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Biogeochemistry and bacteriology of ferrous iron oxidation in geothermal habitats

GL03807

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Abstract—A survey of hot, acid springs in Yellowstone Park has shown that high concentrations of ferrous and ferric iron are often present. Total ionic iron concentrations in different springs ranged from less than 1 ppm to greater than 200 ppm, and up to 50% of the ionic iron was in the ferrous form. Some of these springs also have high concentrations of reduced sulfur species (S^{2-} and S^0). Significant populations of the bacterium *Sulfolobus acidocaldarius*, an autotrophic organism able to live and oxidize sulfur compounds at low pH and high temperature, were present in most of these springs. The role of this organism in the oxidation of ferrous iron was investigated by incubating natural samples of water and assaying for disappearance of ferrous iron. Controls in which bacterial activity was inhibited by addition of 10% NaCl were also run. Bacterial oxidation of ferrous iron occurred in most but not all of the spring waters. The temperature optimum for oxidation varied from spring to spring, but significant oxidation occurred at temperatures of 80–85°C, but not at 90°C. Thus, 85–90°C is the upper temperature at which bacterial iron oxidation occurs; a similar upper limit has previously been reported for sulfur oxidation in the same kinds of springs. The steady-state concentrations of ferrous and ferric iron are determined by the rate at which these ions move into the spring pools with the ground water (flow rate), by the rate at which ferric iron is reduced to the ferrous state by sulfide, and by the rate of bacterial oxidation. The bacterial oxidation rate is faster than the flow rate, so that the rate of reduction of ferric iron is probably the rate-controlling reaction. In several springs, no decrease in ferrous iron occurred, even though high bacterial populations were present. It was shown that in these springs, ferrous iron oxidation occurred but the ferric iron formed was reduced back to the ferrous state again. These springs were all high in suspended sediment and the reductant was shown to be present in the sediment. X-ray diffraction revealed that the sediment contained three major ingredients, elemental sulfur, natroalunite, and quartz. Chemical analyses showed a small amount of sulfide, too little to reduce the ferric iron. Elemental sulfur itself did not reduce ferric iron but when elemental sulfur was removed from the sediment by CS_2 extraction, the activity of the sediment was abolished. It is hypothesized that the sulfide present in the sediment (possibly bound to natroalunite) reacts with elemental sulfur to form a reductant for ferric iron. The results show that bacteria can have a profound influence on the ferrous/ferric ratios of geothermal systems, but that temperature and mineral composition of the water may significantly influence the overall result.

INTRODUCTION

THE RELATIVE stability of ferrous iron in the presence of oxygen at low pH is well established (STUMM and MORGAN, 1970), as is the role of the bacterium *Thiobacillus ferrooxidans* in the oxidation of ferrous iron under these conditions (SINGER and STUMM, 1970). In the absence of this bacterium the rate of oxidation of ferrous iron in dilute solutions at pH values around 2 is slower by a factor of 10^6 (SINGER and STUMM, 1970), so that in the absence of bacterial activity ferrous iron can be considered to be essentially stable. To date, most work on the bacterial oxidation of ferrous iron has been done in systems of normal temperature, such as pyrite-rich coal materials (BELLY and BROCK, 1974), copper leaching dumps (BECK, 1967), and acidic streams draining coal mining regions (TEMPLE and DELCHAMPS, 1953). Ferrous iron is present in many geothermal systems, especially those which are of low pH. Such low pH, high temperature systems arise in hydrothermal areas where volume of water flow is low but vapor and heat flow

to the surface are high. WHITE et al. (1971) have characterized these hydrothermal systems as vapor-dominated. If the vapor is rich in hydrogen sulfide, oxidation at or near the Earth's surface leads to the formation of large amounts of elemental sulfur in soil and rock, and bacterial oxidation of this elemental sulfur (BROCK and MOSSER, 1975) leads to the formation of sulfuric acid. Attack on rock by sulfuric acid and steam leads to extreme weathering, and soils in these acidic areas are usually bleached white due to extensive leaching. In such systems, bacteria are present and active both in soil (FLIERMANS and BROCK, 1972) and water (MOSSER et al., 1973). In vapor-dominated systems the springs present generally have no surface outflow, but receive and discharge their acid water primarily by seepage. Such springs are generally of high temperature, receiving their heat primarily by steam jets within the spring pools (BROCK and MOSSER, 1975). Such springs are in reality drowned fumaroles. Hot, acid springs may be small or large in surface area, but in spite of the absence of surface flow, exchange of water occurs at quite

rapid rates. By experimentally enriching springs of this type with chloride ions and measuring the dilution rate with time, BROCK and MOSSER (1975) were able to measure the turnover time of water. They found that in the small springs (volumes of 20–2000 l.), turnover times (time for chloride to dilute by one-half) were of the order of 8–24 hr, whereas in the large springs (volumes of around 10^6 l.), turnover times were of the order of one month. In all of these springs, significant populations of the bacterium *Sulfolobus acidocaldarius* were present. This organism, which grows only at low pH (range 1·0–5·0) and high temperature (range 55–90°C), is able to oxidize large amounts of elemental sulfur to sulfuric acid (MOSSER *et al.*, 1973), and is responsible for part of the sulfuric acid formed in geothermal systems. Since BRIERLEY and BRIERLEY (1973) had shown that cultures of this bacterium were able to oxidize ferrous iron, it was of interest to assess the importance of this bacterium in the oxidation of ferrous iron in the natural geothermal systems. In the present work, we show that oxidation of ferrous iron by this bacterium is of widespread occurrence, but does not occur at temperatures above 85–90°C. We also show that in certain springs high in suspended sediment, the ferric iron formed is reduced back to the ferrous form by some component in the sediment, thus giving an apparent inhibition of ferrous iron oxidation. The results suggest that even at high temperature, oxidation of ferrous iron in low pH environments is primarily a bacterial process, but that there are environmental limits beyond which the bacterial process does not occur.

Although total ionic assays have been reported on large numbers of springs from around the world, very few reliable assays of both ferrous and ferric iron have been made. We report in this paper the most extensive set of analyses of these two iron species, and show that both ferrous and ferric iron are present in quite high concentrations in many, but not all, acid hot springs.

MATERIALS AND METHODS

Habitats

The locations of the springs studied have been described by BROCK and MOSSER (1975). They are in three separate regions of Yellowstone National Park: Norris Geyser Basin, Sylvan Springs area, and Mud Volcano area.

Environmental measurements

Temperatures were measured with thermistor probes (Yellow Springs Instrument Co.). Water samples were collected in plastic bags or plastic bottles and analyses were made within one or two days of collecting at a field laboratory in W. Yellowstone, Montana. pH was measured with a glass electrode after the water had cooled to room temperature. Sulfate was assayed turbidimetrically following the AMERICAN PUBLIC HEALTH ASSOCIATION (1971). Elemental sulfur was assayed by the method of PACHMAYR (1960), which involves extracting with trichloroethylene and reading the absorbance at 276 nm. Sulfide was assayed on samples preserved with zinc acetate at alkaline pH, a

known volume of NaOH being added to the zinc acetate-treated sample at the time of collection. The hydrogen sulfide was liberated by acidification and distilled under nitrogen into zinc acteate and the collected sulfide was assayed colorimetrically by the method of PACHMAYR, as described by BROCK *et al.* (1971). Ferrous iron was assayed by the phenanthroline method following the AMERICAN PUBLIC HEALTH ASSOCIATION (1971) after the sample had been stabilized with HCl. Total ionic iron (ferrous plus ferric) was assayed after reducing all the iron to the ferrous state with hydroxylamine, again following the AMERICAN PUBLIC HEALTH ASSOCIATION (1971). Bacteria were counted by direct microscopy. If numbers were high, a Petroff–Hausser counting chamber was used, but when numbers less than about 10^7/ml were present, water samples were filtered through black membrane filters, the bacteria on the filters stained with acridine orange, and counts made on the filters using a vertical illuminator on a fluorescence microscope.

Measurement of ferrous iron oxidation

Water samples were returned to the laboratory within an hour or so of collection and 50 ml samples transferred to 6 ounce screw-capped bottles. If desired, ferrous iron levels were increased by adding small amounts of a solution of ferrous sulfate. Samples were removed for an initial assay of ferrous iron and the bottles placed in a water bath at the desired temperature for incubation. Periodically additional samples were removed for ferrous iron assay.

RESULTS

General characteristics of the acidic hot springs

Physical and chemical data for the springs studied are given in Table 1. The hydrology of these acidic hot springs is described in detail by BROCK and MOSSER (1975). None of the springs have significant surface outflow, yet measurement of dilution rate of chloride ions added experimentally to the water revealed that movement of water through the springs via subsurface seepage is in most cases quite rapid. The water turnover times taken from the data of BROCK and MOSSER (1975) are given in Table 1. BROCK and MOSSER (1975) showed that the large and small springs differed significantly in the degree to which bacterial activity contributed to the *in situ* sulfuric acid production. In the small springs, with high water turnover, a small amount of sulfuric acid is produced *in situ* by bacterial oxidation of reduced sulfur compounds but most of the sulfuric acid is derived from downslope movement of acidic water from the adjacent acid-altered soil. In the large springs, most of the sulfuric acid is produced *in situ* by bacterial action. [In both cases, the ultimate source of sulfuric acid is bacterial activity, since bacteria are responsible for sulfuric acid production in the acid-altered soil (FLIERMANS and BROCK, 1972).]

As also seen in Table 1, most of the springs have significant populations of *Sulfolobus*, the only bacterium able to grow at high temperature and low pH (BROCK *et al.*, 1972). (One spring in Table 1, 56-3, is of lower temperature and has a rod-shaped bacterium, probably a species of *Thiobacillus*, instead of *Sulfolobus*.)

Table 1. Characteristics of the acidic hot springs studied

Site	Temp.	pH	Fe^{++}ppm	Fe^{+++}ppm	$S^=$ppm	S^oppm	SO_4^{2-}ppm	Cell#/ml*	Water Turn-over time	Water volume,l.
Norris Geyser Basin										
Locomotive	88.5	2.1	12.9	75.0	1.1	0.3	495	1.2×10^7	16 hr	2,400
White Bubbler	92.0	2.1	1.7	0	0.24	0.2	772	2.5×10^6	10 hr	50
Vermillion	92.5	1.9	6.7	41.8	0.56	1.9	882	3.3×10^7	17 hr	270
26-5	91.0	2.1	1.5	0.22	0.46	0.1	701	1.2×10^7	13 hr	260
21-1	92.5	2.0	2.7	0.84	5.0	0.08	665	1.2×10^7	14 hr	100
Sylvan Springs area										
59-1	90.5	1.9	3.0	6.9	2.8	21.5	1052	2.0×10^7	27 hr	450
59-3	90.5	1.8	5.8	69.1	35	635	1498	1.3×10^7	-	-
54-5	82.0	1.9	4.9	13.6	3.6	319	919	8.8×10^6	20 hr	600
56-3	57.5	1.9	4.5	24.0	4.4	7.0	751	3.4×10^6	8 hr	95
58-3	84.5	1.7	29.7	165.9	2.8	1.4	2217	7.2×10^7	23 hr	80
Evening Primrose	55.5	1.1	8.43	12.4	14	1635	6014	1.0×10^8	-	-
Mud Volcano area										
Moose Pool	69.5	1.5	57.7	215.2	35	2310	3238	1.6×10^8	28 days	1,100,000
Sulfur Caldron	67.0	1.4	13.8	227.4	50	1675	2925	1.1×10^8	35 days	760,000

All data except volumes and turnover times determined in summer 1974. Volumes and turnover times measured by the chloride dilution technique described by BROCK and MOSSER (1975). Turnover time is given as the time necessary for added chloride ions to be diluted by one-half. Other analyses determined by K. Noguchi (personal communication, 1971) are: NH_4^+, Moose Pool 1·15, Sulfur Caldron 0·21; NO_3^-, Moose Pool 0·39, Sulfur Caldron 0·12; PO_4^{3-}, Moose Pool 1·28, Sulfur Caldron 1·93; As, Moose Pool 0·01, Sulfur Caldron 0·00. In a pool which is similar to Sulfur Caldron, (called Old Sulfur Caldron by WHITE et al., 1971), ALLEN and DAY (1935) found 146 ppm Al.

* All bacteria are Sulfolobus except spring 56-3, which has a rod-shaped bacterium, probably Thiobacillus.

Ferrous and ferric iron contents of acidic hot springs

Wide variations in iron contents were found in the hot springs studied (Table 1). Within a given spring there were also fairly wide temporal variations (not shown), although in general high iron springs remained high and low iron springs remained low. Two sources for soluble iron can be envisaged, the minerals of the immediate spring basin, and the water entering the spring by underground seepage. That the latter source is significant was shown by a simple experiment. Two small springs, 58-3 and 56-3, were drained completely by siphoning (see BROCK and MOSSER, 1975), and samples of the first water re-entering the springs were collected for iron assay. This experiment was repeated twice, with about 1 week to allow for recovery between experiments. Ferric iron concentrations of the first water re-entering the drained springs was two- to four-fold higher than the steady-state concentration, and ferrous concentrations were lower. Because of the low pH of the underground water entering the springs, considerable soluble iron (both ferrous and ferric) should be held in solution, derived from breakdown of minerals of the slope above. The relative proportions of ferrous and ferric iron in the entering water are probably determined partly by the possibilities for bacterial oxi-

dation in the upslope seepage area, and partly by the possibilities for contact of the water with reducing agents, primarily hydrogen sulfide. We conclude that much of the soluble iron enters via underground seepage, primarily in the form of ferric ions. Some of the ferric ions are probably reduced to ferrous ions by H_2S in the spring pool, so that the steady-state concentrations of ferrous and ferric ions are determined by the relative rates of the reduction reaction brought about by H_2S and of the oxidation reaction brought about by bacteria. Some of the soluble iron in the spring may also be derived from breakdown of minerals in the spring basin itself, but in view of the rapid water turnover times, this is probably a small part of the total. We should note that the springs listed in Table 1 should not be considered to have iron values typical for all Yellowstone springs. In the Geyser Creek area of Yellowstone (in the same basin as Sylvan Springs but on the opposite side of the valley), iron contents of acidic springs are considerably lower, as shown by RAYMSHASHAY (1966) and confirmed here. Also, in a survey of a large number of Yellowstone springs in 1971, K. Noguchi (personal communication) found wide variation in iron content, some acidic springs having total soluble iron values as low as 0·5 $\mu g/ml$. The springs listed in Table 1 were selected by us because of their iron values.

General observations on bacterial iron oxidation

When samples of water were returned to the laboratory and assayed periodically during incubation for ferrous iron, it could readily be shown that the ferrous iron rapidly disappeared. To increase the accuracy with which oxidation rates could be measured, small amounts of ferrous iron were usually added to the spring water, generally to raise the concentration of Fe^{2+} to around 50–60 $\mu g/ml$. If the Fe^{2+} concentration in a spring water was already of that magnitude, enrichment was not done. To show that Fe^{2+} oxidation was due to bacterial action, some type of control was needed in which bacterial activity was inhibited. In earlier work with sulfur oxidation (Mosser *et al.*, 1973), formaldehyde served as a suitable poison, but this agent could not be used here because it interfered with the ferrous iron assay. Mercuric bichloride was tried and also interfered with the assay. In searching for an inhibitor of bacterial activity which was relatively nonreactive, 10% NaCl was used. It had been shown in unpublished experiments that *Sulfolobus* was sensitive to NaCl, growth being completely inhibited by concentrations around 1%. The concentration was raised to 10% NaCl to ensure complete and immediate inhibition. This concentration did not interfere with the ferrous iron assay. Figure 1 shows an experiment in which ferrous iron oxidation was followed in two parallel samples, to one of which 10% NaCl was added. As seen, no Fe^{2+} oxidation occurs in the presence of the salt, whereas rapid oxidation occurs in its absence. In all subsequent experiments, controls containing 10% NaCl were always run, and always showed negligible oxidation during the incubation periods used. For simplicity, these controls are not shown in the rest of the figures.

Survey of springs for bacterial iron oxidation

To determine how widespread bacterial oxidation of ferrous iron was in acidic hot springs, a survey of a number of high-iron springs was carried out. The results are summarized in Fig. 2. As seen, bacterial

Fig. 2. Bacterial oxidation of ferrous iron in waters from various Yellowstone hot springs. Temperature of incubation, 80°C except 56-3 and Evening Primrose, which were 70°C. Controls which had 10% NaCl added showed no oxidation in any case. Ferrous sulfate was added to all waters except Moose Pool to give the 0 time values reported.

oxidation of ferrous iron occurs in a number of springs, but not in all of them, and there is wide variation in rate of oxidation. In some springs, such as Locomotive, Vermillion, and 54-5, oxidation is not only rapid but begins immediately without a lag, and within 24 hr all of the ferrous iron has been oxidized. In other springs, such as 21-1, 26-5, 59-1 and 56-3, there is a distinct lag before iron oxidation begins, but the rate after the lag is rapid. In three springs, Moose Pool and Sulfur Caldron and 59-3, iron oxidation appears to be virtually absent. The reason for this apparent absence of iron oxidation in the two named springs is presented below.

The oxidation studies presented in Fig. 2 were carried out at 80°C, except for springs 56-3 and Evening Primrose which had lower natural temperatures and for which the studies were carried out at 70°C. Thus the oxidation rates given are not the actual rates which would occur in the springs. As analyzed in detail by Mosser *et al.* (1974a), temperature optima of *Sulfolobus* populations in these springs do not always parallel the temperatures of the springs. But in most cases, 80°C was about the median average temperature, so that the survey was carried out at that temperature for convenience. If oxidation was low or absent in the survey of Fig. 2, temperature was probably not the limiting factor and oxidation did not occur for some other reason.

Temperature optimum for ferrous iron oxidation

The temperature optima for iron oxidation were determined for three springs by incubating parallel samples at different temperatures. As seen in Fig. 3, the optimum for Locomotive Spring was 80–85°C, for spring 58-3 it was 70°C and Moose Pool (after removal of sediment, see below) it was 60° or lower. In all three springs, significant oxidation occurred at temperatures of 80–85°C, but no oxidation occurred at 90°C, at least during the time period used (incubations at 90°C were continued for at least 90 hr, at
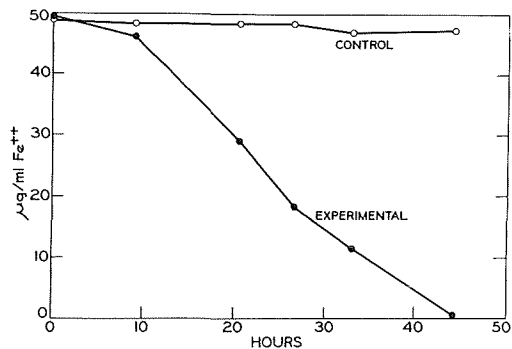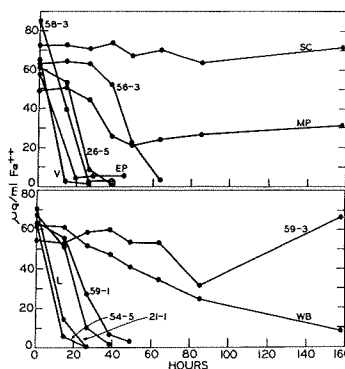

Fig. 1. Bacterial oxidation of ferrous iron in Locomotive Spring water. The concentration of ferrous iron was raised from 15 to 49 ppm at 0 time. The control had 10% NaCl added to inhibit bacterial activity. Temperature of incubation, 80°C.
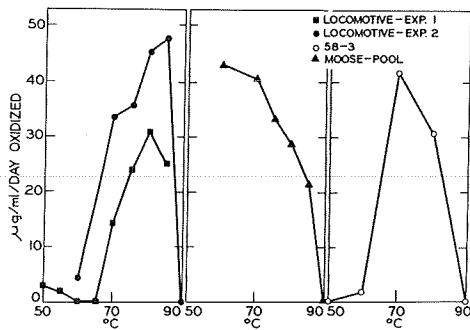
Fig. 3. Temperature optima for ferrous iron oxidation in waters from several hot springs. Ferrous iron was added at 0 time to Locomotive Spring and 58-3 water to raise the initial concentration to 60 ppm. The natural concentration of Moose Pool water was 55 ppm. Controls to which 10% NaCl was added showed no oxidation at any temperature. In Moose Pool, inhibitory sediment was removed (see below).

which time all of the ferrous iron in the samples incubated at optimum temperature had been oxidized). In Evening Primrose, oxidation rate was measured at 50°C (habitat temperature) and 70°C; oxidation was much more rapid at the latter temperature. The temperature optima for iron oxidation can be compared with those for sulfur oxidation. MOSSER et al. (1974a) found a temperature optimum for sulfur oxidation in Moose Pool of 80°C, and here the most rapid iron oxidation was at 60°C, with significant oxidation up to 80°C. In the case of Evening Primrose, MOSSER et al. (1974b) found an optimum of 70°C, even though the habitat temperature was 50°C, and in the present study oxidation proceded much more rapidly at 70 than 50°C, although a temperature optimum was not determined. MOSSER et al. (1974a) showed that Sulfolobus exists as a series of temperature strains, and strains were found with optima of 65–68°, 74–75°, and 80°C. They showed that the temperature optimum of a population in a given spring was not always close to the temperature of that spring. However, in no case did they find strains adapted to 90°C, nor did they find significant sulfur
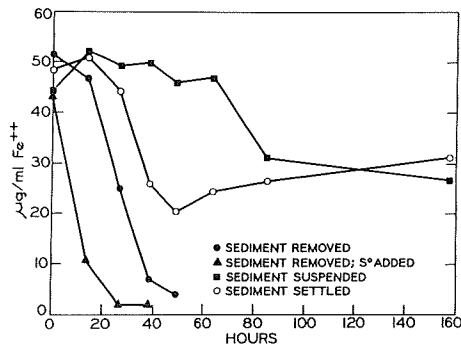

Fig. 4. Effect of Moose Pool sediment on ferrous iron oxidation. Sediment was removed from some samples by decanting after 1 hr settling. In one series, elemental sulfur (Sargent–Welch precipitated sulfur) was added at a concentration of 1000 ppm. Temperature of incubation, 80°C.

oxidation at this temperature. A given strain of Sulfolobus will probably oxidize both iron and sulfur, as has been shown by BRIERLEY and BRIERLEY (1973) for their strain. Thus, the variation in temperature optima we find for iron oxidation are in agreement with those already reported for sulfur oxidation.

Effect of sediment on iron oxidation. As seen in Fig. 2, ferrous iron oxidation did not occur at significant rates in Moose Pool and Sulfur Caldron. This was surprising, since both of these springs had shown active bacterial sulfur oxidation (MOSSER et al., 1973, 1974b). However, a chance observation led to the conclusion that sedimentary material suspended in the water was affecting the results in some way. If this sediment was removed, active iron oxidation could be detected.

The effect of suspended sediment on iron oxidation in Moose Pool is shown in Fig. 4. It can be seen that if the sediment is removed by allowing it to settle out for several hours before the experiment is performed, reasonably active iron oxidation occurred. If the sediment was present throughout the incubation, and was kept in suspension by occasional shaking, no decrease in ferrous iron occurred. Iron oxidation was somewhat better if the sediment was allowed to settle to the bottom of the bottle, but it was still not as rapid as if the sediment was removed completely. Similar results (not shown) were obtained at Sulfur Caldron.

One of the main ingredients of the sediment was elemental sulfur. However, elemental sulfur by itself did not affect iron oxidation in Moose Pool water, but actually stimulated it, as shown in Fig. 4. BRIERLEY and BRIERLEY (1973) also reported that sulfur did not inhibit iron oxidation by their strain of Sulfolobus, but stimulated it.

Nature of the sediment which affects iron oxidation

Not all of the springs studied had significant amounts of suspended sediment. However, the three springs which showed very low to negligible apparent iron oxidation rates, Moose Pool, Sulfur Caldron, and 59-3, all had large amounts of suspended sediment. Sediment is held in suspension by the marked turbulence of the water, created primarily by rising

Table 2. Interpretation of X-ray powder diffraction patterns of inhibitory sediment from Moose Pool and Sulfur Caldron

Mineral	Moose Pool	Sulfur Caldron
Sulfur (orthorhombic)	I_{obs}= 100	I_{obs}= 100
Natroalunite $(Na,K)Al_3(SO_4)_2(OH)_6$	78	29
Quartz	61	37
Kaolinite	6	7
Illite and montmorillonite	traces	traces

X-ray diffraction pattern prepared by Walter Yang, College of Engineering, University of Wisconsin, using a CuK-alpha target. Interpretation by Stephen Hall, Sr., Department of Geology, University of Wisconsin.

steam and gas. To characterize the sediment which was responsible for inhibition of iron oxidation, it was partially purified and analyzed by X-ray diffraction. Purification was accomplished by allowing the sediment to settle overnight, followed by washing three times with deionized water. The sediment was then dried at room temperature. Analysis for elemental sulfur by measuring weight loss following extraction with carbon disulfide revealed that Sulfur Caldron sediment had 65% elemental sulfur and Moose Pool sediment 37% S^0. X-ray diffraction of dried powder revealed patterns which could be interpreted as indicated in Table 2. As seen, in addition to elemental sulfur, the main components found were natroalunite $(Na,K)Al_3(SO_4)2(OH)_6$, and quartz. Small amounts of kaolinite, illite and montmorillonite were also present.

Studies on the mechanism by which the sediment affects ferrous iron oxidation

In the initial studies, it was assumed that the sediment was inhibiting ferrous iron oxidation in some way. However, a large number of studies uncovered no obvious inhibitors. Spectrographic analyses were performed on dried sediment to see if some toxic element might be present, and these results are presented in Table 3. The only likely toxic element present was copper, but from the concentration of copper present in the sediment and the concentration of sediment present in the spring water (1·3 g/l in Moose Pool and 1·9 g/l in Sulfur Caldron, on a dry weight basis), the copper concentration in the spring water would be about 0·2 μg/ml, too low to inhibit bacterial activity, even if all of the copper were in solution rather than bound to the sediment. Also, since the sediment did not affect sulfur oxidation (see above), it seemed unlikely that it would be inhibitory to iron oxidation.

Since iron was present in the dried sediment, as seen in the spectrographic analysis, one possibility was that ferrous iron could have leached out of the sediment during the incubation and replaced that oxidized by the bacteria, thus giving an apparent inhibition. However, the iron concentration was only between 1 and 2 μg/ml of water (at the sediment concentrations present in the iron oxidation assays), much lower than the 25–80 μg/ml ferrous iron present in the waters at the beginning of incubation. Further, experiments showed that no ferrous iron was leached out of sediments incubated in pH 1·3 H_2SO_4 at 65 or 80°C.

Table 3. Mineral analysis of sediments from Moose Pool and Sulfur Caldron

Sediment source	P %	K %	Ca %	Mg %	Na %	Al ppm	Fe ppm	B ppm	Cu ppm	Zn ppm	Mn ppm
Moose Pool	.390	2.35	<.2	.046	.365	45,800	1460	51.5	22.3	28.0	15.4
Sulfur Caldron	.277	1.27	<.2	<.04	.158	20,800	1100	36.6	91.3	32.6	11.4

Analyses performed by the Soil Science Department, University of Wisconsin, Madison.

Another possible explanation was that ferrous iron oxidation occurred normally in the sediment-containing waters, but that something in the sediment was able to reduce the ferric ions formed back to the ferrous form. A direct experiment was set up to test this hypothesis. A solution of $FeCl_3$ containing about 200 μg/ml Fe^{3+} was made up in pH 1·6 H_2SO_4 and mixed with sediment from either Moose Pool or Sulfur Caldron. After incubation overnight at 65°C most of the ferric iron had been reduced to the ferrous form. When incubation occurred at room temperature, the reduction occurred much more slowly, still being incomplete after several days. Thus, it seemed clear that the sediment was affecting the ferrous iron concentration not by inhibition of the microbial oxidation process, but by reduction of ferric iron formed by the bacteria.

What is the reductant in the sediment responsible for conversion of ferric to ferrous iron? The most likely reductant in the sediment appeared to be elemental sulfur, but, as shown above, this did not affect the ferrous iron oxidation process. Also, a direct experiment using ferric chloride and elemental sulfur in pH 1·6 H_2SO_4 showed no reduction of Fe^{3+} to Fe^{2+} when incubation occurred at 65, 80 or 90°C. One other component of the sediment, not seen in any of the above assays, was sulfide. Sulfide was initially detected when sediments from Moose Pool and Sulfur Caldron were incubated in Locomotive Spring water, which is low in ferric iron, and an odor of hydrogen sulfide was detected after a few days incubation. The actual amount of sulfide present in the sediments from these two springs was measured by acidification and distillation under nitrogen, using the Pachmayr procedure (PACHMAYR, 1960), but acidifying with the stannous chloride–hydrochloric acid reagent of BLOOMFIELD (1969). A concentration of sulfide of about 1 μg/mg wet sediment was found in both Moose Pool and Sulfur Caldron, so that the sulfide concentration in the spring water from this source was about 2·5 μg/ml for Moose Pool (which had 2·5 g/l wet weight of sediment) and 3·8 μg/ml for Sulfur Caldron (3·8 g/l wet weight of sediment). As seen in Table 1, the total sulfide concentration in these two pools is much higher, but all of the sulfide dissolved in the water is quite labile, volatilizing during the transport of water samples to the laboratory. As noted in Materials and Methods, the soluble sulfide was preserved for assay by adding zinc acetate and raising the pH with NaOH. On the other hand, the sulfide bound to the sediment is stable at room temperature at the pH of the spring water, although as noted above it is slowly liberated during incubation at high temperature. It is not known whether this sulfide is present in the sediment as iron sulfide (there is enough iron present to react with all of the sulfide) or is part of the natroalunite crystal lattice.

Although it seemed obvious that sulfide liberated from the sediment could reduce ferric iron back to the ferrous state and hence give results resembling

an inhibition, there is insufficient sulfide present to account for the results. Assuming the reaction:

$$H_2S + 8Fe^{3+} + 4H_2O = 8Fe^{2+} + H_2SO_4 + 8H^+$$
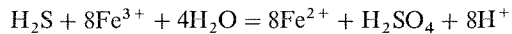
and concentrations of sulfide of 2 $\mu g/ml$ (60 μM) and Fe^{3+} of 70 $\mu g/ml$ (1·3 mM), the sulfide could reduce only 480 μm of Fe^{3+}, about 1/3 of that present. Further, the sulfide is used up in the process, and eventually ferric iron reduction should cease and net ferrous iron oxidation occur. However, as shown in Figs. 2 and 4, ferrous iron does not disappear in Moose Pool and Sulfur Caldron for many days, suggesting that another reductant must be functional.

Several lines of study suggest that the principle reductant in the sediment is elemental sulfur, activated in some way by the sulfide present. As noted earlier, elemental sulfur itself does not cause an apparent inhibition of ferrous iron oxidation, nor does it reduce ferric iron under the conditions used. However, when the elemental sulfur was removed from dried sediment by carbon disulfide extraction, the extracted material no longer affected ferrous iron oxidation. Thus, since neither elemental sulfur nor sediment sulfide are able to reduce ferric iron separately, it appears that both components must be together for the process to occur.

DISCUSSION

These results show clearly that *Sulfolobus acidocaldarius* is able to oxidize large amounts of ferrous iron in the hot, acid springs. However, the rates of oxidation vary widely, from essentially negligible to almost 100% of the ferrous iron oxidized in 24 hr. These data provide a clear indication of the upper temperature limit for bacterial iron oxidation, since no significant oxidation occurred at 90°C but good oxidation occurred at 85°C. No other acidophilic bacterium is known capable of growing at temperatures as high as *Sulfolobus* (BROCK *et al.*, 1972; BROCK and DARLAND, 1970), so that it can be concluded that any oxidation of ferrous iron at temperatures above 90°C is probably nonbiological.
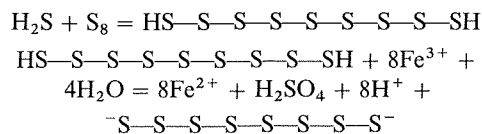
Although the sodium chloride controls never showed any significant ferrous iron oxidation at any temperature employed, casual observations in the laboratory suggest that non-bacterial oxidation of ferrous iron at low pH and high temperature can occur. For instance, if large amounts of ferrous sulfate (of the order of 1000 ppm Fe^{2+}) are added to sterile culture medium at pH 2, after incubation at 80–90°C for several weeks a reddish ferric hydroxide precipitate is formed. No precipitation of ferric hydroxide is observed when the same culture medium is incubated for the same time at room temperature. Thus, although the bacteria greatly accelerate the rate of ferrous iron oxidation, spontaneous oxidation definitely occurs at high temperatures.

The rate at which ferrous iron is oxidized is about the same as the rate at which elemental sulfur is oxidized under the same conditions. Thus, MOSSER *et al.* (1973) reported rates of elemental sulfur oxidation

in different Yellowstone areas of 10–100 $g/m^2/day$. We calculate from the data in Fig. 2 and Table 1 that oxidation rate in Locomotive Spring is about 6 $g/m^2/day$ and in spring 54-5 it is about 17 $g/m^2/day$ (these two springs have natural temperatures similar to those used in the laboratory incubations, so that laboratory rates should be comparable to natural rates). It is likely that ferrous iron, a soluble substrate, is more readily oxidized than elemental sulfur, but in nature elemental sulfur concentrations often are much higher than ferrous iron concentrations, and rates of sulfur oxidation are accordingly higher.

In springs which are low in elemental sulfur, such as all of the Norris springs studied, it is likely that *Sulfolobus* populations are maintained with ferrous iron as energy source, since no other energy source is apparent. In this respect, some casual observations on Locomotive Spring may be relevant. In the early June of 1974, the *Sulfolobus* population density in Locomotive Spring was markedly lower than it had been in the summers of 1972 and 1973, and at the same time the ferrous iron concentration was also lower. By the end of June 1974 the ferrous iron concentration had risen to previous values and the *Sulfolobus* population density had also risen.

The apparent inhibition of ferrous iron oxidation found in several pools with large amounts of sediment has been shown to be an artifact. Ferrous iron oxidation occurs in these pools, but the ferric iron formed is reduced back to the ferrous state by reductants present in the sediment. It was shown that the principal reductant was most likely elemental sulfur, activated in some way by the small amount of sulfide bound to the sediment. There was insufficient sulfide to act alone, and elemental sulfur by itself did not reduce ferric iron under the conditions used, but since no other reductant was found, it is assumed that the sulfide in some way activates elemental sulfur so that it is able to react with ferric ions. According to ROY and TRUDINGER (1970), the S_8 rings of elemental sulfur are opened by sulfides and thiols. It is postulated that the following series of reactions takes place:

$$H_2S + S_8 = HS—S—S—S—S—S—S—SH$$

$$HS—S—S—S—S—S—S—SH + 8Fe^{3+} +$$
$$4H_2O = 8Fe^{2+} + H_2SO_4 + 8H^+ +$$
$$^-S—S—S—S—S—S—S—S^-$$

The polysulfide formed could then reduce further Fe^{3+}. Because of the high concentration of elemental sulfur in the sediment, there will always be sufficient sulfur to keep the iron in the reduced form, and once the S_8 rings are broken, further reaction with ferric iron could take place without participitation of sulfide. In this way, sulfide would be an essential ingredient of the process even though insufficient sulfide is present to react directly with the ferric iron.

The concentrations of ferrous and ferric iron in the spring waters should thus be determined by several factors: (1) the concentrations of ferrous and ferric

iron in the incoming water, (2) the rate of reduction of ferric to ferrous iron by reductants present, (3) the rate at which bacterial oxidation occurs, and (4) the flow rate of water through the spring. It is likely that in springs where bacterial oxidation is rapid (Fig. 2), the rate of ferrous iron oxidation occurs more rapidly than the flow rate. Thus, the steady-state concentrations of ferrous and ferric iron should be determined primarily by the rate at which iron formed by bacterial action is reduced by reductants in the water.

REFERENCES

Allen E. T. and Day A. L. (1935) Hot springs of the Yellowstone National Park. *Carnegie Inst. Wash. Publ.* **466,** 525 pp.

American Public Health Association (1971) *Standard Methods for the Examination of Water and Wastewater,* 13th edition.

Beck J. V. (1967) The role of bacteria in copper mining operations. *Biotechn. Bioeng.* **9,** 487–497.

Belly R. T. and Brock T. D. (1974) Ecology of iron-oxidizing bacteria in pyritic materials associated with coal. *J. Bacteriol.* **117,** 726–732.

Bloomfield C. (1969) Sulphate reduction in waterlogged soils. *J. Soil Sci.* **20,** 207–221.

Brierley C. L. and Brierley J. A. (1973) A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. *Can. J. Microbiol.* **19,** 183–188.

Brock T. D. and Darland G. K. (1970) Limits of microbial existence: temperature and pH. *Science* **169,** 1316–1318.

Brock T. D., Brock M. L., Bott T. L. and Edwards M. R. (1971) Microbial life at 90°C: the sulfur bacteria of Boulder Spring. *J. Bacteriol.* **107,** 303–314.

Brock T. D., Brock K. M., Belly R. T. and Weiss R. L. (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch. Microbiol.* **84,** 54–68.

Brock T. D. and Mosser J. L. (1975) Rate of sulfuric acid production in Yellowstone National Park. *Bull. Geol. Soc. Amer.* **86,** 194–198.

Fliermans C. G. and Brock T. D. (1972) Ecology of sulfur-oxidizing bacteria in hot acid soils. *J. Bacteriol.* **111,** 343–350.

Mosser J. L., Mosser A. G. and Brock T. D. (1973) Bacterial origin of sulfuric acid in geothermal habitats. *Science* **179,** 1323–1324.

Mosser J. L., Mosser A. G. and Brock T. D. (1974a) Population ecology of *Sulfolobus acidocaldarius.* I. Temperature strains. *Arch. Microbiol.* **97,** 169–179.

Mosser J. L., Bohlool B. B. and Brock T. D. (1974b) Growth rates of *Sulfolobus acidocaldarius* in nature. *J. Bacteriol.* **118,** 1075–1081.

Pachmayr F. (1960) Vorkommen und Bestimmung von Schwefelverbindungen im Mineralwasser. Doctoral dissertation, Ludwig-Maximilians-Universität. Munch, Germany.

Raymahashay B. C. (1966) Hot springs and hydrothermal alteration in the Paint Pot Hill area, Yellowstone Park. Ph.D. thesis, Harvard University, Cambridge, Mass.

Roy A. B. and Trudinger P. A. (1970) *The Biochemistry of Inorganic Compounds of Sulphur,* 400 pp. Cambridge University Press.

Singer P. C. and Stumm W. (1970) Acidic mine drainage: the rate-determining step. *Science* **167,** 1121–1123.

Stumm W. and Morgan J. J. (1970) *Aquatic Chemistry,* 583 pp. John Wiley.

Temple K. L. and Delchamps E. W. (1953) Autotrophic bacteria and the formation of acid in bituminous coal mines. *Appl. Microbiol.* **1,** 255–258.

White D. E., Muffler L. J. P. and Truesdell A. H. (1971) Vapor-dominated hydrothermal systems compared with hot-water systems. *Econ. Geol.* **66,** 75–97.