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Bacterial Disproportionation of Elemental Sulfur Coupled to Chemical Reduction of Iron or Manganese

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A new chemolithotrophic bacterial metabolism was discovered in anaerobic marine enrichment cultures. Cultures in defined medium with elemental sulfur (S^0) and amorphous ferric hydroxide (FeOOH) as sole substrates showed intense formation of sulfate. Furthermore, precipitation of ferrous sulfide and pyrite was observed. The transformations were accompanied by growth of slightly curved, rod-shaped bacteria. The quantification of the products revealed that S^0 was microbially disproportionated to sulfate and sulfide, as follows: $4S^0 + 4H_2O \rightarrow SO_4^{2-} + 3H_2S + 2H^+$. Subsequent chemical reactions between the formed sulfide and the added FeOOH led to the observed precipitation of iron sulfides. Sulfate and iron sulfides were also produced when FeOOH was replaced by $FeCO_3$. Further enrichment with manganese oxide, MnO_2, instead of FeOOH yielded stable cultures which formed sulfate during concomitant reduction of MnO_2 to Mn^{2+}. Growth of small rod-shaped bacteria was observed. When incubated without MnO_2, the culture did not grow but produced small amounts of SO_4^{2-} and H_2S at a ratio of 1:3, indicating again a disproportionation of S^0. The observed microbial disproportionation of S^0 only proceeds significantly in the presence of sulfide-scavenging agents such as iron and manganese compounds. The population density of bacteria capable of S^0 disproportionation in the presence of FeOOH or MnO_2 was high, $>10^4$ cm^{-3} in coastal sediments. The metabolism offers an explanation for recent observations of anaerobic sulfide oxidation to sulfate in anoxic sediments.

In aquatic sediments sulfur undergoes bacterial redox transformations which, in their entirety, are known as the microbial sulfur cycle. The reduction of sulfate to hydrogen sulfide (H_2S) is catalyzed by the strictly anaerobic, sulfate-reducing bacteria. The microbiology of sulfate reduction has been studied intensively (37, 48, 49), and quantification of the process by radiotracer techniques has proved it to be a major pathway of carbon mineralization in marine sediments with significance for the global carbon cycle (21, 23, 42).

In the oxic zone of sediments the formed H_2S, or other partially reduced sulfur compounds, is reoxidized to sulfate by chemolithotrophic bacteria such as *Beggiatoa* and *Thiobacillus* spp. (27, 34). In the absence of O_2, some of these bacteria may use nitrate as the terminal electron acceptor (e.g., see reference 29).

There are several indications, however, that sulfide is oxidized completely to sulfate in the absence of oxygen and nitrate in marine and limnic sediments.

(i) The depth in sediments at which H_2S accumulates is often several centimeters below the oxic surface layer (23). The separating suboxic zone is characterized by the accumulation of Mn^{2+} and Fe^{2+} from metal oxide reduction in the pore water (19, 22, 43). Sulfate reduction continuously produces H_2S in the suboxic zone, but the concentrations are kept low (<1 μM), presumably by reactions involving iron and manganese oxides (4, 9, 43).

(ii) Addition of manganese oxide to anoxic salt marsh sediments caused the oxidation of solid-phase sulfides to sulfate with apparent biological catalysis (1, 28). Addition of ferric hydroxide only resulted in slight sulfate production. The depth distribution of manganese and FeS in Danish coastal sediments also suggested manganese oxide to be an important oxidant for FeS-derived sulfur (10). So far, however, bacteria capable of oxidizing reduced sulfur compounds with iron or manganese at circumneutral pH have not been described.

(iii) A strong indication of an anaerobic oxidation of H_2S to sulfate came from recent radiotracer studies. In anoxic, nitrate-free sediments, added radiolabeled H_2S was oxidized to sulfate (14, 16, 24–26). The mechanism was not revealed, but thiosulfate was the major immediate oxidation product. The most important process in the further transformation of thiosulfate was bacterial disproportionation (3).

The studies mentioned above inspired us to search for bacteria oxidizing reduced sulfur species with manganese or iron oxide. Enrichment for bacteria oxidizing H_2S with manganese or iron oxides is complicated by the rapid chemical reaction between these compounds, yielding S^0 as the major product (8, 38, 39). To avoid this, we chose elemental sulfur as the sulfur source in our enrichments. The outcome of the enrichment studies is presented here.

MATERIALS AND METHODS

The procedures used for preparation of culture media and cultivation of anaerobic bacteria were basically those used for sulfate-reducing bacteria described by Widdel and Bak (49).

Medium. The basal medium had the following composition (in grams per liter of distilled water): NaCl, 15; $NaHCO_3$, 2.5; $MgCl_2 \cdot 6H_2O$, 2; $CaCl_2 \cdot 2H_2O$, 0.4; KCl, 0.5; NH_4Cl, 0.25; and KH_2PO_4, 0.2. The medium was autoclaved and cooled under an atmosphere of O_2-free N_2–CO_2 (88:12, vol/vol), after which the following components were added aseptically from sterile stock solutions (per liter): 30 ml of 1 M $NaHCO_3$, 2 ml of trace element solution without chelating agent, and 1 ml each of three different vitamin

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solutions (49). The pH was adjusted to 7.3. The medium was dispensed aseptically into 50- or 250-ml airtight screw-cap bottles. Elemental sulfur and manganese or iron oxide were added directly before inoculation.

Flowers of sulfur were thoroughly ground in a mortar together with distilled water, and the suspension was autoclaved at 114°C for 30 min. Sulfur was added in excess (ca. 0.3 mmol liter^{-1}) to the medium with a sterile spatula.

Red, amorphous ferric hydroxide (FeOOH) was prepared by the method of Lovley and Phillips (30), and its uncrystalline nature was confirmed by x-ray diffraction analysis.

Ferrous carbonate ($FeCO_3$) was synthesized by mixing Na_2CO_3 powder into an anoxic solution of an equimolar amount of $FeCl_2$ (0.5 M) with vigorous stirring under oxygen-free N_2. The pale grey $FeCO_3$ precipitate was washed in anoxic distilled water until the NaCl concentration of the suspension was approximately equal to that of the medium. Dissolution of the $FeCO_3$ in 0.5 N HCl (see below) showed <10% Fe(III).

Manganese oxide (MnO_2) was prepared by the method of Burdige (7) with the modification that the amount of NaOH was doubled. X-ray diffraction analysis showed no lines, indicating poor crystallinity of the MnO_2. The average oxidation state of Mn determined by the oxalate method (33) was 3.98 ± 0.05 (n = 13). The FeOOH and MnO_2 suspensions contained ca. 0.5 mol of Fe or Mn liter^{-1} and were stored at 4°C and added to the culture medium under O_2-free N_2.

Enrichment and cultivation. Completely filled 50-ml screw-cap bottles were used for enrichments. Medium supplemented with elemental sulfur and FeOOH (40 mmol of Fe liter^{-1}) or MnO_2 (30 mmol of Mn liter^{-1}) was inoculated with marine sediment from the 1- to 5-cm depth interval. The sediment was collected at a 16-m-deep location in Aarhus Bay, Denmark. Inoculation and subsequent transfers were made with 5 ml of inoculum into 50-ml screw-cap bottles with no headspace. Incubation was stationary at 30°C in the dark.

Determination of stoichiometries. For determination of stoichiometries, the enrichments were subcultured into 250-ml screw-cap bottles. These were supplied with S^0 and different amounts of MnO_2, FeOOH, or $FeCO_3$ or with S^0 only. Uninoculated controls were made in parallel.

Samples were withdrawn with sterile glass pipettes after vigorous shaking. The gas phase was immediately flushed with N_2-CO_2 (88:12), and after the first sampling, the screw-caps were replaced by black rubber stoppers. To avoid loss of accumulating free H_2S because of flushing with N_2-CO_2, the volume of liquid removed from subcultures of the S^0-MnO_2 enrichment with S^0 only was replaced by pieces of glass and the bottles were sealed again immediately.

Separations. For determination of SO_4^{2-}, H_2S, Fe^{2+}, and Mn^{2+} and of particulate Mn(II), an aliquot was filtered through a 0.45-μm cellulose acetate filter under N_2. For analysis of SO_4^{2-} and H_2S, filtrate was collected in 2% $ZnCl_2$ to stabilize sulfide as ZnS. The rest of the filtrate was acidified with HCl for analysis of dissolved Fe^{2+} or Mn^{2+}.

The Mn(II) in the precipitate [adsorbed Mn(II), $MnCO_3$] remaining on the filter was measured after dissolution. After the filter was washed with distilled water, Mn^{2+} was dissolved and washed out with 2 ml of H_2SO_4 and 2 ml of distilled water. The acid and water from the last wash were mixed and analyzed for Mn^{2+}.

Ferric iron was dissolved by 16-h extraction in 0.5 N HCl. This treatment dissolved amorphous FeOOH completely. Ferric iron was determined as the difference between total Fe and Fe^{2+} in the solution.

For determination of sulfide precipitates, 2 ml of culture was initially fixed in 1 ml of 20% zinc acetate. The sulfide precipitates were extracted by three slightly different sulfide distillation procedures.

Samples from the incubations with $FeCO_3$ were analyzed by a two-step distillation that separated acid-volatile (AVS; H_2S and FeS) and chromium(II)-reducible (CRS; S^0 and pyrite, FeS_2) sulfides (15). Since S^0 caused a high background of sulfide during chromium reduction, iron sulfides distilled in this step were quantified indirectly as iron dissolved. The $FeCO_3$ dissolved completely in the acid distillation step.

Two-step distillation was also used with the final samples from the time course experiments with $FeCO_3$ and FeOOH cultures. Here, however, S^0 in the samples was removed by extraction with CS_2 prior to chromium distillation. Thus, the CRS measured only represented iron sulfides such as pyrite. Control experiments confirmed that the CS_2 treatment dissolved all S^0 in the samples.

Samples from the incubations with MnO_2 were analyzed by acid distillation only. To reduce oxidized manganese, which could interfere by oxidizing the dissolving sulfide (28), the samples were mixed with a solution of 1% $NH_2OH \cdot HCl$ for 10 min prior to acidification. Using this treatment, no loss of sulfide could be detected during control distillations of a defined mixture of ZnS and MnO_2.

Analyses. Sulfate was measured by nonsuppressed anion-exchange chromatography (pump model 510, IC-PAK anion column, conductivity detector model 430; Waters). In a few cases, SO_4^{2-} was measured by $BaSO_4$ precipitation and turbidometry (12). Sulfide was quantified by the methylene blue method (11) with a detection limit of 1 μM. Soluble and HCl-extracted Fe^{2+} were measured spectrophotometrically by reaction with Ferrozine (44) (0.02% in 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7), and total HCl-extractable Fe was measured similarly with reducing Ferrozine (Ferrozine with 1% hydroxylamine hydrochloride) (36). Iron dissolved during chromium distillation and Mn^{2+} were determined by flame atomic absorption spectroscopy (Perkin-Elmer 370 A spectrometer).

Cell counts. Since the Mn and Fe precipitates did not allow growth measurement by the monitoring of increases in optical density, cells were counted directly by staining with acridine orange. Culture samples were preserved with glutaraldehyde (2.5% final concentration). Before counting, manganese precipitates were removed with 1.5 M $NH_2OH \cdot HCl$ in 0.25 N HCl while iron precipitates were dissolved by a dithionite solution (sodium dithionite, 50 g liter^{-1} in 0.2 M sodium citrate–0.35 M acetic acid [9]). Then, 50 to 500 μl of preserved sample was added to 2 ml of the extractants. After 5 min, the mixture was filtered through a 0.2-μm polycarbonate filter, which was subsequently stained with 0.01% acridine orange for 1 min. The staining solution was then filtered off and the filter was washed in distilled water. All solutions were sterilized by filtering (0.2-μm filter). Cells were counted by epifluorescence microscopy.

Viable counts in sediment samples. To enumerate bacteria metabolizing S^0 in the presence of either FeOOH or MnO_2 in natural sediments, we used most-probable-number (MPN) dilutions (2). The dilution series were prepared as described by Bak and Pfennig (4). The medium used was identical to the enrichment medium. The tubes for counting were incubated for 6 months at 20°C and checked for bacterial growth every second week. Grown cultures were tested for H_2S and

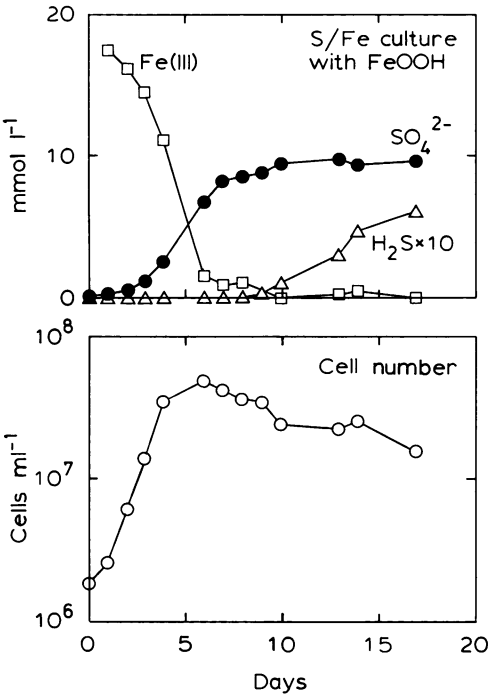

FIG. 1. Time course of SO_4^{2-} and H_2S production, Fe(III) reduction, and bacterial growth in the S/Fe enrichment culture incubated with amorphous FeOOH: average of two parallel cultures. (Top) Chemical analyses; note that H_2S concentrations are multiplied by 10. (Bottom) Cell number; note logarithmic scale.

SO_4^{2-} formation. With S^0 and MnO_2, counts were made with marine sediment from three locations around Denmark: Aarhus Bay at 16-m depth, a salt marsh on the west coast of Jutland, and manganese-rich sediment from the deepest part of Skagerrak at 695-m depth. With S^0 and FeOOH, enumerations were done in sediment from the salt marsh.

RESULTS

With both FeOOH and MnO_2, cultures that metabolized S^0 with concomitant reduction of the metal oxide were obtained. The two enrichment cultures were designated S/Fe culture and S/Mn culture, respectively. The cultures were routinely transferred every other week when growth had ceased (see below). Neither growth nor reduction of metal oxides was detected within 2 months when the cultures were pasteurized (75°C, 20 min), not inoculated, or inoculated with an anoxically autoclaved enrichment culture. Pasteurization of growing cultures terminated all measured processes immediately. The determination of stoichiometries was carried out after more than 20 subcultures.

S/Fe culture. The enrichment culture with S^0 and amorphous ferric hydroxide grew and reduced the added FeOOH within 1 week, with concomitant SO_4^{2-} production (Fig. 1). A lag phase was not observed. The culture was dominated by rod-shaped, slightly curved cells, attached to the particulate phase. The growth rate during the exponential phase was about 1 day^{-1}. During the iron reduction phase, the concentration of free Fe^{2+} did not exceed 200 μM. The red color of the FeOOH precipitate changed to black, and the yellow sulfur grains received a grey coating. Formation of black precipitates consisting of magnetite (Fe_3O_4) has been described for cultures of dissimilatory Fe(III)-reducing bacteria (32). However, the black precipitate observed in our cultures was not magnetite but ferrous sulfides. The iron sulfides were recovered partly as AVS and partly as CRS (Table 1). In the precipitate of grown cultures, pyrite and S^0 were the only compounds detectable by x-ray diffraction analysis.

Free H_2S was not detected in the growth phase, but after depletion of FeOOH, SO_4^{2-} production continued briefly and H_2S accumulated to a final concentration of 0.5 to 1 mM.

The pH of the culture medium decreased during growth. In the cultures sampled for the time course experiment, the pH declined to 6.5, while in grown cultures with larger amounts of Fe, values down to pH 4.5 were measured. Even when the cultures reached such low pH values, growth resumed shortly after transfer.

The molar ratio of Fe(III) added to SO_4^{2-} produced was close to 2 (Table 1). A minor SO_4^{2-} production was seen in the absence of FeOOH.

When the S/Fe culture was transferred to medium containing ferrous carbonate instead of ferric hydroxide, again bacterial growth and production of sulfate and iron sulfides occurred (Fig. 2 and Table 2). The rate of SO_4^{2-} production was lower than in the presence of FeOOH. Growth ceased after 5 days, while SO_4^{2-} production continued at a constant rate for more than 15 days. After this, H_2S accumulated to ca. 1 mM.

When $FeCO_3$ was added in relatively low amounts (10 to 25 mmol $liter^{-1}$), H_2S accumulated to 0.5 to 1 mM after a few

TABLE 1. S/Fe cultures with amorphous ferric hydroxide: stoichiometry and bacterial growth

Fe(III) reduced (mmol $liter^{-1}$)[a]	SO_4^{2-} produced (mmol $liter^{-1}$)[a]	AVS produced (mmol $liter^{-1}$)[a]	CRS produced (mmol of S $liter^{-1}$)[a,b]	Fe(III)/SO_4^{2-} (mol:mol)[a]	Cells formed (ml^{-1})[c]
17.4	9.1	9.0	9.7	1.91	5.1×10^7
18.6	9.8	8.0	14.7	1.90	4.1×10^8
42	19.7	ND[d]	ND	2.13	ND
37.5	15.7			2.39	
14.6	6.7			2.18	
7.3	4.1			1.78	
0[e]	0.2			0	
0[e]	0.7			0	

[a] Grown cultures; all Fe(III) reduced to Fe(II).
[b] S^0 removed by CS_2 extraction.
[c] Difference between day 0 and maximum cell number.
[d] ND, not determined.
[e] No FeOOH added.

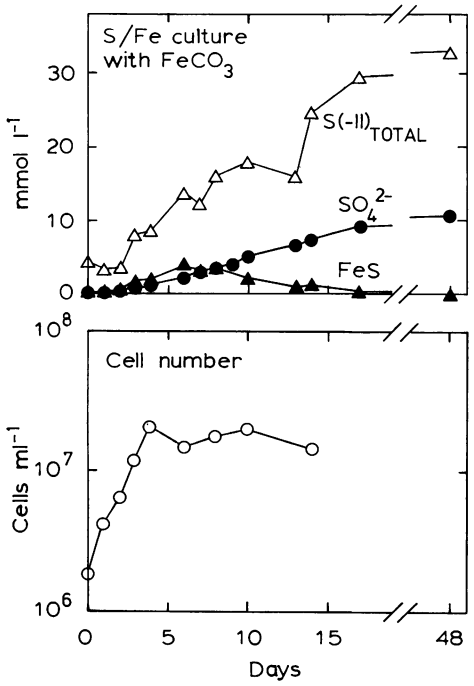

FIG. 2. Time course of SO_4^{2-} and sulfide production and bacterial growth in the S/Fe culture incubated with $FeCO_3$. (Top) Chemical analyses. (Bottom) Cell number; note logarithmic scale. $S(-II)_{TOTAL}$ includes both sulfide bound in FeS and CRS.

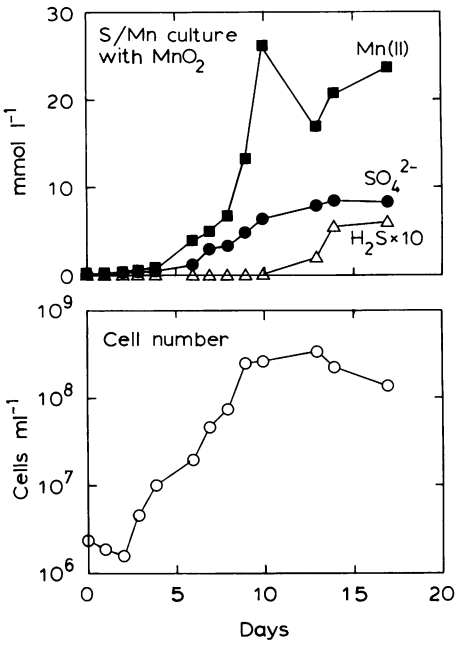

FIG. 3. Time course of SO_4^{2-}, H_2S, and Mn(II) production and concomitant bacterial growth in the S/Mn enrichment culture incubated with MnO_2: average of two parallel cultures. (Top) Chemical analyses; note that H_2S concentrations are multiplied by 10. (Bottom) Cell number; note logarithmic scale. Mn(II) includes both particulate and soluble forms. MnO_2 added: 23 mmol $liter^{-1}$.

days of incubation. At this time, the sum of AVS and CRS was about 2 mmol $liter^{-1}$. Thus, most of the $FeCO_3$ remained unchanged and coexisted with H_2S in these cultures. In cultures with more $FeCO_3$ (37 mmol $liter^{-1}$), the ratio of Fe(II) added to sulfate produced was close to 3 (Table 2).

S/Mn culture. In the culture grown with elemental sulfur and manganese oxide, all Mn(IV) was reduced to Mn(II) within 2 weeks (Fig. 3). A simultaneous production of sulfate was observed. The Mn reduction and SO_4^{2-} production typically started after a lag phase of 1 to 2 days and were accompanied by exponential bacterial growth. The culture was dominated by small, nonmotile, rod-shaped cells that were mainly attached to the particulate phase. The growth rate during the exponential phase was 1 day^{-1}, similar to that of the S/Fe culture. Reduction of MnO_2 changed the color of the precipitates from dark brown to whitish rosy-red. Soluble Mn^{2+} never exceeded 1.2 mM, indicating that most Mn(II) was associated with the solid phase. The mineral formed was presumably $MnCO_3$, as reported from dissimilatory Mn-reducing cultures (31). No sulfide precipitates were detected by distillation. After depletion of the oxide, bacterial growth and the production of SO_4^{2-} ceased and a further release of about 1 mM H_2S (final concentration) was observed before all transformations stopped.

The pH of the medium increased. In the time course experiments it rose to 7.5, and in cultures with larger amounts of MnO_2, values of about 8.5 were measured. At this high pH, the medium turned yellow, presumably due to the presence of polysulfides, and some manganous sulfide might have formed, as indicated by the sulfide smell produced upon acidification of the precipitate.

The molar ratio of Mn(II) to SO_4^{2-} produced was 2.7 on average (Table 3). Control cultures without addition of MnO_2 concomitantly produced SO_4^{2-} and H_2S (Fig. 4 and Table 3). The SO_4^{2-} production ceased when H_2S reached a concentration of approximately 1.5 mM. The ratios of H_2S to SO_4^{2-} produced were 3.61 and 3.68 in two parallel cultures. However, growth was insignificant in the absence of MnO_2. The maximum cell number generally increased with increasing additions of MnO_2 (Table 3).

Enumeration of bacteria in sediments. Viable counts of bacteria metabolizing S^0 in the presence of either FeOOH or MnO_2 were made by using the MPN method. The results are given in Table 4. The highest numbers were found with

TABLE 2. S/Fe cultures with ferrous carbonate: stoichiometry and bacterial production

$FeCO_3$ added (mmol $liter^{-1}$)	SO_4^{2-} produced (mmol $liter^{-1}$)[a]	AVS produced (mmol $liter^{-1}$)[a]	CRS produced (mmol of S $liter^{-1}$)[a,b]	$FeCO_3/SO_4^{2-}$ (mol:mol)	Cells formed (ml^{-1})[c]
37.6	13.2	0.13	50.8	2.85	9.3×10^6
37.6	11.2	0.11	43.4	3.36	1.8×10^7

[a] 48 days of incubation.
[b] S^0 removed by CS_2 extraction.
[c] Difference between day 0 and maximum cell number.

TABLE 3. S/Mn cultures: stoichiometry and bacterial growth

Mn(II) produced (mmol liter^{-1})[a]	SO_4^{2-} produced (mmol liter^{-1})[a]	Mn(II)/SO_4^{2-} (mol:mol)[a]	Cells formed (ml^{-1})[b]
24.7	8.1	3.05	4.8×10^8
22.6	8.5	2.66	2.3×10^8
14.8	5.1	2.90	8.2×10^7
8.0	3.1	2.22	6.9×10^7
0[c]	1.6	0	1.7×10^6
0[c]	0.47	0	ND
0[c]	0.43	0	

[a] Grown cultures; all Mn(IV) reduced to Mn(II).
[b] Difference between day 0 and maximum cell number. ND, not determined.
[c] No MnO_2 added.

FeOOH in the salt marsh sediment, 10^5 to 10^6 cells per cm^3. With MnO_2, the numbers obtained in the Aarhus Bay and salt marsh sediments were about 10^4 to 10^5 cells per cm^3, while the numbers found in Skagerrak were significantly lower, <100 cm^{-3}.

DISCUSSION

Sulfur transformations in enrichment cultures. In marine enrichment cultures established in anoxic bicarbonate-buffered defined medium with elemental sulfur and either ferric hydroxide or manganese oxide, sulfur was transformed by biologically catalyzed processes, during which both sulfate and sulfide were formed (Fig. 1 and 3). The sulfur metabolism sustained bacterial growth which, since no organic compounds except vitamins were added, most likely involved fixation of bicarbonate.

The observed formation of sulfide, either in the form of ferrous sulfide minerals or as free H_2S in late growth phases or in cultures without metal oxide additions (Fig. 1, 3, and 4), must be the result of a reduction of part of the added elemental sulfur. However, since no external electron donors, such as organic compounds or H_2, were added, the measured sulfide cannot be the product of a dissimilatory microbial sulfur reduction. Thus, a microbial disproportionation of elemental sulfur, similar to the recently described disproportionation of thiosulfate and sulfite by sulfate-reducing bacteria (3), is the only plausible explanation for the formation of sulfide in our enrichment cultures. The formed H_2S will react spontaneously with amorphous FeOOH, as expected by the following equation (e.g., see references 38 and 39):

$$3H_2S + 2FeOOH \rightarrow S^0 + 2FeS + 4H_2O \quad (1)$$

TABLE 4. Viable counts of bacteria in sediments disproportionating S^0 in the presence of MnO_2 or FeOOH[a]

Site depth (cm)	MnO_2 (cells per cm^3)			FeOOH (salt marsh; cells per cm^3)
	Aarhus Bay	Salt marsh	Skagerrak	
0–1	4.3×10^4	2.4×10^5	93	2.4×10^5
1–2	9.3×10^4	2.4×10^5	4	4.6×10^5
2–3[b]	1.5×10^4	4.6×10^4	23	$>1 \times 10^6$
4–6				1.1×10^6

[a] The 95% confidence interval is 0.14 to 5.4 times the MPN or less.
[b] The site depth was 2 to 4 cm for FeOOH in this sampling.

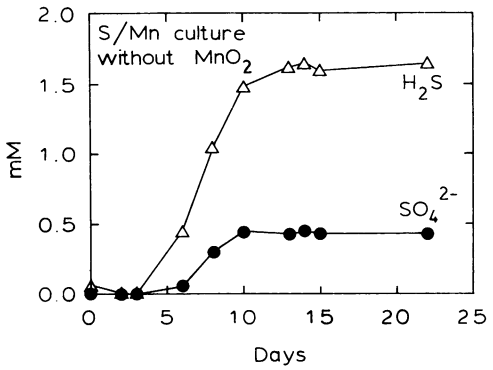

FIG. 4. Time course of concomitant H_2S and SO_4^{2-} production in the S/Mn enrichment culture in the absence of MnO_2.

A complete spontaneous oxidation of H_2S to SO_4^{2-} as recently reported with hematite (α-Fe_2O_3) (13) is not likely since this pathway was found to be dependent on undersaturation with respect to FeS, whereas FeS precipitated in our cultures.

A stoichiometric microbial disproportionation of S^0 to H_2S and SO_4^{2-} is described by equation 2:

$$4S^0 + 4H_2O \rightarrow 3H_2S + SO_4^{2-} + 2H^+ \quad (2)$$

The combination of equations 1 and 2 yields the overall reaction for the disproportionation of S^0 in the presence of FeOOH:

$$3S^0 + 2FeOOH \rightarrow SO_4^{2-} + 2FeS + 2H^+ \quad (3)$$

The determined ratio of Fe(III) to SO_4^{2-} of 2 (Table 1), the observed precipitation of iron sulfide, and the decrease of pH in the medium of the S/Fe culture are all in agreement with this equation, and we thus conclude that this is indeed the net reaction that occurred in this culture.

Accumulation of both AVS (H_2S and FeS) and CRS was observed (Table 2). Since x-ray diffraction analysis indicated the presence of pyrite in the culture, it is likely that this compound mainly contributed to the CRS fraction when S^0 had been removed. The acid-resistant grey coatings observed on sulfur grains could be pyrite, similar to that described by Berner (5). Pyrite may form by reaction of FeS and S^0 or by reaction of Fe^{2+} with polysulfides (5, 40). Since only half of the sulfur in pyrite is in the oxidation state of sulfide, S(-II), the amount of sulfide accumulated in the cultures can be calculated as the sum of AVS and (½ × CRS). Thus, the ratio of S(-II) to SO_4^{2-} accumulated in the two cultures in Table 2 was 1.54 on average; i.e., 23% less S(-II) was recovered than theoretically expected (equation 3). This discrepancy is probably due to difficulties in sampling the rapidly settling sulfur grains on which iron sulfides were seen as coatings.

When the S/Fe culture was incubated with $FeCO_3$ as an alternative scavenger of H_2S, the calculated ratio of Fe(II) to SO_4^{2-} (Table 2) agreed well with that predicted from the combination of S^0 disproportionation (equation 2) and FeS precipitation:

$$4S^0 + 3FeCO_3 + 4H_2O \rightarrow \quad (4)$$

$$SO_4^{2-} + 3FeS + 3HCO_3^- + 5H^+$$

In the cultures which received the highest amounts of $FeCO_3$, almost all sulfide was found as CRS. We have not identified the compound contributing to this fraction. As-

suming it to be pyrite, the ratio of S(-II) to SO_4^{2-} was 1.92 on average for the two cultures presented in Table 2, 65% of that expected from equation 4. Particle discrimination in sampling might again explain part of the discrepancy. However, the measurements of iron released during chromium reduction (Fig. 2) indicate an iron sulfide of a composition other than pyrite. These iron concentrations should be equal to the amount of S(-II) bound in the CRS fraction. The ratio of iron released to sulfur recovered during the chromium reduction step at the end of the time course was 0.74, which corresponds to the Fe/S ratio of greigite (Fe_3S_4) rather than that of pyrite. If this was indeed the composition of the ferrous sulfide in the CRS fraction, the ratio of S(-II) to SO_4^{2-} is 2.91, which is close to 3, the expectation from equation 4.

The S/Mn culture produced Mn^{2+} and SO_4^{2-} with an average ratio of 2.7 (Fig. 3 and Table 3). This implies an oxidation of S^0 by MnO_2 by the reaction:

$$S^0 + 3MnO_2 + 2H_2O \rightarrow SO_4^{2-} + 3Mn^{2+} + 4OH^- \quad (5)$$

The concomitant increase in pH supports this stoichiometry. In the culture, Mn^{2+} precipitated as $MnCO_3$. However, the simultaneous production of sulfate and sulfide observed when the culture was incubated without MnO_2 (Fig. 2) again implies a disproportionation of S^0. The measured ratio of sulfide to sulfate (3.7 on average) is in the range of that expected from equation 2.

In the presence of MnO_2, H_2S is rapidly oxidized to S^0 (8):

$$H_2S + MnO_2 \rightarrow S^0 + Mn^{2+} + 2OH^- \quad (6)$$

A combination of a biologically catalyzed disproportionation (equation 2) and a spontaneous sulfide oxidation (equation 6) would be stoichiometrically identical to a one-step S^0 oxidation with MnO_2 (equation 5). For that reason, these two pathways are indistinguishable in our experimental setup. However, since sulfur is disproportionated in the S/Mn culture when MnO_2 is absent, it is most likely that S^0 is also disproportionated in the presence of MnO_2. The H_2S concentration is kept below detection by the rapid reoxidation. The appearance of H_2S after oxide depletion supports this interpretation (Fig. 3).

Abiological disproportionation of elemental sulfur to sulfide and sulfate in aqueous solution occurs at temperatures above 80°C (6, 20). Biologically catalyzed disproportionation of S^0 to sulfide and sulfate has, to our knowledge, only been reported as a photochemical process in CO_2-free cultures of *Chlorobium limicola* (35). The authors suggested that *Chlorobium* sp. also disproportionates S^0 in the presence of CO_2 as a first step in the oxidation of S^0 to SO_4^{2-}. In analogy to our S/Mn culture, the intermediate formation of H_2S only becomes visible in the absence of the oxidant, here CO_2.

Thermodynamic considerations. The observed microbial dismutation of elemental sulfur to sulfide and sulfate represents a new chemolithotrophic metabolism. The disproportionation (equation 2) is endergonic at pH 7 and under standard conditions, $\Delta G^{\circ\prime} = 41$ kJ mol^{-1} (45). The ΔG is, however, strongly dependent on the activities of H_2S and H^+. Thus, at very low concentrations of H_2S, ΔG is sufficiently low that energy may be gained through the reaction. The process can therefore sustain bacterial growth as long as the H_2S is removed, e.g., by reaction with MnO_2, FeOOH, or $FeCO_3$. With activities of H_2S and SO_4^{2-} of 10^{-7} and 10^{-2} M, respectively, $\Delta G'$ is -92 kJ mol^{-1}. This is sufficient for synthesis of at least 1 mol of ATP (45). The disproportionation of S^0 is thus dragged by the abiological removal of the H_2S produced.

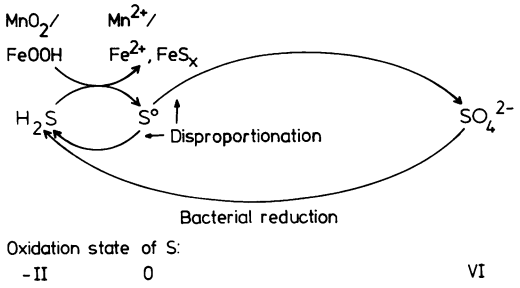

FIG. 5. Possible role of S^0 in anaerobic oxidative pathways of the sulfur cycle. See text for details.

Ecological considerations. Disproportionation of elemental sulfur as reported here is a novel pathway in the biogenic transformations of sulfur. Significant amounts of elemental sulfur are found in both marine and freshwater sediments (42, 46, 47). The concentration is often highest in the suboxic zone of the sediment where S^0 probably derives from abiological oxidation of H_2S with Mn(IV) or Fe(III) (equations 3 and 5). Although H_2S is produced at significant rates by sulfate reduction, the pore water concentration of H_2S in the suboxic zone is often below 1 μM (9). Thus, the suboxic environment is relatively similar to that of the growing enrichment cultures described herein, and this is the environment in which disproportionation of elemental sulfur would be expected to be of importance.

The sediment sections used for MPN counts (Table 4) were, except for the oxic surface layer, all from the suboxic zone of the sediments. Thus, the high numbers of S^0-disproportionating bacteria found in the two coastal sediments support the idea that the process could be of importance here. The low numbers in the manganese-rich Skagerrak sediment could indicate that the process is not ubiquitous or that S^0-disproportionating bacteria at this site require different conditions for growth.

The MPN counts for the coastal sediments (Table 4) are comparable to those of both $S_2O_3^{2-}$-disproportionating and SO_4^{2-}-reducing, hydrogen- or acetate-oxidizing bacteria found in Danish marine sediments (26). The authors suggested that the different sulfur transformations are carried out by largely the same groups of bacteria. This could also be true for S^0 disproportionation.

Elemental sulfur may have a role similar to that of $S_2O_3^{2-}$ as an intermediate in the anoxic transformations of sulfur in sediments (24). In analogy to the thiosulfate shunt, a small cycle may exist between H_2S and S^0 on the basis of H_2S oxidation by iron or manganese oxides and bacterial S^0 disproportionation (Fig. 5). For every 4 mol of H_2S oxidized to S^0, 1 mol of S is transferred to the SO_4^{2-} pool (equation 2). This pathway could explain the observations of anoxic oxidation of ^{35}S-labeled H_2S to SO_4^{2-} (e.g., see references 16 and 26). Even in the absence of an oxidant for $H_2{}^{35}S$, $^{35}S^0$ may form rapidly after $H_2{}^{35}S$ addition by isotope exchange reactions (17, 18). In the sediment studies, $S_2O_3^{2-}$ was found to be the major immediate oxidation product. We cannot exclude $S_2O_3^{2-}$ as a rapidly metabolized intermediate in our enrichment cultures.

A major difference between the effects of Fe and Mn on the pathways in Fig. 5 is the loss of sulfur through FeS and FeS_2 precipitation. When sulfur is limiting and FeS or FeS_2

precipitates, the SO_4^{2-} production is expected to be much smaller when coupled to FeOOH reduction rather than to MnO_2 reduction. Working with salt marsh sediments, Aller and Rude (1) found SO_4^{2-} production by biological catalysis after MnO_2 addition, while the SO_4^{2-} concentration only increased slightly after the addition of amorphous FeOOH. Bacterial disproportionation of S^0 might explain these observations.

In unmanipulated sediments, processes are often in a steady state, where substrates are supplied and metabolites are removed continuously. Thus, iron reduction might not lead to supersaturation with respect to FeS or FeS_2 and precipitation of these. Therefore, the efficiency of ferric iron as an oxidant for sulfide, e.g., by the pathway suggested here, might be greater than indicated by batch-type experiments (1, 14, 28).

In conclusion, another twist has been added to the sulfur cycle. The disproportionation of S^0 coupled to Mn or Fe reduction can explain at least some of the observations of anaerobic sulfide oxidation in sediments. The discovery raises new questions concerning both the ecological significance and the microbiology of the process.

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