sulphide with no O_2 limitation. This is evidence that thiosulphate is further oxidized by the symbionts, as clams held in captivity for a longer period (and thus with a smaller or less-viable symbiont population) showed greatly increased levels of thiosulphate accumulation (Fig. 6B) when incubated under the same conditions. In addition, the oxidation of thiosulphate by the bacteria is O_2-dependent, as thiosulphate concentrations rose significantly in the blood of clams under O_2-limiting conditions (Fig. 6A). Thus, sulphide oxidation in the animal tissues appears to perform at least three functions: detoxification of sulphide and resultant protection of the host and bacteria (Powell & Somero, 1985), concurrent production of ATP by the host (Powell & Somero, 1986), and supply of the non-toxic product (thiosulphate) for use by the bacteria. In contrast to the sulphide-binding proteins found in the blood of the vent tubeworm *Riftia pachyptila* (Arp & Childress, 1983) and vent clam *Calyptogena magnifica* (Arp, Childress & Fisher, 1984), protection mechanisms in *S. reidi* do not involve a blood-borne sulphide-binding protein (J. J. Childress, unpublished data).

All forms of stored reduced sulphur (including elemental sulphur and thiosulphate measured in this study) appear to be of little importance in the overall carbon flux of the intact animal. We did not find evidence of CO_2 uptake (i.e. decreased CO_2 production with respect to O_2 consumed) in respiration studies in the absence of exogenous sulphide or thiosulphate in even the most recently caught animals. This is consistent with the data on free-living sulphur bacteria, which show that even with elemental sulphur stores of 30% DM [the maximum value found in pure cultures of *Beggiatoa* (Nelson & Castenholz, 1981) and twice that found in field samples (Jorgensen, 1977)], the maximum contribution of the oxidation of such stores to bacterial carbon biomass (assuming complete oxidation to sulphate) would be only 6% (Nelson *et al.* 1986). Given these estimates, oxidation of the mean level of

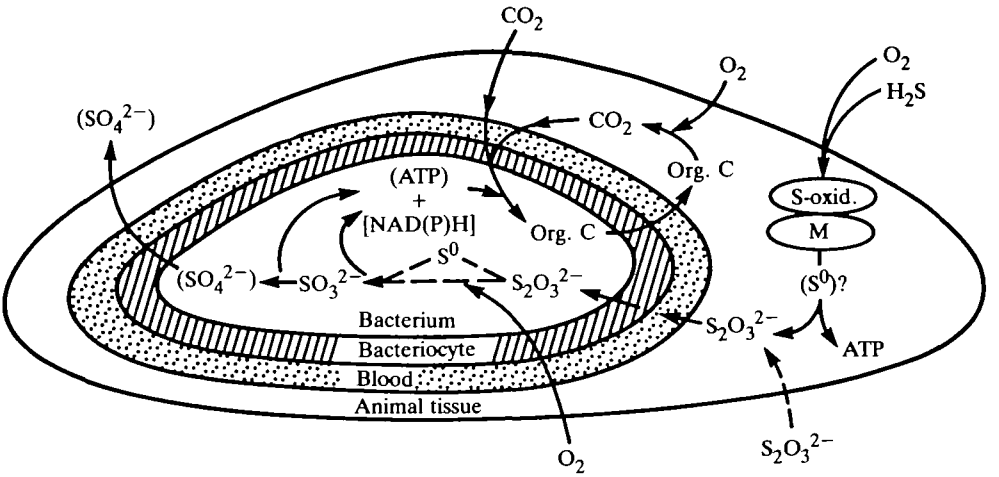

Fig. 7. Whole animal model for *Solemya reidi*, indicating the sites and products of sulphide (and thiosulphate) oxidation, site for animal (and probable site for bacterial) ATP production, and coupling to CO_2 fixation. Diagram based upon results of this study and those of Felbeck (1983), Fisher & Childress (1986) and Powell & Somero (1985, 1986). Org.C, organic carbon; M, mitochondrion; S-oxid., sulphur oxidation.

elemental sulphur that we found in a pooled sample of gills of experimental clams, 0·29% DM, would thus be expected to support far less of the total CO_2 flux (determined in 100 μmol l^{-1} sulphide over 12 h) than the 6% suggested for isolated bacteria with stores of 30% DM and to be below detection in our respirometry system.

Whole animal model

Our data, as well as those published by other investigators, suggest an overall model (Fig. 7) for the pathways of sulphide metabolism and concurrent mechanisms for nutritional input of symbiont-fixed carbon in the intact *S. reidi*. Briefly, the detoxification of sulphide, an oxygen-dependent process, occurs in the animal tissues and results in the production of ATP (Powell & Somero, 1986). A major product of this oxidation, thiosulphate, can also be taken up across the body surfaces from the medium, and is carried in the blood to the gill symbionts. There, thiosulphate is further oxidized by the bacteria, again dependent upon availability of free O_2, to sulphite and, probably, sulphate. This oxidation yields ATP and NAD(P)H to power the fixation of CO_2 *via* the Calvin–Benson cycle, as evidenced by the coupling between the addition of reduced sulphur species and net CO_2 uptake. The CO_2 for this fixation is potentially derived from ambient sea water as well as from animal respiration. Under conditions of limiting O_2, or sulphide levels that exceed the capacity of the sulphide detoxification processes, sulphide diffuses into the animal tissues and blood and inhibits O_2 and sulphide consumption and CO_2 uptake by inhibiting both animal and bacterial cytochrome *c* oxidases. Under these O_2-limiting conditions, it is likely that the inhibition of the complete oxidation of sulphide results

in the storage of elemental sulphur, which was detected in many of the freshly captured and experimental animals.

Molar CO_2: O_2: sulphur compound ratios

The simultaneous measurements of CO_2, O_2 and sulphide by gas chromatography in the respiration studies allow us to pursue a unique line of inquiry with our data. Microbiologists typically use the molar CO_2: sulphur compound ratios determined for long-term steady-state growth of various free-living chemolithoautotrophic bacteria to estimate the efficiency with which the energy derived from oxidation of reduced sulphur compounds is used to fix CO_2. O_2 consumption: sulphur compound ratios, if determined at all, are from separate, short-term respiration studies. Using the concurrent flux rates of CO_2, O_2 and sulphide found in our respiration studies, we were able to calculate such efficiency ratios for the intact *S. reidi*.

It is interesting to compare the ratios derived for this intact symbiosis, in which only a small percentage of the biomass is bacteria, with those reported for free-living, sulphur-oxidizing bacteria (Table 3). The first three listings in Table 3 represent net molar ratios based upon growth yields (*Beggiatoa*) or net CO_2 uptake (this study), and are calculated as net CO_2 fixed: total O_2 consumed: total sulphide oxidized. These net ratios determined for *S. reidi* at 50–100 μmol l^{-1} sulphide are very similar to those based upon net growth of *Beggiatoa* cultures on sulphide. These ratios indicate comparable growth efficiencies in this symbiosis and in free-living bacteria.

Using the calculation methods of Kelly (1982), the thermodynamic efficiencies implicit in these ratios for CO_2 fixation *via* sulphide oxidation energy 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 sulphide to sulphate, the resulting yields are 10·4 % and 20 % at 50–80 and 100 μmol l^{-1} sulphide, respectively. These ratios can easily be adjusted to reflect the CO_2: thiosulphate ratios (2 sulphide = 1 thiosulphate), perhaps more reflective of the true net efficiencies, as the substrate used by the bacteria for CO_2 fixation is almost certainly thiosulphate rather than sulphide. The calculated net CO_2: thiosulphate ratios are 0·3: 1 and 0·58: 1, and corresponding thermodynamic efficiencies are 16 % and 31 %, respectively (given a $\Delta G = -936$ kJ for the oxidation of thiosulphate to sulphate). It is necessary to point out that these net ratios do not estimate the potential total CO_2 fixed per sulphide oxidized, as net CO_2 uptake or growth measurements have not been corrected for the respiratory CO_2 loss of aerobic metabolism.

The next four listings are estimates of the molar ratios of total CO_2 fixed: sulphur compound oxidized for various sulphur-oxidizing bacteria, and represent 'maximum' or 'true' growth yields (Y_{max}) (Table 3). These values were obtained by adjusting observed net growth yields (data not listed) for the energy of maintenance [which varies from very low to moderately high, depending upon the bacterium (Beudeker, Gottschal & Kuenen, 1982)] by means of the Pirt (1965) equation (Kelly, 1982).

Aerobic metabolism in *S. reidi* is maintained in the presence of 50–100 μmol l^{-1} sulphide, as is indicated by the y-intercept in Fig. 5A, continuing gill ciliary beating at these sulphide concentrations, and absence of anaerobic substrates and products

Table 3. *Estimates of molar CO_2:O_2:sulphur compound ratios for* Solemya reidi *and comparisons with ratios of various chemolithoautotrophic sulphur-oxidizing bacteria*

Substrate	[Substrate] (μmol l^{-1})	Efficiency ratios (molar equivalents) CO_2:O_2:sulphur compound	Species studied	Reference
Net ratios				
Sulphide	—	0·35:1·71:1	*Beggiatoa* spp.†	Nelson, Jorgensen & Revsbach (1986)
Sulphide	50–80	0·15:2·0:1	*S. reidi*‡	This study
Sulphide	100	0·30:1·4:1	*S. reidi*‡	This study
Estimated total ratios				
Miscellaneous free-living bacteria§				
Thiosulphate	—	0·26:ND:1	*Thiobacilli* spp. (aerobic)	Kelly (1982)
Thiosulphate	—	0·42:ND:1	*T. denitrificans* (anaerobic)	Justin & Kelly (1978), Timmer-ten Hoor (1981), Kelly (1982)
Thiosulphate	—	0·55:ND:1	*Thermothrix thiopara*	Mason, Kelly & Wood (1987)
Thiosulphate	—	0·58:ND:1	*T. denitrificans* (aerobic)	Justin & Kelly (1978), Kelly (1982)
Solemya reidi¶				
Sulphide	50–80	* 0·86:1	*S. reidi*	This study
Sulphide	100	* 0·92:1	*S. reidi*	This study

* The true value for total CO_2:sulphide, uncertain due to difficulties estimating the levels of CO_2 production by background respiration, probably lies between 0·30 (maximum net value) and 0·69 CO_2:sulphide (see Discussion).

† Based on net growth (increase in C biomass) on sulphide.

‡ Ratios calculated from net CO_2 uptake in the presence of sulphide (see Discussion); net CO_2 uptake: total O_2 consumed: sulphide.

§ 'Total' or maximum growth estimates; based upon net growth data adjusted by Pirt (1965) equation to correct for energy of maintenance.

¶ Estimated total ratios, based upon net ratios adjusted to include background aerobic metabolism in clam tissues (see Discussion for details); total CO_2 uptake: O_2 used for sulphide oxidation alone: sulphide.

ND, not determined.

in the tissues of clams exposed to $100\,\mu mol\,l^{-1}$ sulphide (A. E. Anderson, in preparation), and such metabolism therefore represents a potentially large fraction of the CO_2 and O_2 fluxes in the intact association. However, it cannot be assumed that endogenous aerobic metabolism continues at a constant rate when the conditions are changed by exposing the animals to sulphide. Thus, to estimate the total CO_2 fixation for *S. reidi* it is necessary to estimate endogenous respiration in the presence of sulphide and to adjust the carbon and oxygen fluxes by this estimate. Using such an estimate, CO_2 fixation [(respired CO_2) + (net (measured) CO_2 uptake) = total CO_2 fixation] and O_2 used for sulphur compound oxidation alone [(O_2 with sulphur) − (O_2 without) = O_2 used to oxidize sulphur compounds] can be calculated.

Our estimation of the background aerobic rates assumes a decline in the requirement by the animal tissues for ATP produced by aerobic metabolism due to the yield of 1 ATP per sulphide oxidized by the animal tissues (Powell & Somero, 1986). This implies lower background rates than those measured in the absence of sulphide (Table 1). If we take the pre-sulphide O_2 consumption as a measure of total ATP needs, we can calculate a decline in these rates (assuming $6\,ATP/O_2$ *via* oxidative phosphorylation) in proportion to the ATP produced by sulphide oxidation at each non-inhibiting sulphide concentration (as predicted by the sulphide oxidation rates, Table 1). The background O_2 consumption rates calculated by this adjustment are 1·71 and $1{\cdot}46\,\mu mol\,g^{-1}\,h^{-1}$ for 50–80 and $100\,\mu mol\,l^{-1}$ sulphide, respectively. In fact, the average of these rates, 1·59, is much closer to the background aerobic rate of 1·56 predicted by the y-intercept of the regression of O_2 on sulphide ($50–100\,\mu mol\,l^{-1}$ sulphide, Fig. 5A) than to the average pre-sulphide O_2 consumption rate of $1{\cdot}97\,\mu mol\,g^{-1}\,h^{-1}$ (Table 1), supporting our assumption that there is a decline in background aerobic metabolism in the presence of low ($50–100\,\mu mol\,l^{-1}$) levels of sulphide. Using these values (1·71 and 1·46), and assuming the maintenance of the pre-sulphide RQ of 1·0, the resulting estimates of total CO_2 fixed:O_2 used for sulphur oxidation:sulphur oxidized for our data are 1·31:0·86:1 and 0·77:0·92:1 for 50–80 and $100\,\mu mol\,l^{-1}$ sulphide, respectively, and are theoretically comparable to the ratios representing 'true' bacterial growth yields (Table 3). The mean O_2:sulphide ratio predicted by these methods, 0·89, is nearly identical to the independent estimate of the incremental O_2:sulphide (0·86:1), the slope of the regression of [O_2] on [sulphide] (Fig. 5A), thus providing additional confidence for our estimates of background O_2 consumption. These results would appear to suggest an efficiency of CO_2 fixation for this intact symbiosis (0·77–1·31:1) that is greater than the highest efficiency yet reported (0·58:1) for a chemoautolithotrophic sulphur bacterium (Justin & Kelly, 1978). In addition, the thermodynamic efficiencies implicit in these ratios (0·77–1·31:1), 90% and 53%, respectively, are very unlikely in a biological system.

One possible source of error that could account for artificially high CO_2:sulphide estimates is our assumption that the pre-sulphide CO_2:O_2 ratio (RQ) of 1·0 is maintained in the presence of sulphide. The slope ($0{\cdot}38 \pm 0{\cdot}31$) of the regression ■ CO_2 uptake on sulphide (see Results), which is not affected by estimates of

background aerobic respiration, is a predictor of the incremental CO_2 fixed per sulphide oxidized and thus provides an alternative method of determining the total CO_2:sulphide ratio, just as the slope of the regression of O_2 on sulphide independently predicted the incremental O_2 per sulphide derived by other methods. Though the data of the molar CO_2:sulphide regression are scattered, the mean total CO_2:sulphide ratio predicted is 0·38:1 at 50–100 μmol l^{-1} sulphide (only slightly higher than the highest net value of 0·30) and the maximum (upper 95 % confidence interval) value is 0·69:1. The range of thermodynamic yields implicit in these ratios is 26–48 %, and 40–73 % if transformed to CO_2:thiosulphate. Given these fixation ratios and thermodynamic yields, it is reasonable to assume that the true yields lie somewhere between the maximum net value (not affected by estimates of background metabolism) of 0·3:1 and the maximum total estimate of 0·69:1. These values are within the range of maximum fixation efficiencies reported for free-living bacteria (Table 3).

These estimates imply a dramatic change in animal carbon metabolism in the presence of sulphide. At the high CO_2:sulphide estimate of 0·69:1, the CO_2 production rates would have been 0·80 and 1·23 μmol g^{-1} h^{-1} at 50–80 and 100 μmol l^{-1} sulphide, respectively. Compare these values with those corresponding to the maintenance of an RQ of 1·0: 1·94 and 2·35 μmol g^{-1} h^{-1}. At the lower estimated CO_2:sulphide ratio (0·38:1), the discrepancies would be even greater. Clearly, a substantial decline in animal CO_2 production relative to O_2 consumption is implied.

One additional potential error is the possibility that the rate of spontaneous (non-biological) sulphide oxidation in the animal chamber is decreased relative to that in the control chamber. This would not be detected in our system and would result in underestimates of sulphide and oxygen consumption rates, and thus overestimates of the CO_2:sulphide ratios.

The ratios of $^{14}CO_2$ fixed:O_2 consumed (0·34:1) reported in Cavanaugh (1985*b*) for *S. velum* are difficult to compare to the ratios listed for *S. reidi* in Table 3. The $^{14}CO_2$ fixed, though reported by Cavanaugh as 'net fixation', is closer to an estimate of total fixation. In fact, it is an underestimate of total CO_2 fixation because it does not account for loss of fixed CO_2 by animal respiration (Muscatine, 1980). In *S. velum*, no increase in O_2 consumption upon the addition of thiosulphate was detected, suggesting conditions of limiting O_2 that would preclude maximal autotrophic rates. Thus, the ratio of 0·34:1 represents total CO_2 fixed:total O_2 consumed, which is not comparable to our ratios because of the presence of background aerobic metabolism in the animals in our studies.

An examination of the estimates of 0·86 and 0·92 O_2:sulphide for 50–80 and 100 μmol l^{-1} sulphide (Table 3) raises an apparent discrepancy between our results and the balanced equation of 2 O_2 per sulphide for complete oxidation to sulphate. Although the majority of bacterial studies estimating CO_2:sulphur compound ratios have not measured O_2 consumption, a few have reported similar O_2:sulphur compound ratios for chemolithoautotrophic bacteria. Peck & Fisher (1962) reported a ratio of 0·82:1 for *Thiobacillus thioparus*, another deviation from the classical

models and very similar to our estimated total ratios of 0·86:1 and 0·92:1. Kelly (1982) reported an O_2:sulphide ratio of 1·47:1 for *T. neapolitanus*. Similarly, Nelson *et al.* (1986) reported an O_2:sulphide ratio of 1·07:1 during exponential growth of *Beggiatoa* on sulphide, during which phase elemental sulphur also accumulated.

The apparent 'missing' oxygen uptake can be partially accounted for by the reducing equivalents from the oxidation of sulphide that must be used to fix CO_2. Following the reasoning of Kelly (1982), each CO_2 fixed to the level of CH_2O *via* the Calvin–Benson cycle requires $4e^-$ and $4H^+$. By Kelly's calculations, for our total fixation estimate of $0{\cdot}38CO_2$:sulphide, CO_2 requires $0{\cdot}38 \times 4(H) = 1{\cdot}52$ of the 8(H) available from complete oxidation of sulphide. Thus, $8 - 1{\cdot}52 = 6{\cdot}48$(H) theoretically remain for the reduction of O_2, and the predicted O_2 uptake would be $6{\cdot}48/8 \times 2 = 1{\cdot}62O_2$, compared with our value of 0·86–0·92 (Table 3). By similar means, at our maximum estimate of $0{\cdot}69CO_2$:sulphide, the predicted O_2 uptake would be $1{\cdot}31O_2$:sulphide. This discrepancy in O_2 consumption could possibly be explained by the use of available reductant for heterotrophic CO_2 fixation, in addition to O_2 reduction and bacterial CO_2 fixation, and might be related to the proposed decrease in animal tissue CO_2 production in the presence of sulphide. Attempts to quantify precisely the complexities of the oxidation of sulphide and its products by both compartments of this symbiotic association are speculative at the present time.

Clearly, the picture is not complete. The pathways and mechanisms of sulphide oxidation in chemolithotrophs are complex and not yet completely understood and cannot be precisely revealed by these methods. Though much has been elucidated, no simple metabolic scheme can yet be proposed that would explain all the data. Nevertheless, the coupling of sulphide oxidation and CO_2 fixation in *S. reidi* does suggest an efficient usage of the chemical energy of sulphide oxidation in this highly integrated symbiosis.

Solemya reidi *in its natural environment*

Though the occurrence of thiosulphate uptake has been demonstrated, the significance of this mechanism in the clam's natural environment, where thiosulphate levels are negligible (R. Vetter, personal communication), is not known. The interstitial sulphide levels in the Hyperion outfall muds are quite variable and can be very high, up to 22 mmol l^{-1} (Childress & Lowell, 1982). It is not known what levels of sulphide are found in the mucus-lined burrows of this clam. Behavioural adaptations may allow these clams to avoid sulphide toxicity and maintain CO_2 fixation at or near optimal levels. Doeller (1984) has suggested temporally separate loading of O_2 and sulphide for *S. velum*. However, the results of our 65-h incubation in 100 μmol l^{-1} sulphide (Fig. 3) show that simultaneous uptake of O_2 and sulphide is possible under these conditions and results in continuous CO_2 uptake. In the field, however, an encounter with potentially toxic levels of sulphide in the burrow might stimulate the clam to: (1) swim away, as these clams are active swimmers (Reid 1980); (2) move to a higher and thus more oxygenated portion of their burrows and

increase ventilation of overlying water; (3) decrease pumping rates; (4) increase stores of elemental sulphur for use when oxygen is available, a possibility also suggested by Nelson *et al.* (1986) for *Beggiatoa*; and/or (5) close their valves completely and undergo short periods of anaerobiosis.

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