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FUNDAMENTAL STUDIES OF AQUEOUS PYRITE OXIDATION AND
THE FORMATION OF METASTABLE SULFUR OXY-ANIONS
UNDER ABIOLOGICAL AND MICROBIAL CONDITIONS AT pH 6.5

by
Cathryn A. Shepard

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A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Master of Science (Geochemistry).

Golden, Colorado

Date Sept 2, 1982

Signed: Cathryn A. Shepard
Cathryn A. Shepard

Approved: DM Updegraff
Dr. David M. Updegraff

Golden, Colorado

Date Sept. 3, 1982

SRD
Dr. George H. Kennedy
Dept. of Chemistry/Geochemistry

A B S T R A C T

Oxidative products of the iron sulfide mineral, pyrite (FeS_2), in a neutral aqueous medium were studied under various conditions. The metastable sulfur species were measured periodically utilizing routine analytical procedures. The results show bacteria are capable of utilizing pyrite as an energy source in the near-neutral pH range of 6.0 to 6.5. The addition of bacteria appears to increase the rate of pyrite oxidation. In a bacterial medium the abundance of intermediate sulfur species relative to total sulfur in solution declined rapidly compared to the results from a simple KCl solution at the same pH.

It is particularly interesting to note that pentathionate ($\text{S}_5\text{O}_6^{2-}$) appeared to be the dominant polythionate in the bacterial medium, whereas tetrathionate ($\text{S}_4\text{O}_6^{2-}$) is dominant in the KCl matrix.

Sulfite (SO_3^{2-}) was detected in experiments utilizing bacteria to oxidize pyrite, although none was detected in the sterile control.

In experiments providing pyrite as an energy source for the bacteria, the predominant sulfur species was sulfate. Where thiosulfate was provided as an energy source for the bacteria, the predominant sulfur species detected

was thiosulfate.

Pyrite oxidation studies involving T. thioparus show that these bacteria influence the rate of intermediate sulfur species produced. Studies involving an enrichment culture produced similar results. In addition, experiments showed that the bacteria catalyze the initial step in the pyrite oxidation process rather than waiting for the intermediate sulfur species to appear in solution.

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I N T R O D U C T I O N

There has been a steady growth of interest in the fundamental processes involved in the geochemical cycling of sulfur and various metallic elements. A great deal of information has been accumulated over the past thirty years on particular facets of the overall process, although a definitive and comprehensive model of the interactions occurring in a sulfidic ore deposit has not yet been unequivocally proven.

The involvement of microbial populations in the accelerated degradation of sulfide minerals has been unequivocally demonstrated by a number of investigators (Rudolfs and Helbronner, 1922; Carpenter and Herndon, 1933; Colmer and Hinkle, 1947; Ralph, 1979). A number of metal sulfides are of geomicrobial interest because they are formed as a result of microbial activity. Under certain conditions sulfur combines directly with hydrogen and with nearly all metals to form sulfides (ZoBell, 1963). Examples of metal sulfide minerals of geomicrobial interest are listed in Table 1 (Ehrlich, 1981).

BIOGEOCHEMICAL CYCLE OF SULFUR

Sulfur occurs in a great variety of chemical compo-

Mineral or synthetic compound	Formula	References
Antimony trisulfide	Sb_2S_3	Silver and Torma(1974); Torma and Gabra(1977)
Arsenopyrite	FeAsS	Ehrlich(1964a)
Chalcocite	Cu_2S	Byrner et al.(1954); Ivanov(1962); Razzell and Trussell(1963); Sut- ton and Corrick(1963, 1964); Fox(1967)
Chalcopyrite	$CuFeS_2$	Bryner and Anderson(1957)
Covellite	CuS	Bryner et al.(1954); Razzell and Trussell (1963)
Galena	PbS	Silver and Torma(1974)
Marcasite, pyrite	FeS_2	Leathen et al.(1953); Silverman et al.(1961)
Molybdenite	MoS_2	Bryner and Anderson (1957); Bryner and Jame- son(1958); Brierley and Murr(1973)
Orpiment	As_2S_3	Ehrlich(1963a)
Nickel sulfide	NiS	Torma(1971)
Pyrrhotite	Fe_4S_5	Freke and Tate(1961)
Sphalerite	ZnS	Ivanov et al.(1961); Ivanov(1962); Malouf and Prater(1961)

Table 1. Metal sulfides of geomicrobial interest
(modified from Ehrlich, 1981)

sitions having oxidation states ranging from -2, as in sulfides (S^{2-}) to +6, as in sulfates (SO_4^{2-}). Intermediate oxidation states include sulfur dioxide (SO_2), sulfites (SO_3^{2-}), thiosulfates ($S_2O_3^{2-}$) and polythionates ($S_nO_6^{2-}$), where n may be any value from two to six.

With the exception of sulfates, the anions of oxyacids of sulfur rarely occur in nature except at relatively low concentrations in transitory states (ZoBell, 1963).

Under most natural conditions only the -2 and +6 valence states are important. These form the sulfide anion and sulfate ion that in combination with metals are the basis of the two classes of sulfur-bearing minerals (Field, 1972).

Sulfur is present in magma and igneous rocks primarily in the form of sulfides and $SO_2(g)$, while sulfate is the predominant form in the hydrosphere and biosphere. Sulfur is among the thirteen most abundant elements in the crust of the earth, ranking fifth, sixth and eighth by weight in abundance in the entire earth, sea water, and living organisms, respectively.

Figure 1 (ZoBell, 1963) outlines some of the major pathways of sulfur as it passes from magma and igneous rocks into the atmosphere, hydrosphere and biosphere. Although the sulfur content of the atmosphere is almost

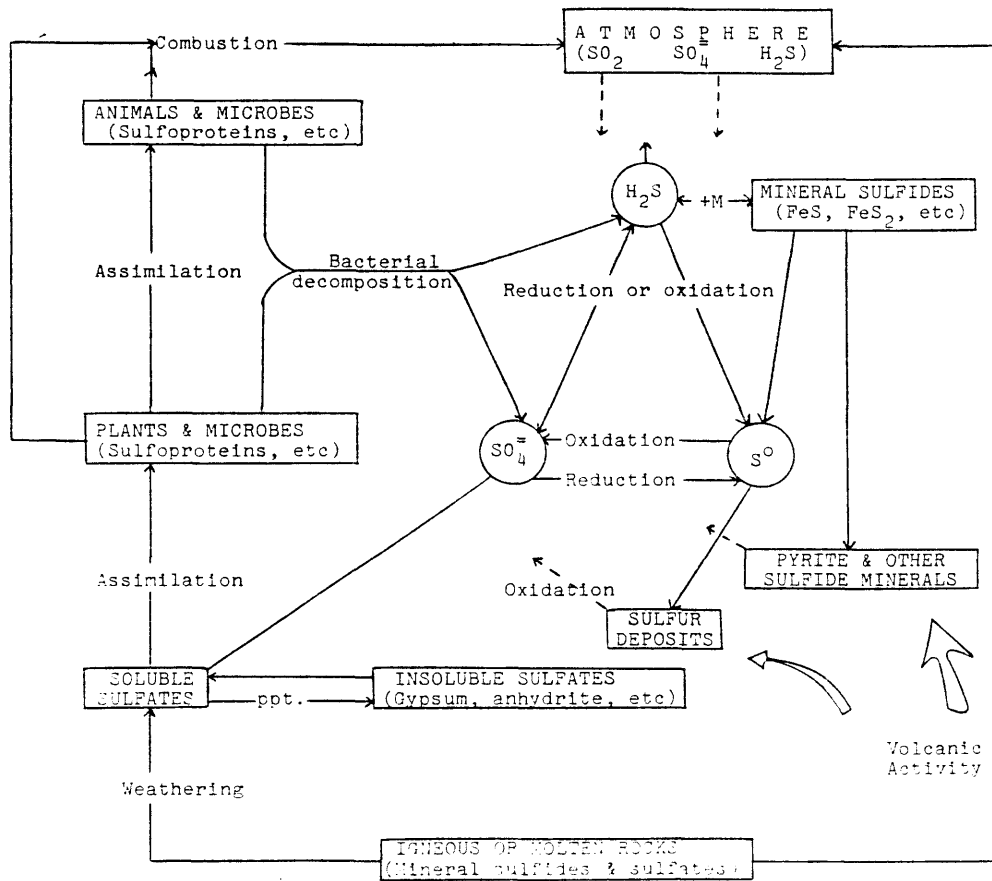


Figure 1. Cycle of sulfur from primeval earth through the biosphere in sediments, soil, water and air (Zobell, 1963).

negligibly small except near the earth's surface, appreciable amounts circulate in the air, mostly as sulfate with lesser quantities of sulfite, hydrogen sulfide, sulfur dioxide, sulfur trioxide, mercaptans and organic sulfur (ZoBell, 1963).

Microorganisms affect the forms of sulfur in water, soil and sediments and are largely responsible for the parts of the sulfur cycle depicted in the center and upper left-hand corner of Figure 1. The variety of microbiological processes within the sulfur cycle can be schematically presented as three main reactions: oxidation of sulfide minerals and native sulfur to sulfuric acid, oxidation of dissolved hydrogen sulfide to sulfur and sulfates, and reduction of sulfates to hydrogen sulfide and sulfides (Ivanov, 1977). The main transformations of sulfur catalyzed by microorganisms are shown schematically in Figure 2. Volumes have been written on the intermediate reactions and governing conditions (ZoBell, 1963).

SULFATE REDUCTION

Sulfates do not give up their oxygen or set sulfur free in any form without the expenditure of energy because of the stability of the sulfur-oxygen bonds. The

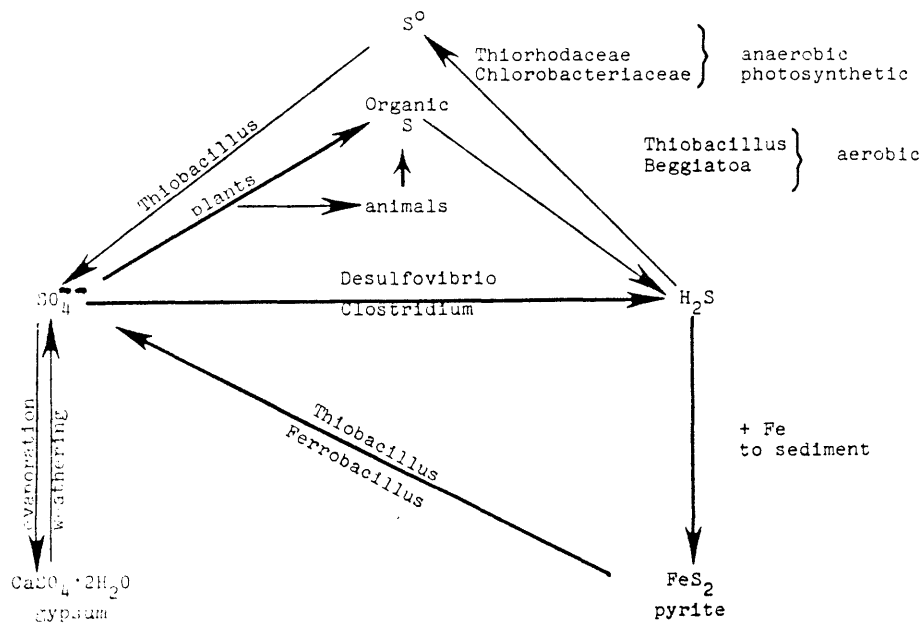


Figure 2. The biological sulfur cycle (Field, 1972).

reduction of one gram-molecular weight of sulfate to sulfide under standard conditions requires almost 200 Kcal (Wiame, 1958). Plants obtain the necessary energy for this reduction from solar radiation. The major products of this kind of sulfate reduction (assimilatory sulfur reduction) are proteins incorporating sulfur-containing amino acids such as cysteine and methionine. This process is briefly summarized by Goldhaber and

Kaplan (1974) and further discussed by Nicholas (1967) and Trudinger (1969).

Certain bacteria can conduct a more rapid reduction of sulfate (dissimilatory sulfur reduction) in which sulfate accepts the electrons generated during the metabolism of carbon compounds that lead to growth. The sulfur is not incorporated into the bacterial structure. Extensive research has been done in this area (Roberts et al., 1955; Butlin and Postgate, 1956; Challenger, 1959; Rees, 1961; Roy and Trudinger, 1970).

SULFATE-REDUCING BACTERIA

The most important class of organisms capable of sulfate reduction is the Schizomycetes in the order Eubacteriales (bacteria). Postgate and Campbell (1966) collected all the existing forms of sulfate-reducing bacteria and examined the taxonomy of these forms. They assign all existing forms into two genera in accordance with their capacity for forming spores: sporeforming - Desulfotomaculum - and non-sporeforming - Desulfovibrio. Table 2, modified from Postgate and Campbell (1966), presents a simplified classification of sulfate-reducing bacteria.

The majority of marine sulfate-reducing bacteria belong to the genus Desulfovibrio. Their most important

Microorganisms	Shape of Cell	Flagella	Spore Formation	Halophilics	Thermophilics
<u>Desulfotomaculum nigrificans</u>	Bacillus	Peritricate	+	-	+
<u>D. orientis</u>	'Bent' bacillus	"	+	-	-
<u>D. ruminis</u>	Bacillus	"	+	-	-
<u>Desulfovibrio desulfuricans</u>	Vibrio	Monotricate	-	-	-
<u>D. vulgaris</u>	"	"	-	-	-
<u>D. salexigens</u>	"	"	-	-	-
<u>D. africanus</u>	Curved bacillus	Lofotricate	-	-	-
<u>D. gigas</u>	Spiral	"	-	+	-

Table 2. Taxonomy of sulfate-reducing bacteria (modified from Postgate and Campbell, 1966)

function in geochemical cycles is the production of available oxygen from sulfate for the oxidation of organic matter (Redfield, 1958). They generally grow best between pH 6.5 and 8 and at a low oxidation-reduction potential (Eh) (Postgate, 1959). They are strict anaerobes, growing and reducing sulfates only in the absence of free oxygen. They require water and an energy source such as organic matter and/or free hydrogen. Sulfate-reducing bacteria are thought to be mostly heterotrophic. However, it has been shown that in the presence of a minimal supply of organic matter, up to 25% CO₂ can be fixed in the presence of molecular hydrogen (Goldhaber and Kaplan, 1974). Under controlled conditions, growth will not occur if CO₂ is the only carbon source (Mechalás and Rittenberg, 1960; Postgate; 1960). They are abundant in marine bottom deposits, soils, sewage, stagnant water basins and lacustrine sediments. They have been found in sulfur deposits from salt domes and in reservoir fluids and borings from oil wells to depths of several thousand feet (ZoBell, 1958). These bacteria are discussed elsewhere (Miller, 1950; Young and Maw, 1958; ZoBell, 1958; Senez, 1962; Postgate, 1965; Kaplan and Friedman, 1970; Trudinger et al., 1972; Goldhaber and Kaplan, 1974; Karaiivko et al., 1977).

PYRITE BIODEGRADATION

Bacteria belonging to the family Thiobacteriaceae are believed to be the most widespread and probably the most important organisms involved in the oxidation of sulfur in deposits of useful minerals. Some of the species of Thiobacilli involved are listed in Table 3. The high-temperature chemoautotroph Sulfolobus is also listed, as it has been found to be capable of leaching molybdenite (MoS_2) and tolerating high concentrations of molybdenum (Brierley, 1974). The genera of gliding bacteria Beggiatoa and Thiothrix are also capable of oxidizing sulfide to native sulfur and are further described by Brock (1974).

All species of the genus Thiobacillus are small, gram-negative, non-sporeforming, polarly flagellated rods able to derive energy from the oxidation of elemental sulfur, metal sulfides, or thiosulfate (Bergey, 1957). Most thiobacilli are considered obligate autotrophs, although many can grow heterotrophically (Brock, 1974).

INORGANIC OXIDATION OF PYRITE

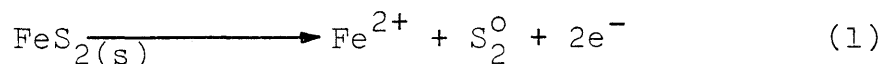
Pyrite oxidation is a complicated process which includes several types of oxidation-reduction reactions, hydrolysis and complex ion formation, solubility and kinetic effects (Nordstrom, 1979). Extensive research and

SPECIES	MOTILITY	NUTRITION	pH RANGE	TEMPERATURE RANGE
<u>T. thioparus</u>	motile by polar flagella	strict autotroph; $S_2O_3^{2-}$, S^0 , H_2S	optimum 6.6-7.0 range 4.5-7.8	8-37°C optimum 28°C
<u>T. neapolitanus</u>	most nonmotile; some motile	strict autotroph but will assimilate organic compounds; H_2S , $S_2O_3^{2-}$, S^0 , $S_4O_6^{2-}$	optimum 6.2-7.0 range 3.0-8.5	8-37°C optimum 28°C
<u>T. thiooxidans</u>	motile	strict autotroph; S^0 , $S_2O_3^{2-}$	optimum 2.0-3.5 range 0.5-6.0	10-37°C optimum 28-30°C
<u>T. denitrificans</u>	motile	strict autotroph; facultative anaerobe (utilizes nitrate) to oxidize H_2S , S , $S_2O_3^{2-}$, $S_2O_6^{2-}$	range 6.0-8.4	8-37°C optimum 28°C
<u>T. ferrooxidans</u>	motile	strict autotroph although has been reported to grow on sucrose; S , $FeSO_4$, $S_2O_3^{2-}$, S^{2-} , $S_4O_6^{2-}$, FeS_2	optimum 2.4-5.8 range 1.4-6.0	15-25°C optimum 15-20°C
<u>T. novellus</u>	nonmotile	facultative autotroph; organic matter suppresses thiosulfate oxidation	optimum 7.5-9.0 range 5.0-9.2	optimum 30°C
<u>T. intermedius</u>	motile	facultative autotroph; oxidizes both organic matter and thiosulfate simultaneously; $S_2O_3^{2-}$, S^0	optimum 6-7 range 1.9-7.0	optimum 30°C
<u>T. perometabolis</u>	motile	obligate mixotroph; oxidizes both thiosulfate and organic matter	range 2.8-6.8	optimum 30°C
<u>Sulfolobus</u> sp.	nonmotile	facultative autotroph	optimum 2.3-4.0 range 0.0-5.8	optimum 70-75°C range 55-85°C

Table 3. Thiobacillus and related sulfur-oxidizing bacteria.

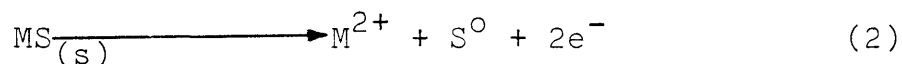
study has begun to unravel many of the more important aspects of this process although many questions remain unresolved.

Measurements of single electrode potentials enabled Sato (1960) to tentatively conclude that the direct aqueous oxidation of a simple sulfide mineral proceeds as a result of the metal atoms moving into the surrounding solution to become aqueous cations, accompanied by a step-wise decrease in the metal to sulfur ratio of the remaining solid phase. Upon oxidation of pyrite, ferrous ions and S₂ molecules appear to be released simultaneously according to equation 1 for pH 3.0 (Sato, 1960; Garrels and Thompson, 1960).

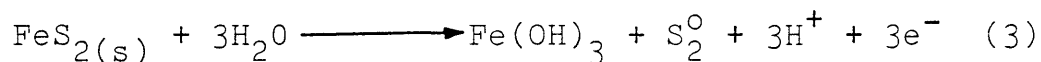


The sulfur in equation 1 is not in a crystalline state, but in diatomic molecular form. The remaining sulfur undergoes a series of reactions to be oxidized to sulfate ion. It is interesting to note the relative resistance of pyrite to oxidation as shown in Table 4 (Malouf, 1972). This may be explained by the high oxidation potential for reaction 1. It is possible this is a result of the crystal structure of pyrite, where two sulfur atoms exist close together, forming -Fe-S₂-Fe- type bonds which apparently require considerable energy to break (Sato, 1960).

Other investigators have shown the dissolution of the metal, with sulfide oxidizing to neutral sulfur as being the first step in the sulfide mineral oxidation process (Burkin, 1966; Wadsworth, 1973). The general stoichiometry for a divalent metal, M, is:



In neutral to basic solutions the hydrolysis of ferric ion to ferric hydroxide must be taken into account (Sato, 1960):



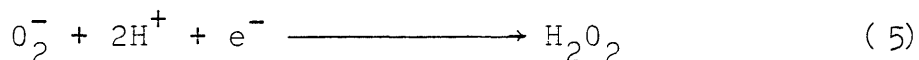
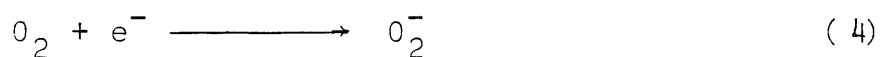
There is evidence for both the presence and absence of elemental sulfur resulting from pyrite oxidation via reaction 2 (Stokes, 1901; Clark, 1966; Smith et al., 1968).

Pyrrhotite	FeS
Chalcocite	Cu ₂ S
Covellite	CuS
Galena	PbS
Arsenopyrite	FeAsS
Sphalerite	ZnS
Pyrite	FeS ₂ (isometric)
Chalcopyrite	CuFeS ₂
Molybdenite	MoS ₂

Table 4. List of minerals according to increasing resistance to oxidation (Malouf, 1972).

Observations by several investigators suggest the relative rates of oxidation of S_2^- sulfur to elemental sulfur and elemental sulfur to sulfate are involved, with sulfide to sulfur being faster (Kinkel et al., 1956; Nordstrom, 1979). Instances which cite the absence of elemental sulfur lend evidence to this hypothesis, which is still open to further critical examinations.

As the pyrite is oxidized, oxygen is reduced through the following reactions:



Reaction 4 is the rate limiting step at $pH < 4$ and reaction 5 is rate-limiting at $pH > 4$ (Biegler et al., 1976; Smith et al., 1968). Combining the oxygen reduction reaction with pyrite oxidation reaction results in equation 6.

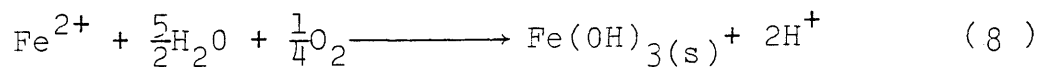


All three products in reaction 6 are unstable. At neutral pH values, the ferrous iron is rapidly oxidized according to the expression:

$$\frac{d[Fe(II)]}{dt} = \frac{k[Fe(II)][O_2]}{[H^+]^2} \quad (7)$$

where $k = 8.0 \times 10^{13} \text{ liter}^2 \text{ mole}^{-2} \text{ atm}^{-1}$ at 25°C . (Stumm and

Lee, 1961; Singer and Stumm, 1968; Ghosh, 1974). Ghosh (1974) indicated this oxidation rate increases with increasing carbonate alkalinity. The iron quickly undergoes hydrolysis and precipitates as ferric hydroxide:

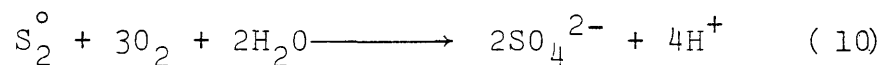


or, if the hydrogen peroxide produced in reaction 6 is the oxidizing agent:



(Nordstrom, 1979). Baker (1972) has observed iron hydroxide on the surface of oxidizing pyrite.

The sulfur is oxidized to sulfate according to:



thus creating a drop in the pH.

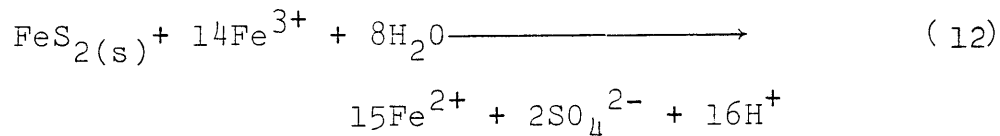
This last reaction is extremely important as it can drop the pH of the solution down significantly (Singer and Stumm, 1968) and ferric hydroxide becomes increasingly more soluble. At pH values below 3.5 ferrous iron oxidation reactions proceeds independently of pH, that is ,

$$-\frac{d[\text{Fe}(\text{II})]}{dt} = k'[\text{Fe}(\text{II})][\text{O}_2] \quad (11)$$

where $k' = 1.0 \times 10^{-7} \text{ atm}^{-1} \text{ min}^{-1}$ at 25°C (Singer and Stumm, 1968).

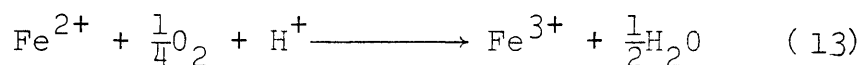
This increases the importance of ferric iron as an oxidizing agent in the oxidation of sulfide minerals.

Ferric iron is a strong oxidizer and rapidly oxidizes pyrite in the absence of oxygen according to the stoichiometry:

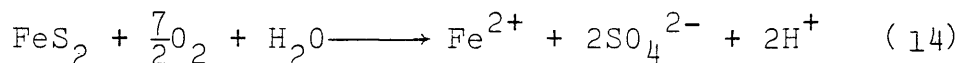


(Garrels and Thompson, 1960; Smith et al., 1968). Figure 3 from Nordstrom (1979) compares the oxidation rates of

a) ferrous to ferric by oxygen,



b) pyrite by oxygen,



and c) pyrite by ferric (equation 12) as a function of pH (Singer and Stumm, 1968, 1969; Smith, et al., 1969). At $\text{pH} < 3.0$ ferric iron oxidizes pyrite more rapidly than oxygen and consequently is the only oxidizer of pyrite of importance at these low pH's. From Figure 3 it can be seen the rate of ferrous oxidation increases rapidly above pH 4.5, but the precipitation of ferric hydroxide decreases the availability of the ferric ion in solution. These studies indicate pyrite is initially oxidized by oxygen and the pH will consequently decrease to a point ($\text{pH} \approx 4.5$) at which ferric iron becomes more soluble and begins to act

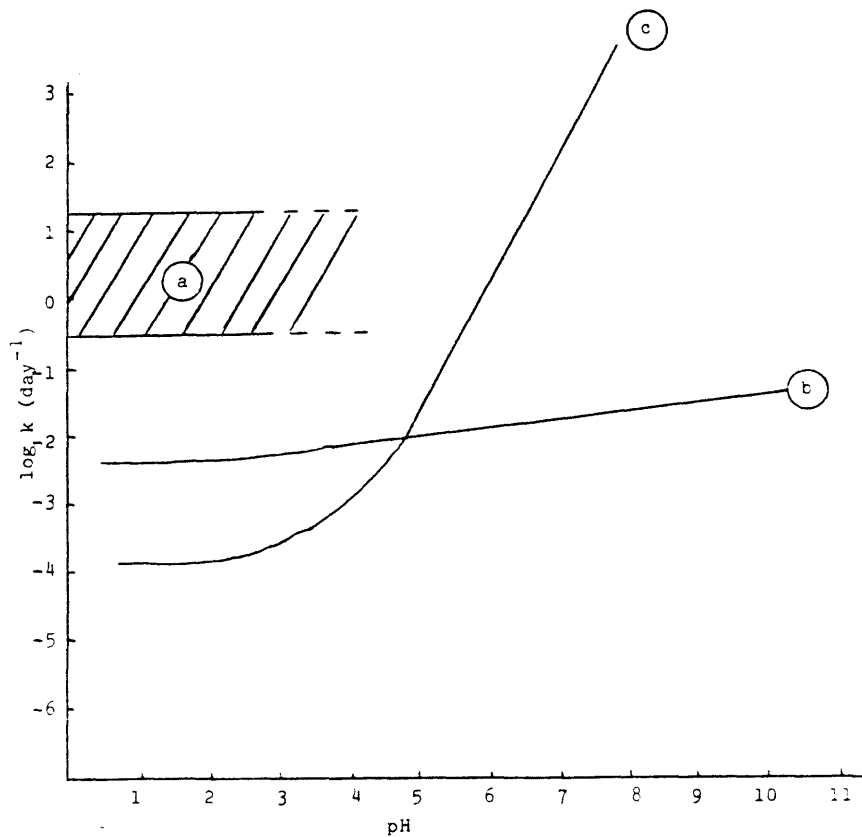


Figure 3. Comparison of rate constants as a function of pH for a) pyrite oxidation by ferric iron, b) pyrite oxidation by oxygen and c) ferrous iron oxidation by oxygen (Nordstrom, 1979).

as an oxidizing agent. The rate of oxidation by ferric iron is independent of the oxygen availability (Singer and Stumm, 1969). However, the rate-determining step appears to be the ferrous to ferric oxidation, as Figure 3 illustrates the rate of reduction of ferric to ferrous by pyrite is much faster than ferrous can be reoxidized to ferric by oxygen (Singer and Stumm, 1970).

Other factors which can affect the inorganic oxidation

rate of pyrite include temperature, available surface area, the presence of impurities such as trace metals and the presence of other minerals such as chalcopyrite, sphalerite, calcite, etc. (Nordstrom, 1979). These factors have been summarized by Clark (1966) and Shumate et al. (1971).

MICROBIAL PYRITE OXIDATION

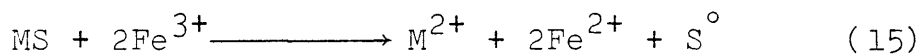
Research dealing with pyrite oxidation usually attempts to elucidate whether an inorganic or microbiological mechanism is more important in controlling the oxidation rate. The latter mechanism may take the form of indirect or direct interaction. In indirect interaction, the microbes are responsible for generating a solution which causes solubilization of the metal sulfide, most commonly through oxidation but under certain conditions, through complexation (Kee and Bloomfield, 1961; Wenberg et al., 1971; Hartmannova and Kuhr, 1974). This mechanism is of primary importance in the solubilization of uraninite (UO_2). In direct interaction, the microbes attack the metal sulfide directly by oxidizing it in its insoluble form, producing the soluble sulfate salt. Silverman (1967) attempted to determine which mechanism was more important, and concluded both mechanisms operated concurrently. However, his

experimental methods were questioned (Nordstrom, 1979) and this controversy remains unresolved.

INDIRECT MECHANISMS

The ferric ion (Fe^{3+}) either alone or complexed, is the most important chemical species involved in the indirect attack mechanisms. This oxidizing agent may be generated from dissolved ferrous iron (Fe^{2+}) at optimal pH values from 3.5 to 5.0 by Metallogenium in a mesophilic temperature range.

At pH's below 3.5, ferrous iron may be oxidized to ferric iron by Thiobacillus ferrooxidans in a mesophilic temperature range, and by Sulfolobus sp. in a thermophilic temperature range (Brierley and Lockwood, 1977). In the presence of pyrite, ferric iron may be generated by T. ferrooxidans (Kuznetsov et al., 1963; Silverman and Ehrlich, 1964; Zajic, 1969). The ferric iron, in acidic media under aerobic conditions, then acts as an oxidant to the metal sulfides (Ehrlich and Fox, 1967). The general reaction (Bryner et al., 1954; Ivanov et al., 1961) may be expressed as:



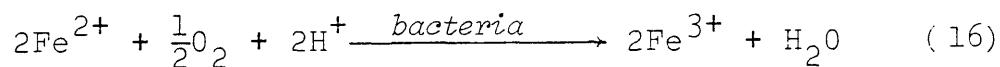
where M may be any metal, which does not always have to be divalent. The sulfide is only oxidized to elemental

sulfur, where further chemical oxidation to sulfuric acid by oxygen proceeds slowly. This reaction is accelerated by T. thiooxidans, T. ferrooxidans and/or Sulfolobus sp.. Reaction 15 is only applicable if the pH of the solution stays below 5.0 in order to maintain enough ferric iron in solution. This acid may be formed chemically through autooxidation of sulfur as in equation 10, or biologically through bacterial oxidation of sulfur or iron.

The formation of several intermediate sulfur species such as thiosulfate and polythionates are possible complications in equation 10. London and Rittenberg (1964) present data which show unequivocally the sequential intermediate formation of thiosulfate, tetra-, and tri-thionate during sulfide (Na_2S) oxidation by extracts of T. thioparus and T. thiooxidans. However, they conclude that inabilities of other researchers to demonstrate enzymatic oxidation of the polythionates resulted from the experimental procedures used. The formation of intermediate sulfur species is dependent on many other external factors, including oxygen concentration, pH, rate of growth, phosphate concentration, trace metals

and possible other unidentified factors (Brock, 1974).

To perpetuate a cycle, T. ferrooxidans can oxidize the ferrous ions produced by the oxidation of a metallic sulfide in equation 15 to ferric ions:

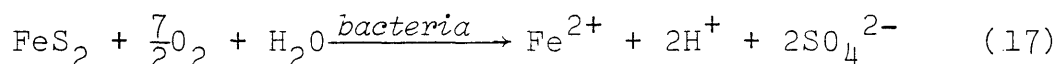


This indirect attack mechanism is twofold: 1) the chemical interaction of ferric ions with the metal sulfide and 2) bacterially catalyzed regeneration of ferric ions. Oxidation of ferrous ions by T. ferrooxidans has been shown to be as much as 5 to 6 orders of magnitude faster than by aeration of a sterile solution at low pH's (Nordstrom, 1979).

DIRECT MECHANISMS

Some investigators (Bryner and Jameson, 1958; Razzell and Trussell, 1963; Duncan and Trussell, 1964; Beck and Brown, 1968) have questioned the indirect oxidative mechanism and have proposed that the microbes make direct contact with the crystal lattice of pyrite oxidizing via enzymatic pathways. This mechanism would neces-

sitate that the bacteria be in direct contact with the mineral they metabolize and precludes any significant contribution by a ferrous-ferric cyclic mechanism. The direct microbial oxidation of pyrite probably proceeds according to the reaction (Nordstrom, 1979):



The ferrous iron generated in this reaction is then oxidized by bacteria according to equation 16, whereby chemical oxidation of pyrite by ferric iron may commence as in equation 15. An extensive review of this mechanism is presented by Ralph (1979).

Recent work indicates the genera Thiobacillus and Sulfolobus are able to reduce ferric iron when grown on elemental sulfur (Brock and Gustafson, 1976). This discovery is geochemically significant in that elemental sulfur is thought to build up on the surface of sulfide minerals as a result of their oxidation by O_2 (Sato, 1960). It had been previously thought that the oxidation of sulfide minerals by ferric iron (equation 1) was strictly nonbiological (Silverman, 1967). Elemental sulfur does not react rapidly with Fe^{3+} , so if these bacteria couple Fe^{3+} as an electron acceptor with oxidation of S^0 , the

rate of oxidation of sulfide minerals might be greatly increased. This is an extremely important point, as it suggests that bacterial catalysis may be significant in Fe^{3+} reactivity. It has also been suggested that this mechanism may permit anaerobic growth and sulfur oxidation by these bacteria (Brock and Gustafson, 1976).

PRACTICAL IMPLICATIONS

The biodegradation of iron sulfide (FeS_2) plays an important role in (a) the formation of acid mine drainage, (b) bacterial leaching of metals from ores, (c) desulfurization of coal and (d) the supergene alteration of uranium ore deposits. Hence, it is important that scientific research strive for a clear understanding of the biogeochemical interactions involved in pyrite oxidation.

Much of the coal in the United States contains a relatively high total sulfur content of 3.0 to 5.5%, much of which is in the form of iron sulfide minerals deposited during coal formation (Dugan, 1978). The sulfur can be found either as organic sulfur, sulfate or pyrite. Combustion of coal oxidizes most of the sulfur to SO_2 which is eventually oxidized to SO_4^{2-} in the atmosphere. These emissions necessitate stack scrubbers because

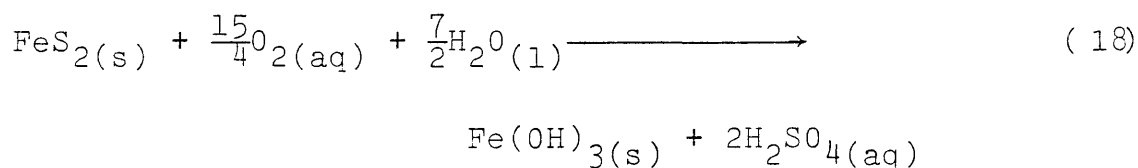
the sulfur content is too high to meet air quality standards.

Mixed enrichment cultures of acidophilic microorganisms have been found to be effective in removing pyritic sulfur from slurries of pulverized coal (Dugan, 1978). The sulfur is removed via the solubilization and leaching process described earlier involving T. ferrooxidans and T. thiooxidans. Neither species alone were proved effective in sulfur removal from coal. Bacterial removal of pyrite from coal has been used successfully on a commercial scale (Capes et al., 1973) and can result in a beneficiation from an average 4.6% total sulfur to about 1.5% total sulfur.

Acid mine drainage has created a serious environmental problem due mainly to its toxicity or otherwise inhibitory effect on most plants and wildlife with which it comes into prolonged intimate contact (McCoy and Dugan, 1968). Relatively few plants and no higher animals can thrive where their environmental pH is less than 3.5. Consequently, a considerable amount of research has been done to understand the principles of acid formation in the mining region (Lackey, 1938; Leathen et al., 1953; Temple and Kohler, 1954; Beck, 1960; Dugan and Lundgren, 1964; Lorenz and Stephan, 1967; Tuttle et al., 1969;

Dugan, 1975).

The overall mechanism of the formation of acid mine water is generally understood to be dissolution of the pyrite resulting in ferrous iron and sulfide. The sulfide rapidly oxidizes to sulfate and the ferrous iron oxidizes to ferric, catalyzed by T. ferrooxidans. Ferric iron oxidizes pyrite and the cycle perpetuates itself in this manner. The overall process describing pyrite oxidation is commonly given by the following incongruent reaction (Nordstrom, 1979):



The drainage water from the mine becomes amber-colored as the iron oxidizes, and subsequently hydrated iron oxides coat the stream bed with a rusty-appearing precipitate (Colmer et al., 1950).

The third important role of biodegradation of sulfide minerals is in bacterial leaching, a widely recognized process of metal recovery. Until recently, ores selected for mining were very accessible, contained a high concentration of the desired metal, and could be recovered with the least amount of energy. Due to the great increase in demand for metals during this century and the likeli-

hood of this demand further increasing in the future, it has now become necessary to look to the lower-grade deposits as well as less accessible ores, as ore resources for the future.

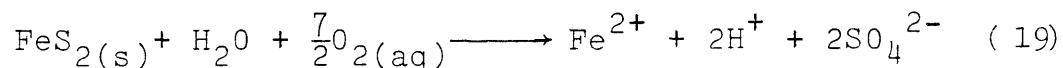
Accessible high-grade deposits for some minerals are already scarce and hence there is a need for technologically and economically feasible processes to recover metals from lower-grade ores. The microbiological leaching technique requires relatively low capital investment and low operation cost. The leach tanks can be built near the mining site and used as a complimentary treatment of the ore prior to the smelting process (Torma, 1978). The microbiological leaching treatment does not require high temperature nor pressure and it is easy to operate and control. This process, if properly operated does not compromise in any way the quality of the environment.

The economic importance of the extraction of metals from low-grade sulfide-bearing ores was first suggested in 1922 when Rudolf et al. reported the microbiological leaching of metal sulfides by an unidentified autotrophic bacterium. However, it was not until 1947 when Colmer and Hinkle first discovered that the microorganism, Thiobacillus ferrooxidans, isolated from a coal mine acid drainage source, was responsible for the oxidation of

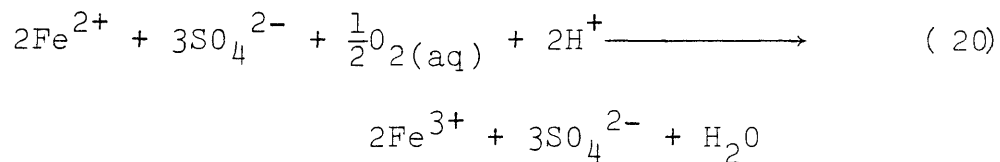
sulfide minerals. Since then the economic and scientific interest in these organisms has steadily grown and their role in the biodegradation of copper sulfide ores has been reviewed by Silverman and Ehrlich (1953), Beck (1967), and more recently by Tuovinen and Kelley (1974) and Dutrizac and MacDonald (1974), among others.

The chemolithotrophic microorganism T. ferrooxidans can be found wherever sulfide minerals occur in acidic environments. The process by which the metal is extracted from the ore is actually a form of solution mining, in which the lixiviant which dissolves and extracts the desired metal is produced by the microorganisms. All species of Thiobacillus actually obtain energy from the oxidation of sulfides or sulfur to sulfuric acid (equation 19) and one particular species, T. ferrooxidans, may obtain energy by oxidizing ferrous iron to ferric iron (equation 20). This latter process results in an acid ferric sulfate solution which acts as a lixiviant to dissolve the desired metal, according to general equation 21, where M represents any metal.

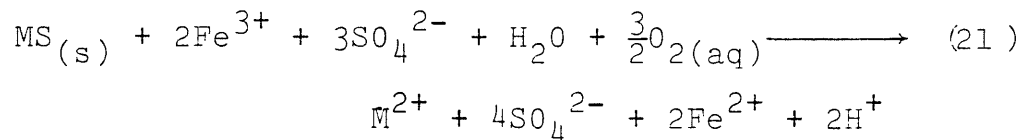
FIRST STEP:



SECOND STEP (T. ferrooxidans only):



THIRD STEP:



These microbiological catalyzed reactions have been applied commercially only to the recovery of copper and uranium by a variety of methods of solution mining, including trickle, dump, heap and vat leaching. However, other metals such as As, Mn, Zn, Mo, Pb and Ni are solubilized by this lixiviant solution (Malouf, 1972) and may thus be amenable to solution mining.

The bacteria involved in current bacterial leaching operations in acid solutions with pH less than 3.0 are the mesophilic species Thiobacillus thiooxidans and Thiobacillus ferrooxidans, and the thermophile Sulfolobus. They are chemosynthetic autotrophic aerobic microorganisms. T. thiooxidans and Sulfolobus are able to oxidize ions of sulfide, sulfur, and other reduced sulfur species to sulfate ion. T. ferrooxidans can utilize both ferrous iron and reduced sulfur compounds as energy

sources.

Lastly, pyrite oxidation plays an important role in uranium ore deposition, although just exactly what that role is has yet to be unequivocally defined. The first of two theories is the biogenic or geochemical cell model, in which bacteria are important in the development of the environment in which uranium deposits occur (Shockey et al., 1968; Rackley et al., 1968; Austin, 1970; Brutt, 1972; Rackley, 1972). There is much less agreement on the role of bacteria in the actual formation of the ore deposits. The cell, illustrated in Figure 4, is a dynamic system of advancing oxidizing fronts which cause changes in Eh, pH, mineralogy, chemistry and microorganisms (Rackley, 1976). Briefly, sulfur is redistributed via bacterially catalyzed oxidation of iron disulfide minerals producing sulfate ions which are carried with uranium-bearing groundwater into reduced (iron sulfide and/or organic carbon-bearing) rock. There, the sulfate is rereduced to aqueous sulfide which in part reacts with available non-sulfide iron to form ore stage iron disulfide minerals (Goldhaber, 1980). The process of uranium migration, oxidation, dissolution, transportation, reduction and deposition, involve both physical and chemical reactions all of which may

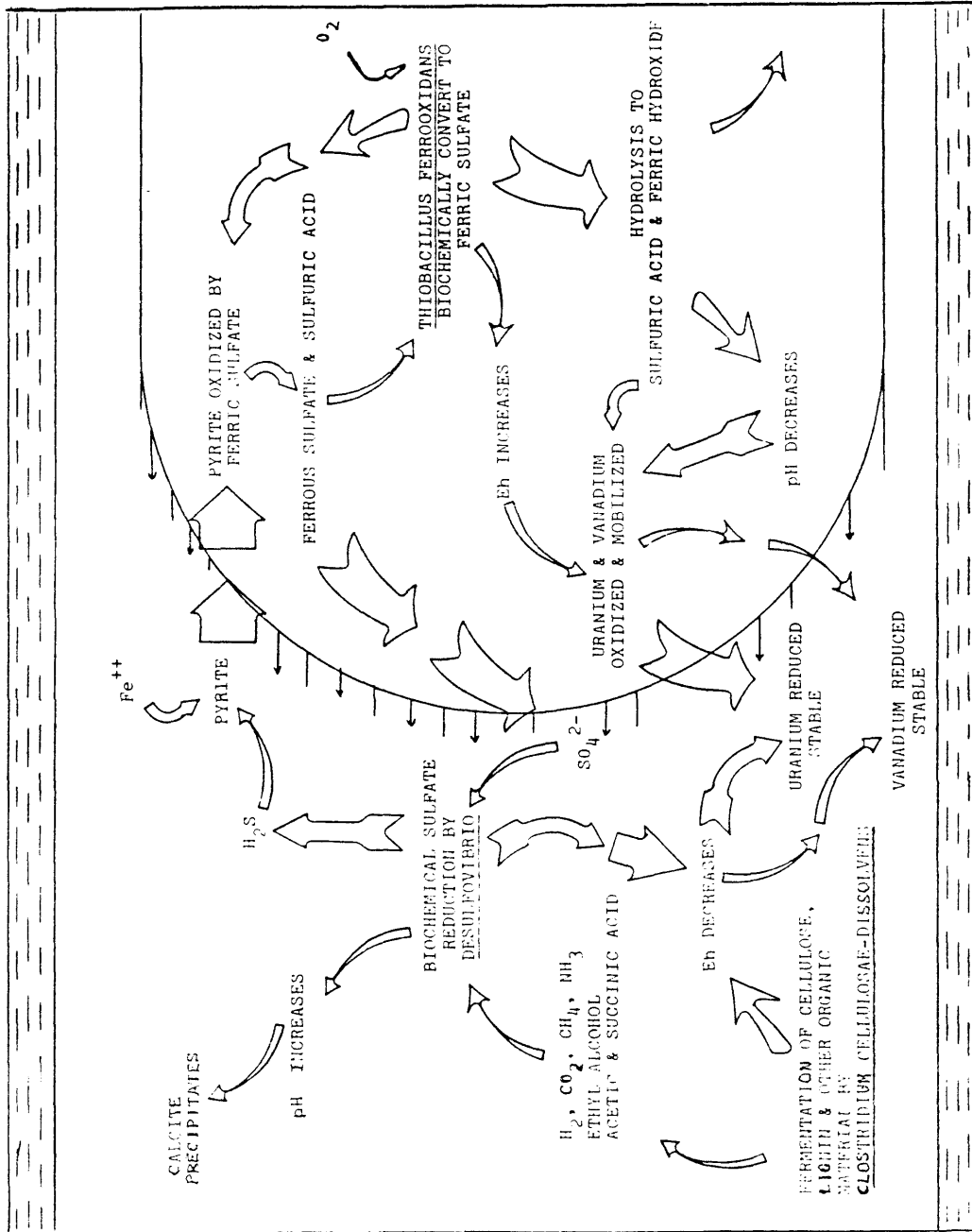


Figure 4. Probable reactions in advancing geochemical cell (Rackley, 1976).

be taking place within an overall distance of a few centimeters.

The second theory set forth by Granger and Warren (1969 and 1974) proposes an abiological chemical process to produce a reduction barrier. The proposed process involves a partial oxidation of sulfide minerals to produce unstable soluble sulfur species of intermediate oxidation state (i.e. thiosulfate, $S_2O_3^{2-}$) at the roll front. These are rapidly moved into the reducing environment, where they spontaneously undergo decomposition by disproportionation, a reaction in which part is oxidized and part is reduced. It is obvious that a thorough understanding of the mechanism of pyrite oxidation would add considerable insight into uranium ore genesis.

EXPERIMENTAL METHODS

It was the purpose of this work to study the oxidation of pyrite under both abiological and microbial conditions in the pH range of the groundwater associated with roll-type uranium deposits, namely 6.5.

All oxidation experiments were conducted under conditions of constant pH, pO_2 and temperature in a thermostated glass reaction vessel, with a magnetic stirrer

suspended from the cap and with two side ports (Figure 5). One side port was equipped with the gas-inlet bubbler and the other side port contained the inlet tube for NaOH titrant and the combination pH electrode. The pH of the sample solution in the reaction vessel was controlled by use of the pH-stat used by Goldhaber (1980). It monitored the pH to within ± 0.03 of the reported value by addition of CO₂-free* NaOH (0.025M).

The concentration of dissolved oxygen was controlled by bubbling water-saturated high purity oxygen through the sample solution. In experiments not requiring CO₂, the gas was scrubbed free of CO₂ prior to entering the solution. When CO₂ was required for the bacteria, a mixture of oxygen and CO₂ was bubbled through the solution. This gaseous mixture contained 3.6% CO₂. Since autotrophic bacteria use carbon dioxide as their sole carbon source, CO₂ was employed in all runs containing T. thioparus or the Thiobacillus-containing enrichment culture. These two cultures were used in all runs involving the bacterial oxidation of pyrite or thiosulfate. In all

* Protected from atmospheric CO₂ by CaCl₂ tubes filled with ascarite

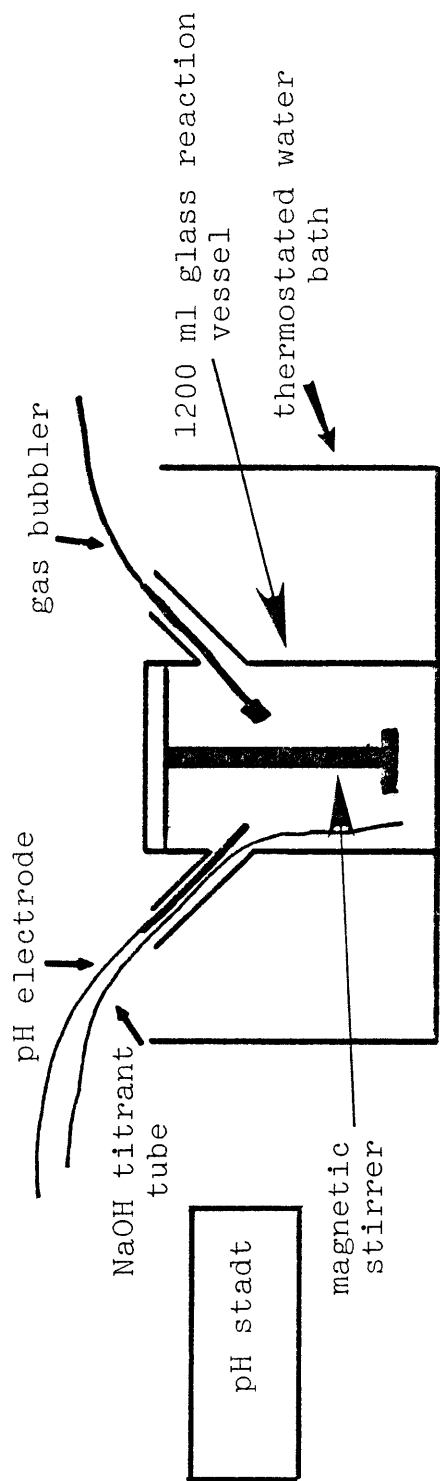


Figure 5. Schematic drawing of experimental set-up.

runs, the total gas-flow rate was 275 cm³ per minute**. When the mixture of gases was used, oxygen was monitored at 265 cm³ per minute and CO₂ at 10 cm³ per minute.

To initiate an inorganic pyrite oxidation run, approximately five grams of pyrite was added to 1200 ml of 0.1M KCl solution which had previously been thermally equilibrated to 30°C and presaturated with the appropriate gas mixture. In a run involving bacteria, Stanier medium was substituted for the KCl solution. The amount of NaOH added to maintain pH was noted as a function of time. At routine intervals a 20-ml sample aliquot was removed, filtered via a 0.45-micrometer membrane filter, and immediately analyzed for a range of aqueous sulfur constituents.

A brief summary of the methods used to determine the relative amounts of all sulfur species present is listed below. Appendix A contains a more detailed description of the analytical procedures.

<u>Sulfur Species</u>	<u>Detection Method</u>
SO ₃ ²⁻	colorimetry
S ₂ O ₃ ²⁻	colorimetry; ion chromatography
S ₄ O ₆ ²⁻	colorimetry

** This rate was determined by Goldhaber (1980) to be sufficiently rapid that oxygen concentration was not limited by mass transfer.

$S_5O_6^{2-}$	calculated from tetrathionate data (see Appendix A)
SO_4^{2-}	calculated as dif- ference between total sulfur and sum of all other sulfur species
Total Sulfur	colorimetry; ion chromatography

Thiobacillus thioparus was grown in medium 290 or in Stanier #1 or #2 medium, and was enumerated by the spread plate method on medium 290B agar (see Appendix A). All culture media were sterilized by autoclaving at 121°C at 15-20 lbs/in² for 15-20 minutes.

Four species of Thiobacillus which grow well at pH 6.5 were evaluated for this study: T. thioparus and T. neapolitanus (strict autotrophs), T. intermedius and T. novellus (mixotrophs). The autotrophs were maintained in medium 290, and the mixotrophs in medium 152. Experiments on pyrite oxidation by Thiobacillus cultures were carried out in Stanier medium #2 containing 0.4% washed ground pyrite (< 44 micrometer).

O X I D A T I O N S T U D I E SP E R F O R M E D

The rationale for the selection of these oxidation studies is presented in the results and discussion section. All experiments were monitored with O_2 at saturation unless $O_2 + CO_2(g)$ is specified.

A. Preliminary examination of the inorganic oxidation of pyrite

A-1. 0.1M KCl, pyrite, pH 6.5

A-2. 0.1M KCl, pyrite, pH 6.0

B. Examination of bacterial medium under oxidative conditions

B-1. Stanier medium alone, pH 6.5

B-2. Stanier medium, yeast extract, pH 6.5

C. Examination of oxidative effects of pyrite in bacterial medium

C-1. Stanier medium, pyrite, pH 6.0

C-2. Stanier medium, pyrite, $O_2 + CO_2(g)$, pH 6.5 (autoclaved control for pyrite oxidation studies)

D. Examination of thiosulfate oxidation in bacterial medium

D-1. Stanier medium, $S_2O_3^{2-}$, $O_2 + CO_2(g)$, pH 6.5 (autoclaved control for thio-

sulfate oxidation studies)

E. Examination of pyrite oxidation in bacterial medium inoculated with bacteria

E-1. Stanier medium, pyrite, $O_2 + CO_2(g)$,
pH 6.5, enrichment culture

E-2. Stanier medium, pyrite, $O_2 + CO_2(g)$,
pH 6.5, T. thioparus

F. Examination of thiosulfate ($S_2O_3^{2-}$) oxidation in bacterial medium inoculated with bacteria

F-1. Stanier medium, $S_2O_3^{2-}$, $O_2 + CO_2(g)$,
pH 6.5, enrichment culture

F-2. Stanier medium, $S_2O_3^{2-}$, $O_2 + CO_2(g)$,
pH 6.5, T. thioparus

These studies are listed in Table 5.

G. Hydrogen Ion Production

H. Iron Studies

I. Microbiological Cultures

Run	Aqueous medium	pH	Pyrite added?	Bacterial inoculation	Added constituents
A-1	0.1M KCl	6.5	yes	none	none
A-2	0.1M KCl	6.0	yes	none	none
B-1	Stanier medium	6.5	no	none	none
B-2	Stanier medium	6.5	no	none	yeast
C-1	Stanier medium	6.0	yes	none	none
C-2	Stanier medium	6.5	yes	none	$O_2 + CO_2(g)$
D-1	Stanier medium	6.5	no	none	$O_2 + CO_2(g), S_2O_3^{2-}$
E-1	Stanier medium	6.5	yes	<u>T. thioparus</u>	$O_2 + CO_2(g)$
E-2	Stanier medium	6.5	yes	enrichment culture	$O_2 + CO_2(g)$
F-1	Stanier medium	6.5	no	enrichment culture	$O_2 + CO_2(g), S_2O_3^{2-}$
F-2	Stanier medium	6.5	no	<u>T. thioparus</u>	$O_2 + CO_2(g), S_2O_3^{2-}$

Table 5. Summary of characteristics for each oxidation study performed (O_2 at saturation unless specified).

R E S U L T S A N D
D I S C U S S I O N

Oxidation Studies in Terms of Metastable Sulfur Species
and Hydrogen Ion Generation

A. Preliminary Examination of the Inorganic Oxidation
of Pyrite

A-1. Pyrite in 0.1M KCl Solution, pH 6.5 and O₂
at Saturation.

The results are shown in Figure 6 and in
Appendix B, Table B-1.

A-2. Pyrite in 0.1M KCl Solution, pH 6.0 and O₂
at Saturation.

The results are shown in Figure 7 and in
Appendix B, Table B-2.

These two runs were performed to 1) ensure that the experimental and analytical techniques utilized were satisfactory by comparing the results to those obtained by Goldhaber (1980) under similar experimental conditions, and 2) provide a baseline study with which to compare oxidation studies at similar pH's but substituting a bacterial medium for the KCl solution. The pyrite used in the run at pH 6.5 was from a different batch than the

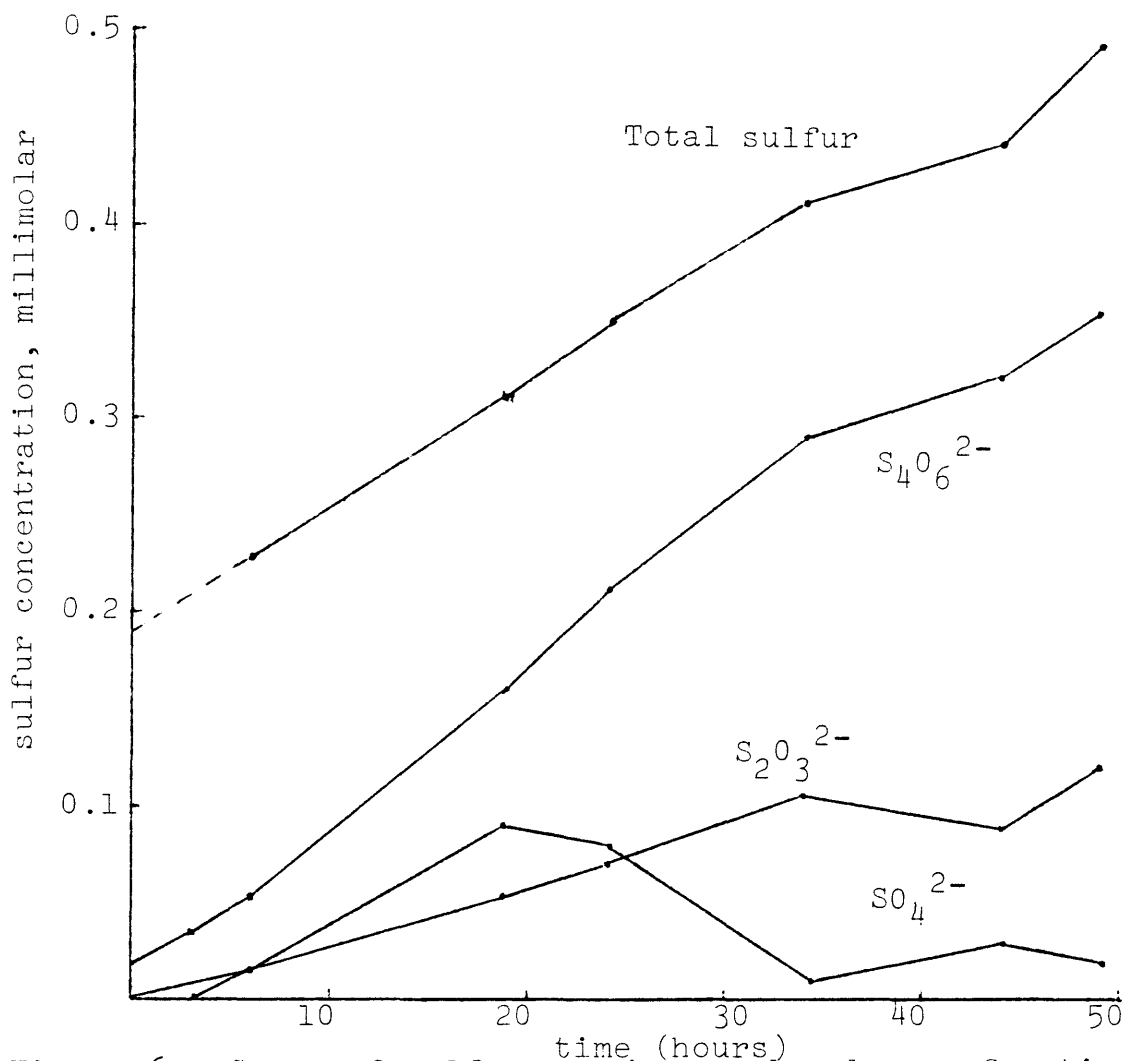


Figure 6. Conc. of sulfur species produced as a function of time in 0.1M KCl during pyrite oxidation at pH 6.5, 30°C, O_2 saturation.

pyrite used in the pH 6.0 experiment, although both batches were sized and washed as described in Appendix A. However, the surface area available for oxidation differs with each batch, so the rate data will not be directly comparable.

The rates of production of soluble metastable sulfur

oxyanions during pyrite oxidation at pH 6.5 shown in Figure 6, are similar to those obtained by Goldhaber(1980) except this experiment indicates the presence of thio-sulfate, which Goldhabers' work reveals only in solutions from pH 7 to 9. This presence of a relatively small amount of thiosulfate is reflected in a proportionately lower percentage of sulfate present in solution, as sulfate is calculated as the difference between the sum of the analyzed constituents and total sulfur. This discrepancy between experiments utilizing similar con-

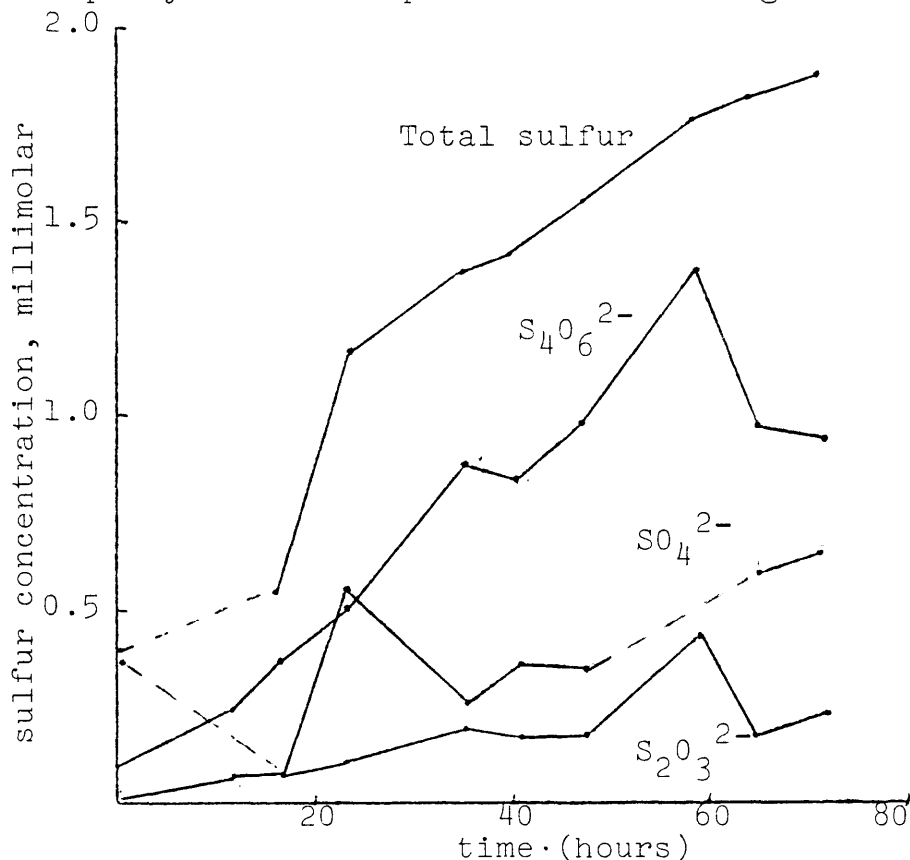


Figure 7. Concentration of sulfur species produced as a function of time in 0.1M KCl during pyrite oxidation at pH 6.0, 30°C, O₂ saturation.

ditions is most likely a result of different methods used to calculate the amount of thiosulfate (Appendix A).

The important results from these two runs are:

1. Tetrathionate is the major sulfur species of total-S in solution (44-76%)
2. Thiosulfate is not a predominant sulfur species (7-26%)
3. Sulfite is relatively minor (<2%)
4. Sulfate is relatively abundant at pH 6.0 (18-48%)

A systematic trend towards an increasing proportion of tetrathionate with time is seen at pH 6.5. Comparison with Goldhabers' results at pH 6.0 was difficult because of the difference in length of run - 10 hours (Goldhaber) versus 71 hours in Figure 7.

B. Examination of a Bacterial Medium Under Oxidative Conditions

B-1. Stanier Medium, pH 6.5.

The results are shown in Figure 8 and in Appendix B, Table B-3.

B-2. Stanier Medium with Yeast Extract, pH 6.5.

The results are shown in Figure 9 and in Appendix B, Table B-4.

The purpose of these two runs was threefold:

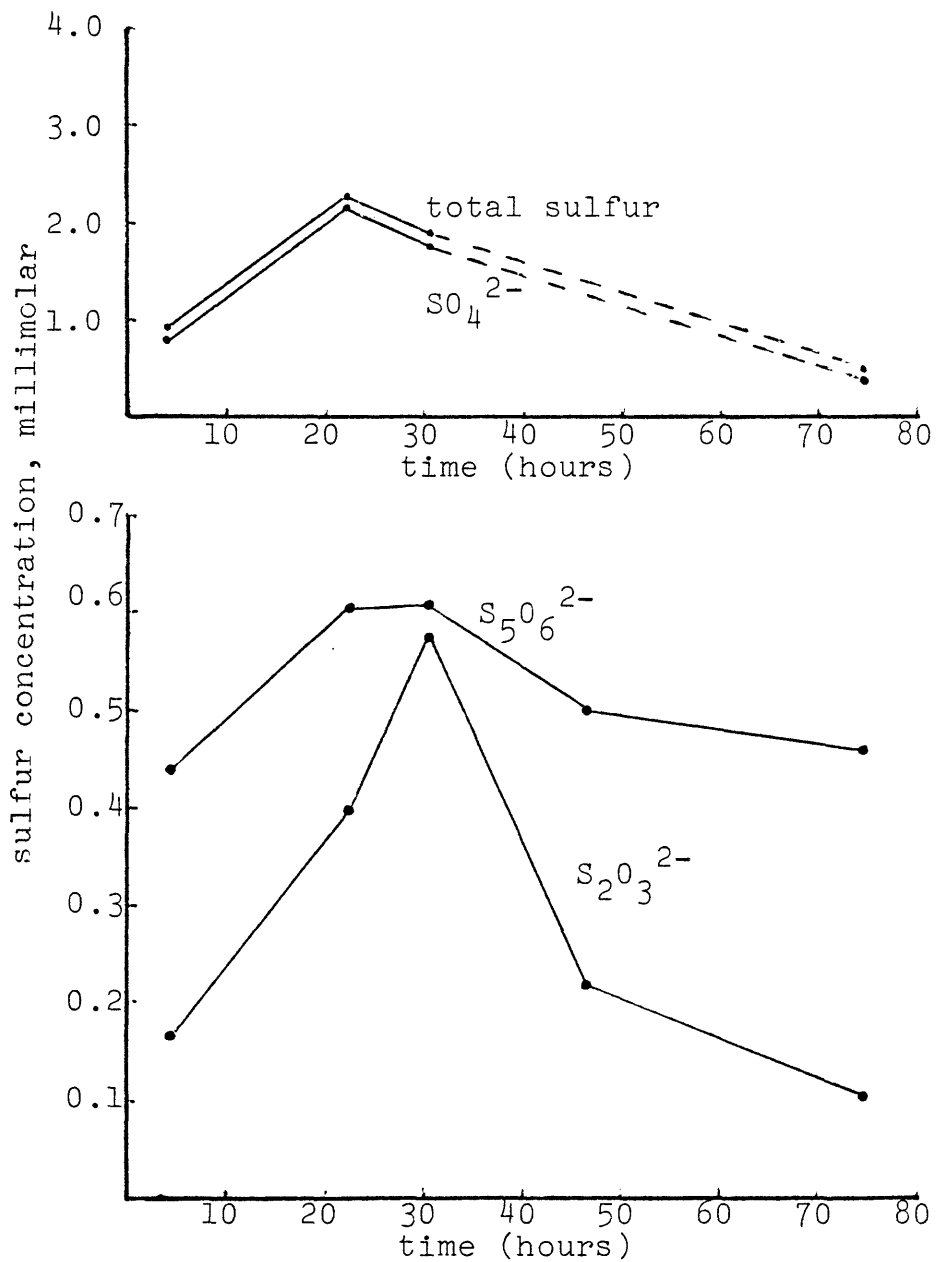


Figure 8. Concentration of sulfur species produced as a function of time in Stanier medium #1 at pH 6.5, 30°C, O_2 saturation.

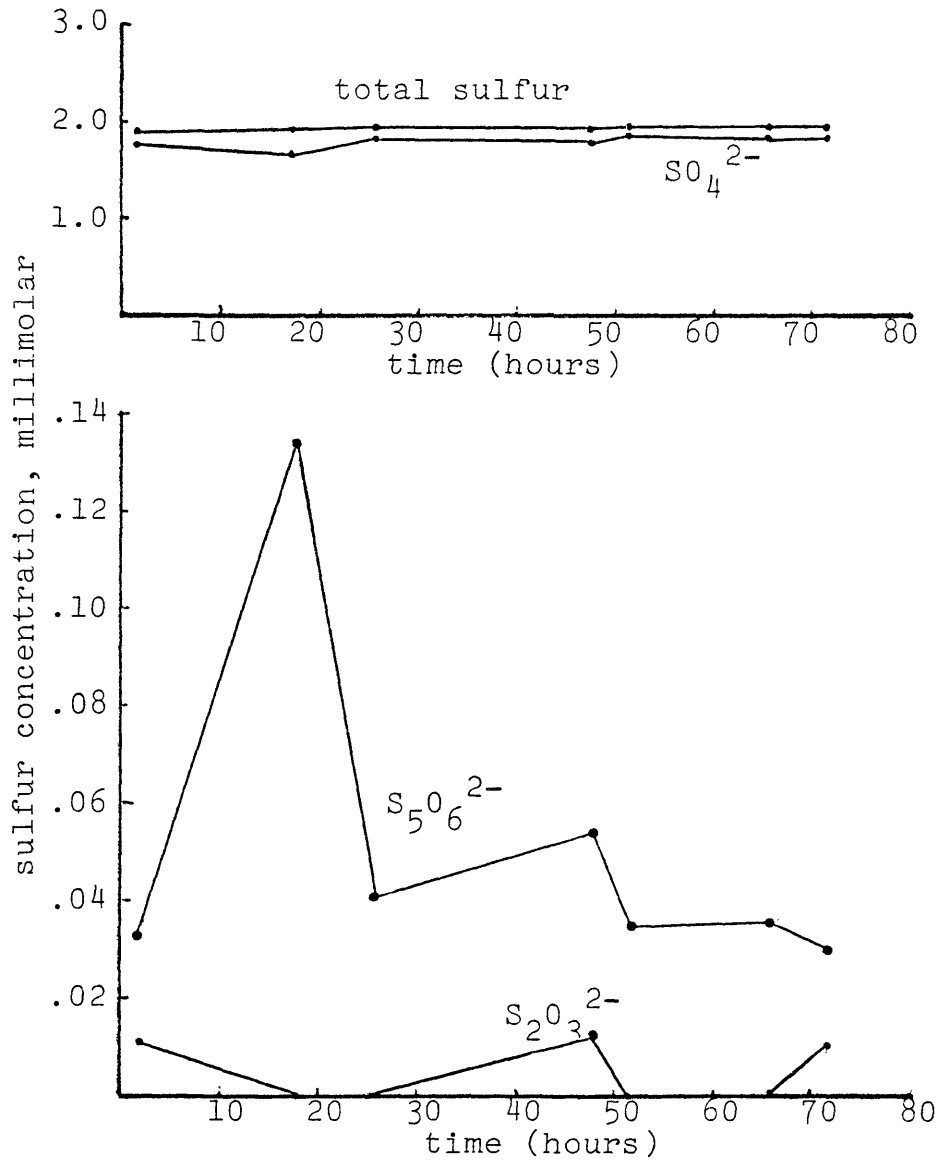


Figure 9. Concentration of sulfur species produced as a function of time in Stanier medium #1 at pH 6.5, 30°C, O₂ saturation with yeast extract

1. To ensure analytical techniques were appropriate for a bacterial medium containing sulfate,
2. To obtain baseline data on the medium alone before pyrite was added, and
3. To determine what effects yeast extract (a source of carbon and vitamins for certain bacteria) would have on the oxidation process.

There are three major differences between the oxidation results of the simple KCl matrix and the more complex Stanier medium. First, the relationships in Figures 8 and 9 indicate sulfate is the dominant sulfur species present in Stanier medium #1. This was a predicted result as the medium alone contains 0.2g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter. Apparently any polythionates present as trace amounts in solution were rapidly oxidized to sulfate.

Second, it is interesting to note that pentathionate ($\text{S}_5\text{O}_6^{2-}$) is the dominant polythionate in the Stanier medium whereas tetrathionate ($\text{S}_4\text{O}_6^{2-}$) is dominant in the KCl matrix (further discussed in Appendix C).

The third and most significant difference is the abundance of the dominant polythionate relative to other forms of sulfur in solution is considerably different in the two mediums. Figs. 8 and 9 show that the abundance of pentathionate, as well as thiosulfate,

decline after approximately 75 hours. This contrasts with the results shown in Figures 6 and 7, and could indicate a catalytic effect in the complex media in which the polythionates were converted to some other product; probably sulfate (Goldhaber, 1980).

It is difficult to determine if this decrease in polythionates is balanced quantitatively by an increase in sulfate because sulfate was not measured analytically, but was calculated as a difference (see Appendix A). Consequently, as the amount of measured polythionate decreases, the amount of sulfate calculated will automatically increase, but by an amount within the uncertainty of the analytical techniques.

The amount of total sulfur should theoretically remain constant, as no pyrite was present to be oxidized. Hence, the decrease in total sulfur shown in Figure 8 could indicate sulfate being obscured from detection by the ion chromatography technique, the sample not being completely oxidized before the analysis, or that some unknown, undetected insoluble sulfur compound was produced.

C. Examination of the Oxidative Effects of Pyrite in a Bacterial Medium

C-1. Stanier Medium, Pyrite, pH 6.0.

The results are shown in Figure 10 and in Appendix B, Table B-5.

This experiment was conducted with the intention of completing more runs at pH 6.0 to compare with the runs at pH 6.5. Due to time constraints, no further oxidation experiments were monitored at pH 6.0. The amount of polythionate sulfur relative to total sulfur decreases after reaching a maximum value. This is similar to the results shown in Figure 8 for a bacterial media run monitored at pH 6.5.

In contrast to the results shown in Figures 8 and 10, the amount of polythionate sulfur relative to other forms of sulfur increases in the KCl solution (Figures 6 and 7). Consequently, run C-1 is useful as another example of the hypothesized catalytic effect appearing in the complex media in which the polythionate sulfur is converted presumably to sulfate.

In addition, this run illustrates that the amount of thiosulfate present is minor relative to that of pentathionate and sulfate as a percentage of the total-S in solution, in agreement with Goldhabers' work with KCl.

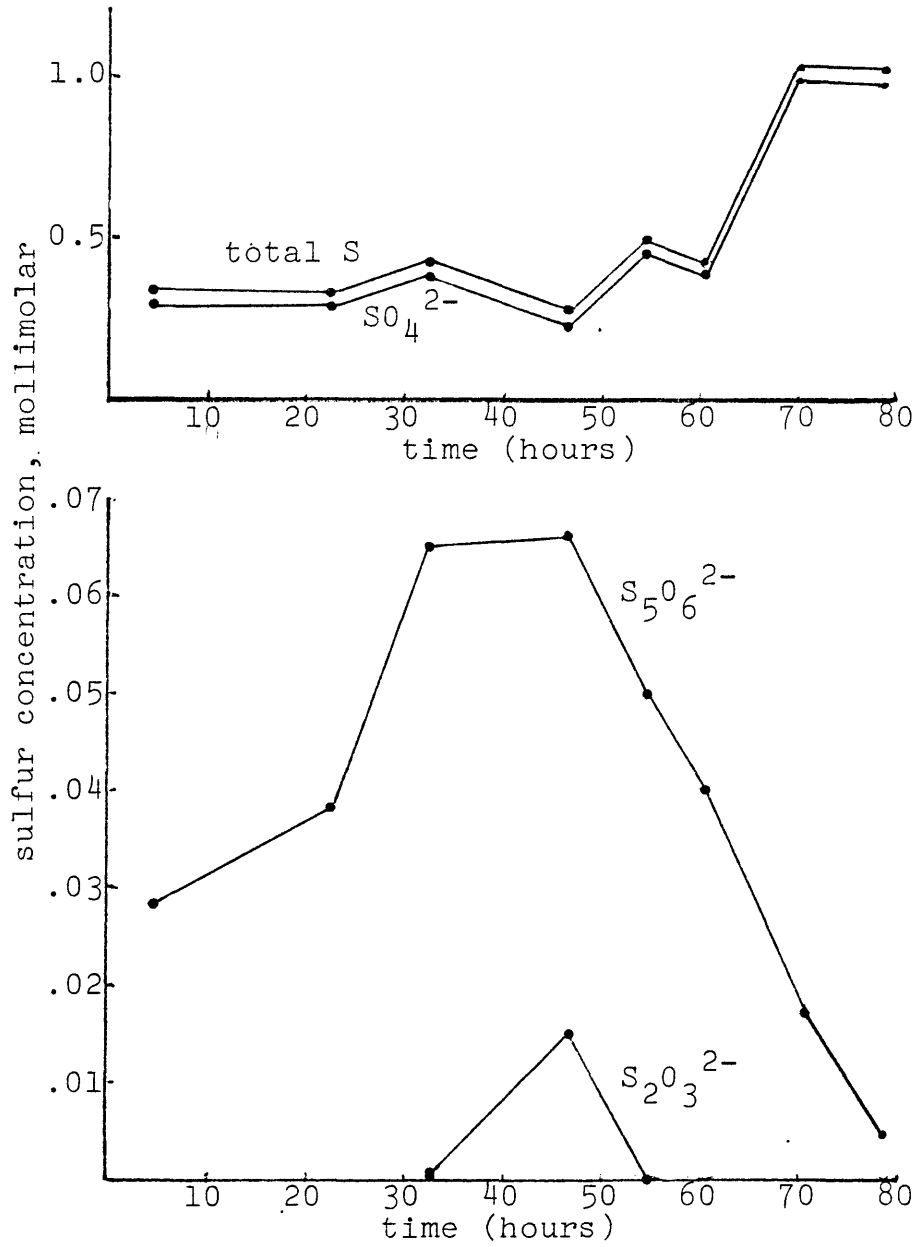


Figure 10. Concentration of sulfur species produced as a function of time in Stanier medium #1 at pH 6.0, 30°C, 0₂ saturation.

C-2. Stanier Medium, Pyrite, CO₂(g), pH 6.5 (CONTROL).

The results are shown in Figure 11 and in Appendix B, Table B-6 .

Figure 11 presents the data used as a control for the set of experiments using bacterial medium and an O₂/CO₂ mixture (Figures 12, 14, 15, 16, 17). The pyrite (washed and sized as discussed in Appendix A) was placed under a nitrogen atmosphere (to prevent air oxidation), weighed and subsequently autoclaved. The reaction vessel, lid, magnetic stirrer and bacterial medium were also autoclaved.

After each sample was taken during the experiment, it was analyzed for S₂O₃²⁻ using the ion chromatograph in addition to the usual chemical procedure (Appendix A). The purpose of this was to verify the precision of the chemical procedure. The results from the chemical procedure were used in Figure 11 so that meaningful comparisons could be made with the experiments in which the chemical procedure was used exclusively.

The amount of total sulfur remains relatively constant during the 90 hour period with the exception of the initial data point. The large amount of total sulfur (and hence, sulfate) detected after 1½ hours is most likely an experimental artifact, because sulfate

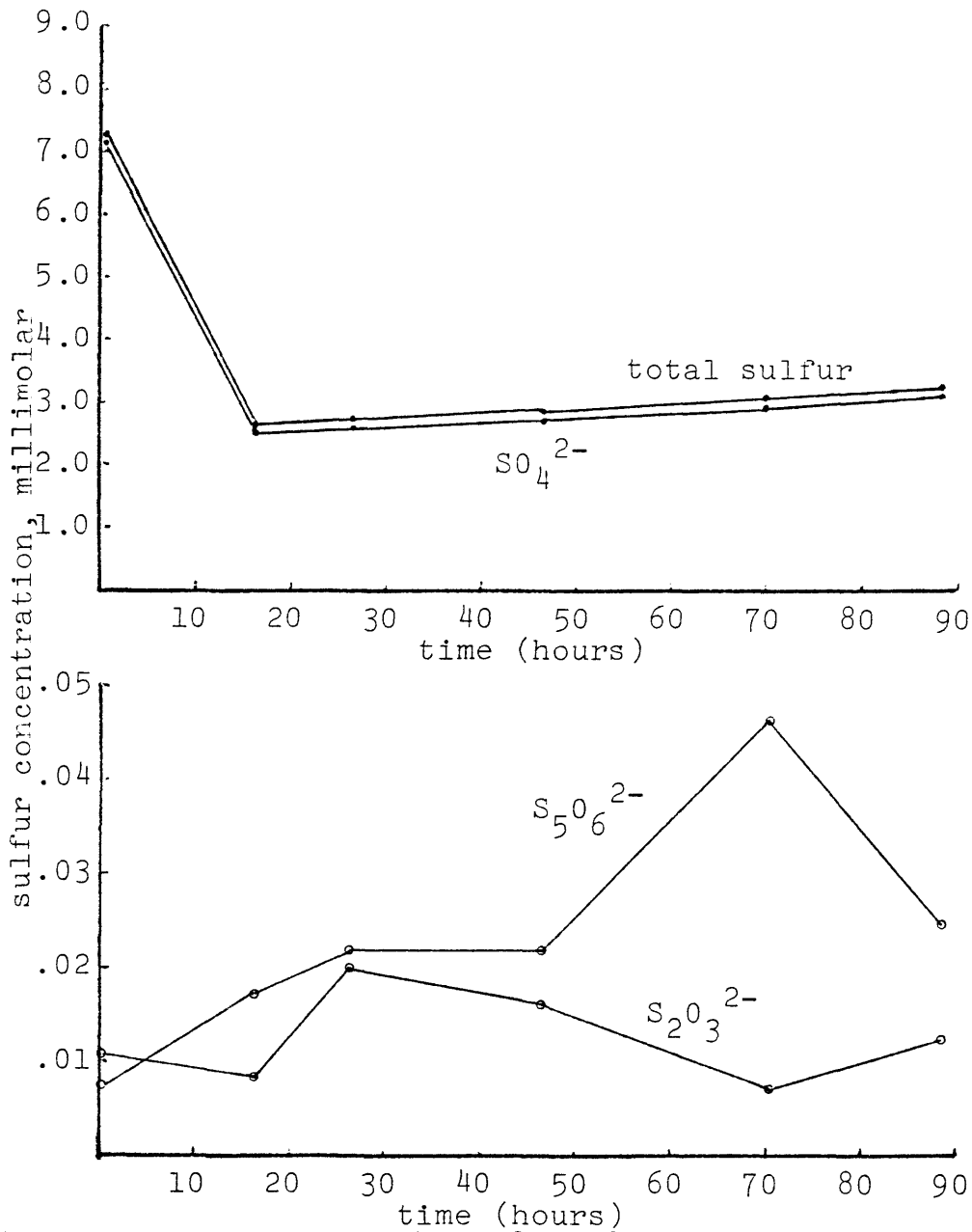


Figure 11. Concentration of sulfur species produced as a function of time in Stanier medium #2 at pH 6.5 during pyrite oxidation, 30°C, with O₂/CO₂ mixture.

would not disappear from solution (Goldhaber, 1980). This anomalous initial concentration was observed in other experiments during this project as well as several done by Goldhaber. These concentrations have been hypothesized to be a result of an initial "pre-oxidation" which occurs during handling of the pyrite in air (Steger and Desjardins, 1978; Goldhaber, 1980). In addition, Goldhaber noted an increase in the intensity of this anomalous concentration when the pyrite was not carefully cleaned and stored.

D. Examination of Thiosulfate ($S_2O_3^{2-}$) Oxidation in a Bacterial Medium

D-1. Stanier Medium, Thiosulfate ($S_2O_3^{2-}$), pH 6.5.

The results are shown in Figure 12 and in Appendix B, Table B-7.

The purpose of this experimental run was to obtain data on the oxidation of thiosulfate in the bacterial medium in order to compare the results to those involving pyrite oxidation in the same medium. This would help determine if the bacteria were actually attacking the pyrite itself, or utilizing soluble oxidation products of inorganic pyrite oxidation, i.e. the thiosulfate, as their energy source.

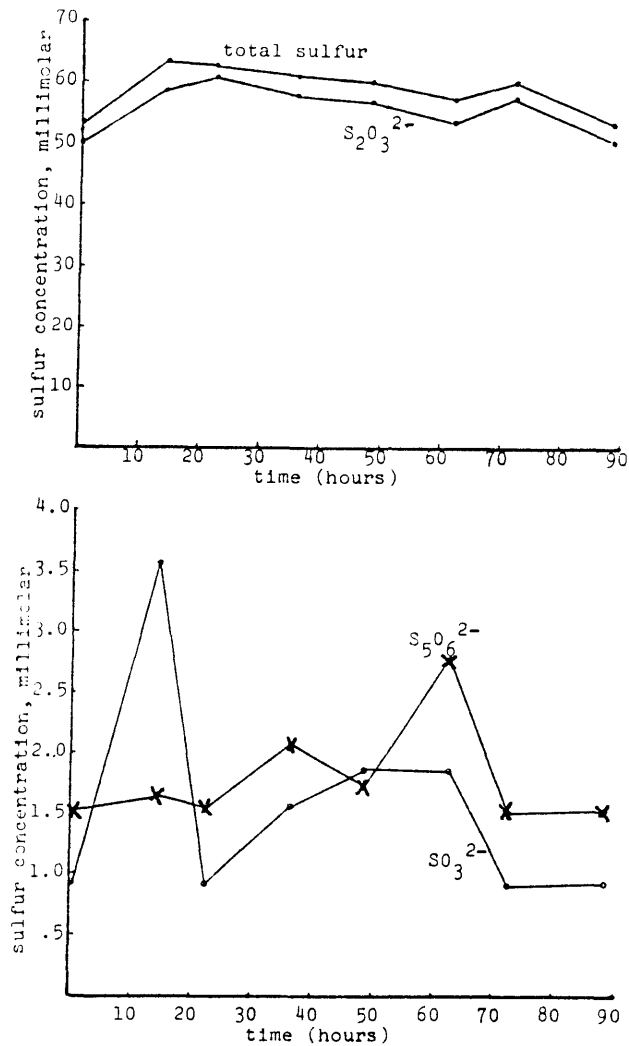


Figure 12. Concentration of sulfur species produced as a function of time in Stanier medium #2 during thio-sulfate oxidation at pH 6.5, 30°C, with O_2/CO_2 mixture.

Bergeys' manual (1957) suggests a 1% solution of thiosulfate as an appropriate energy source for Thio-bacillus sp.. However, the pH proved difficult to stabilize and the suggested concentration necessitated

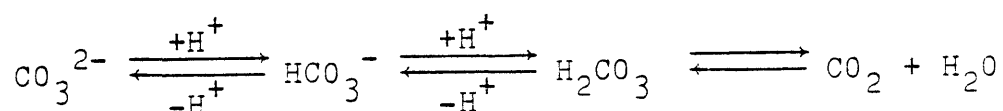
a time-consuming dilution of the samples before analysis could proceed. This was subsequently reduced to a 0.35% thiosulfate solution, which still provided an adequate energy source for the bacteria as evidenced by viable bacterial counts discussed in Section I.

The amount of total sulfur present in each sample was measured by the ion chromatograph but produced erratic results (see Appendix A for discussion), and hence no total-S or SO_4^{2-} -S values, are given. Summation of the amounts of sulfur present as SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$ and $\text{S}_5\text{O}_6^{2-}$ for each sample would result in a minimum value (no SO_4^{2-} value available) representing the total amount of sulfur present in the solution. The significance of these results are discussed and compared with results in Section F.

E. Examination of Pyrite Oxidation in a Bacterial Medium Inoculated With Bacteria

One aspect of bacterial cultivation is the provision of gaseous reactants such as CO_2 and molecular oxygen. The concentration of CO_2 in the atmosphere is only 0.03% and, while not actually prohibiting growth, it is quite slow unless artificially enriched with CO_2 .

The soluble carbonates, such as Na_2CO_3 and NaHCO_3 , cannot be used in media exposed to air, since they lose CO_2 to the atmosphere, causing the medium to become very alkaline (Stanier, 1963). This sequence of reactions, all of which are freely reversible, can be summarized as follows:



In contrast to the soluble carbonates, insoluble carbonates, such as CaCO_3 and MgCO_3 , can be very useful under certain experimental conditions, providing a CO_2 source while preventing the accumulation of acid.

CaCO_3 is the carbonate generally used for this purpose and hence was the one selected for the first experimental run. A pyrite oxidation experiment was run in Stanier medium with an enrichment culture at pH 6.5. A 0.5% CaCO_3 suspension was used, suggested in Stanier (1963, pg. 456). Calcium carbonate is insoluble and does not create strongly alkaline conditions in the medium. When acid is produced by the oxidation of the pyrite, the carbonate is decomposed with the evolution of CO_2 gas. It thus acts as a neutralizing agent for

any acids that may appear in solution by converting them to their calcium salts (Stanier, 1963). A problem was encountered in the monitoring of this experiment at the desired pH value of 6.5. For the first half (approximately 40 hours) of this experiment the pH of the medium slowly drifted upward and dilute HCl had to be added at routine intervals to maintain the desired pH. It appeared to stabilize after 40 hours at about pH 6.6.

As a result of the problem discussed above, all subsequent experimental runs were conducted using a gaseous source of CO_2 , pumped into the reaction vessel in the same manner as the aeration technique described earlier. The trap which was used to scrub CO_2 from the oxygen tank for the KCl experiments was replaced with distilled water only, to saturate the oxygen. The O_2 and CO_2 gas-flow rates were fixed at 265 and 10 cm^3 per minute, respectively.

E-1. Stanier Medium, Pyrite, O_2/CO_2 Mixture,
pH 6.5, Enrichment Culture.

E-2. Stanier Medium, Pyrite, O_2/CO_2 Mixture,
pH 6.5, *T. thioparus*.

The results are shown in Figures 13 and 14 and in Appendix B, Tables B-8 and B-9, respectively.

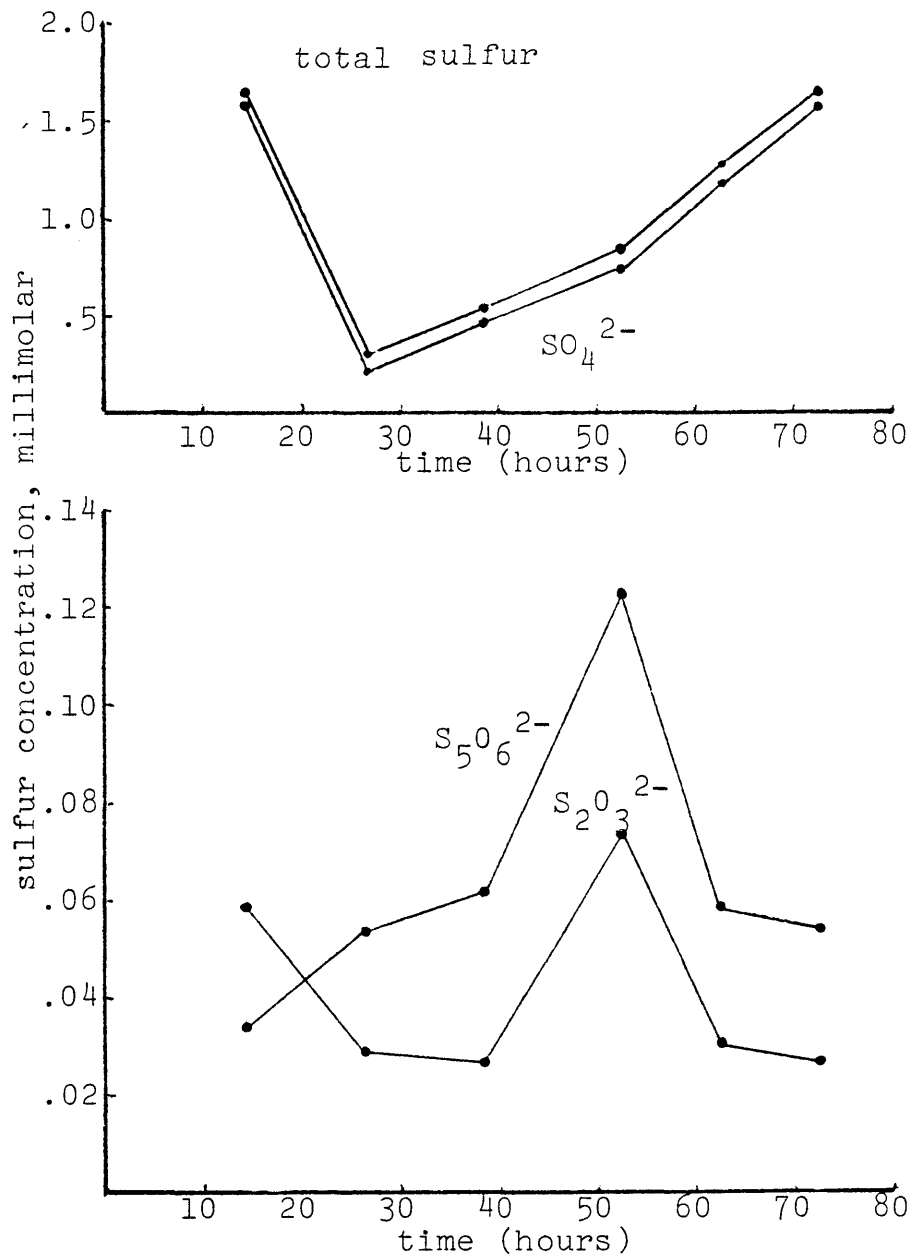


Figure 13. Concentration of sulfur species produced as a function of time in Stanier medium #2 during pyrite oxidation with enrichment culture, pH 6.5 and O₂/CO₂ mixture, 30°C.

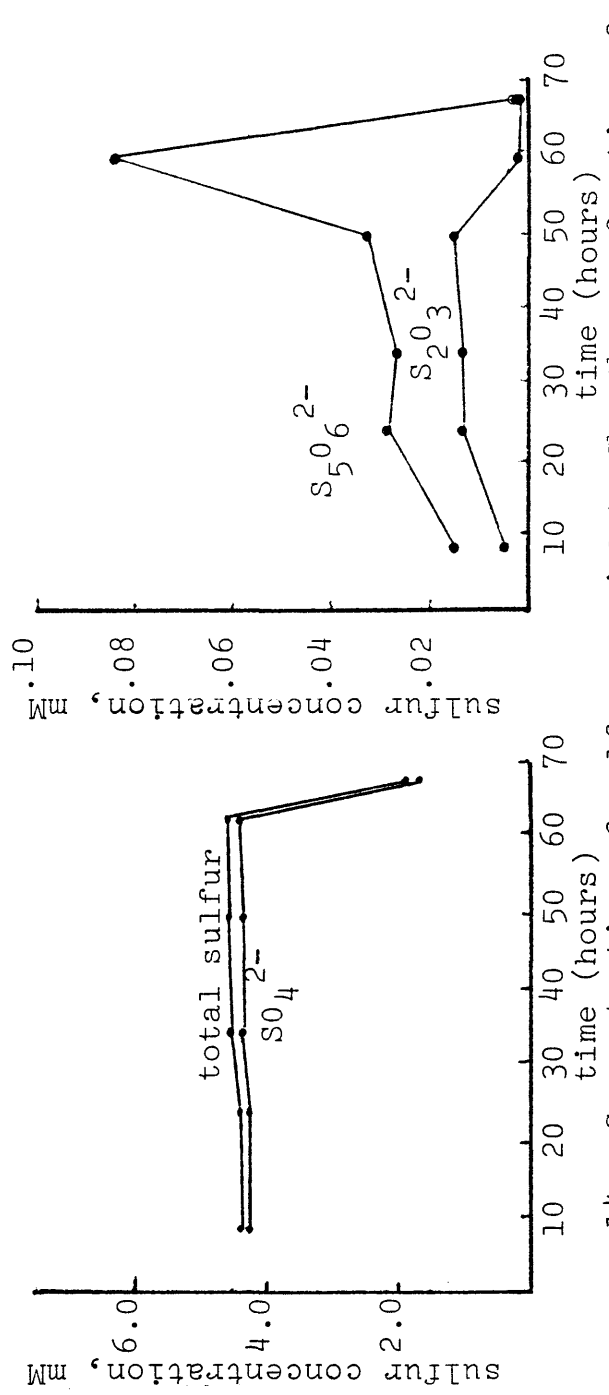


Figure 14. Concentration of sulfur species produced as a function of time in Stanier medium #2 during pyrite oxidation with O_2/CO_2 mixture, T. thioparus, pH 6.5, $30^\circ C$.

The purpose of these two experiments was to determine what effect T. thioparus and an enrichment culture would have on the rates of production of metastable sulfur species and hydrogen ion during pyrite oxidation. Three important points resulted from these particular runs.

First, a comparison of Figures 14 and 11 (the control) reveals that T. thioparus has a definite influence on the rate of sulfur species production under this particular set of experimental conditions. This is an extremely important point as the effect of bacteria on pyrite oxidation is the major focus of this research project. The run inoculated with T. thioparus developed a greater pentathionate concentration than the control run over the same time interval. In addition, the amount of sulfate present in the inoculated run was larger throughout the experiment than the amount present in the control run. This result indicates the presence of T. thioparus influences the conversion of pyritic sulfur to sulfate. This hypothesis is further supported by acid production data presented in Section G.

Second, addition of an enrichment culture increases the rate of total sulfur and sulfate production over that of the control run (7×10^{-3} mM/hr for the control

run versus 3×10^{-2} mM/hr for the enrichment culture run). This is readily apparent by comparing Figure 13 with the control run (Figure 11).

It is also interesting to note the amount of sulfur detected as intermediate sulfur species relative to total-S was greater in the enrichment culture run (5-32%) than in the control or the T. thioparus run (approximately 1%). This would indicate that T. thioparus had a greater influence than the enrichment culture on the conversion of intermediate sulfur species to sulfate.

The third important result of these two experiments requires a comparison with the KCl experiments. The amount of sulfur detected as intermediate sulfur species relative to total-S was considerably greater in the KCl experiment illustrated in Figure 6 (32-97%) than either bacterial run. It appears the intermediate sulfur species were more quickly oxidized to sulfate in the sterile Stanier medium and even more so with the addition of either T. thioparus or an enrichment culture.

- F. Examination of Thiosulfate ($S_2O_3^{2-}$) Oxidation in a Bacterial Medium Inoculated With Bacteria
- F-1. Stanier Medium, pH 6.5, $S_2O_3^{2-}$, O_2/CO_2 Mixture, Enrichment Culture.
- F-2. Stanier Medium, pH 6.5, $S_2O_3^{2-}$, O_2/CO_2 Mixture, T. thioparus.

The results are shown in Figures 15 and 16 and in Appendix B, Tables B-10 and B-11, respectively.

Thiosulfate is, by virtue of experimental design, the major component of the total sulfur present in solution in both experiments. In F-1, the values obtained for total sulfur using the ion chromatographic technique were consistently smaller than the thiosulfate numbers and hence the total sulfur values are not reported. There does not appear to be any major difference between these two runs and the one which maintained the same experimental conditions with bacteria (Figure 12). Pentathionate ($S_5O_6^{2-}$) sulfur represented approximately 3-4% of the total-S in all three runs. The experiment without bacteria and the one with enrichment culture (Figure 15) show sulfite

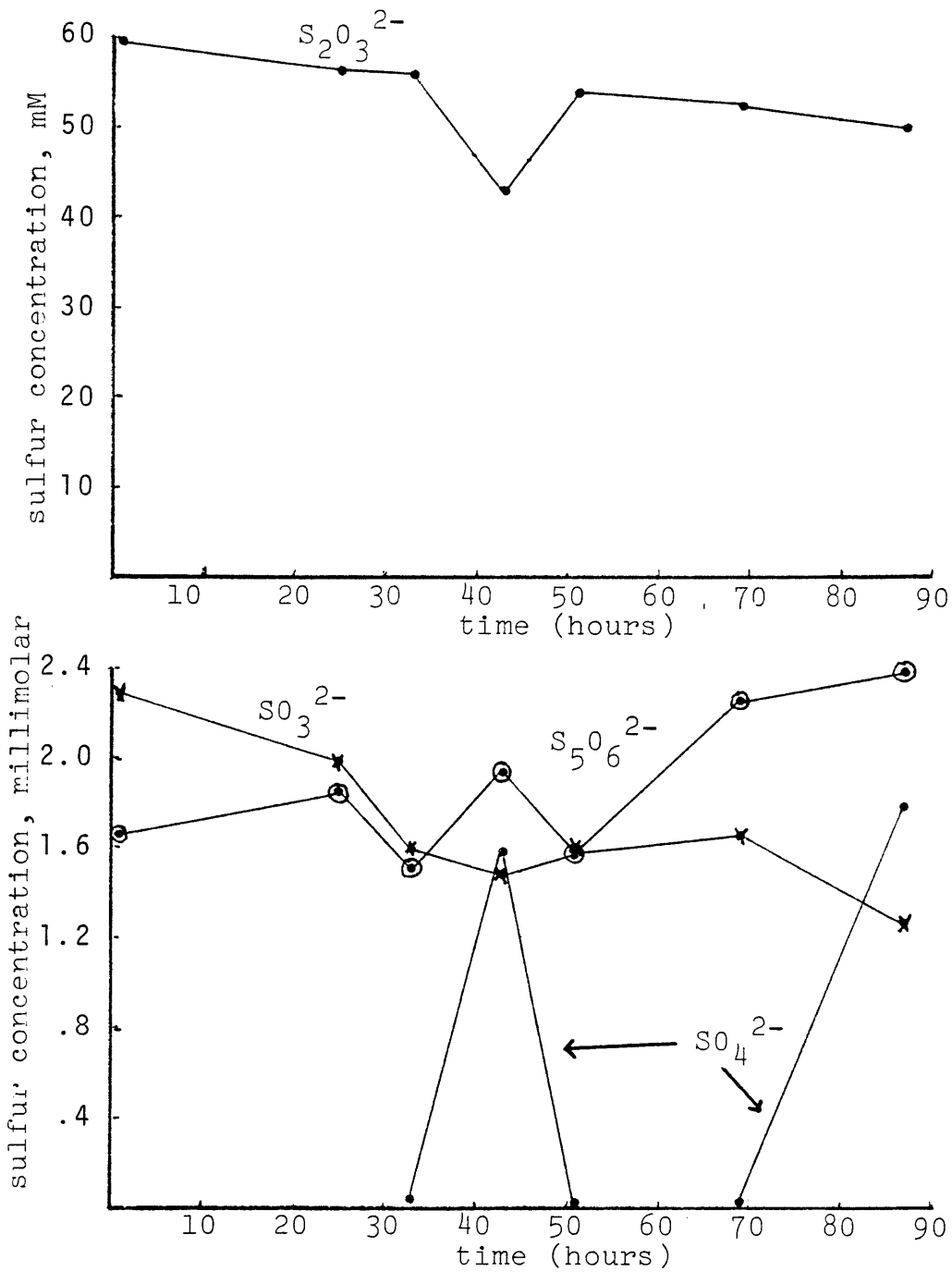


Figure 15. Concentration of sulfur species produced as a function of time in Stanier medium #2 during thiosulfate oxidation with enrichment culture, O_2/CO_2 mixture at pH 6.5, $30^\circ C$.

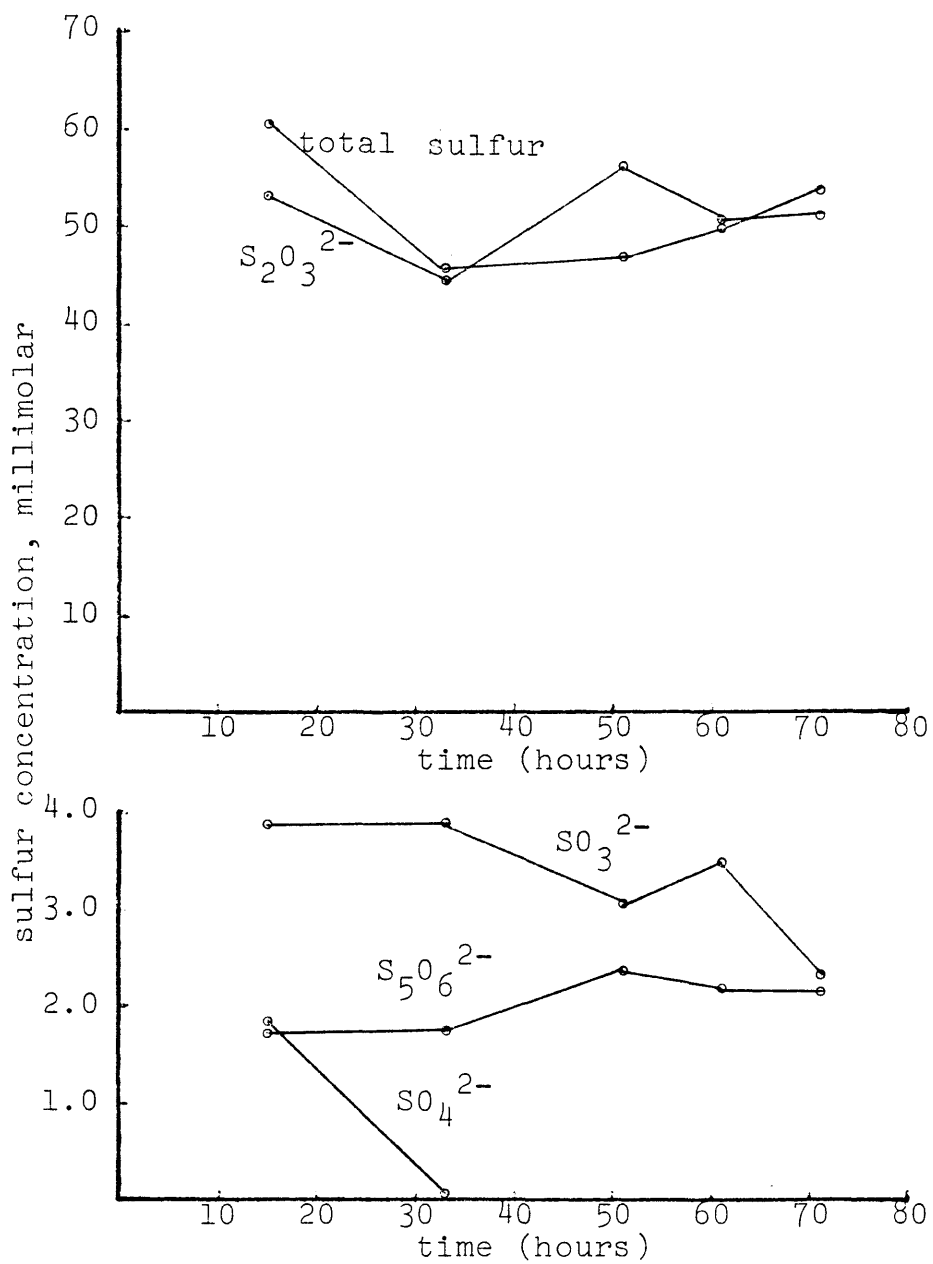


Figure 16. Concentration of sulfur species produced as a function of time in Stanier medium #2 during thiosulfate oxidation with *T. thioparus*, O_2/CO_2 mixture, pH 6.5, $30^\circ C$.

(SO_3^{2-}) sulfur representing 2-3% of the total-S, while the experiment with T. thioparus (Figure 16) show SO_3^{2-} to be about 6-8% of the total-S. No other significant differences were observed between runs with bacteria, and the run without bacteria.

However, there is a significant difference between the bacterial runs with thiosulfate as an energy source, and the bacterial runs with pyrite as an energy source, that being the rates of oxidation. Pentathionate and thiosulfate increase dramatically in the pyrite runs as illustrated in Figures 13 and 14. However, Figures 15 and 16 do not show any definitive increase or decrease in any of the intermediate sulfur species except sulfite, which decreases slightly over time.

G. Hydrogen Ion Production Using a Bacterial Medium

In addition to the production of metastable sulfur species, hydrogen ion production is an important factor during pyrite oxidation (as discussed in the introduction). The rate of hydrogen ion production was measured experimentally by the NaOH consumption rate, which is the slope of the NaOH versus time curves

(Figure 17). The relationship between hydrogen ion production (NaOH consumption) and rate of pyrite oxidation is dependent upon particular sulfur oxidation product (Goldhaber, 1980). However, as the purpose of this research was solely to determine if bacteria had an effect on pyrite oxidation, a change in NaOH consumption upon inoculation with bacteria would suggest that 1) the bacteria were viable and 2) they affected the rate of hydrogen ion production.

G-1. Pyrite Oxidation in Stanier Medium #2 Inoculated With Bacteria

The results are shown in Figures 17 and 18 and Appendix B, Tables B- 8 and B- 9.

Figure 17 illustrates the marked difference in hydrogen ion production between the control run and the inoculated runs. The experiment utilizing T. thioparus consumed the most NaOH at the fastest rate, followed by the run inoculated with enrichment culture, with the control run showing the slowest rate of NaOH consumption. The rate of NaOH addition expressed as milliequivalents per gram of pyrite per hour is shown for all three runs in Figure 18.

It is important to note that these differences

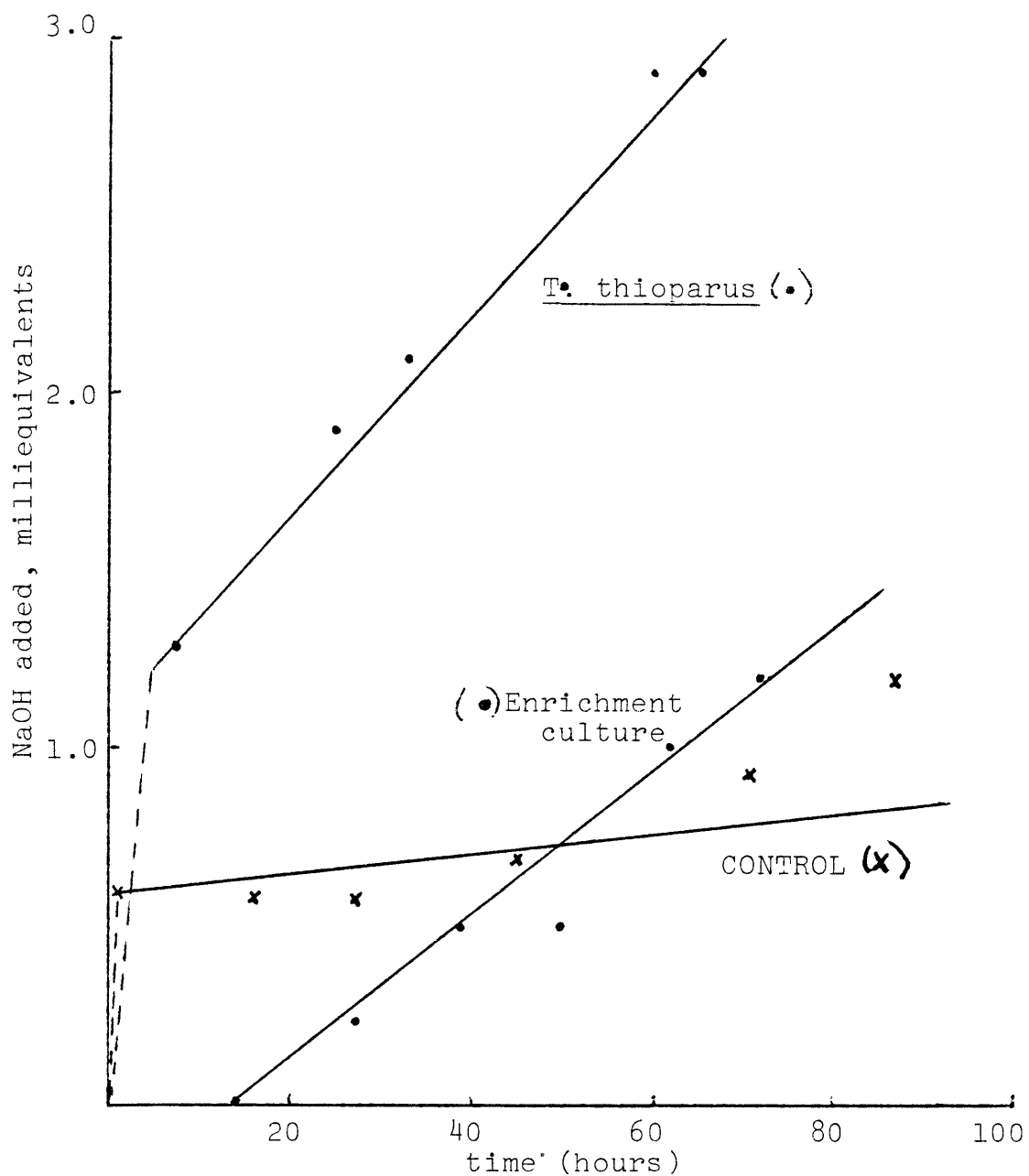


Figure 17. NaOH consumption as a function of time during pyrite oxidation in Stanier medium #2 at pH 6.5 with O_2/CO_2 mixture at $30^\circ C$.

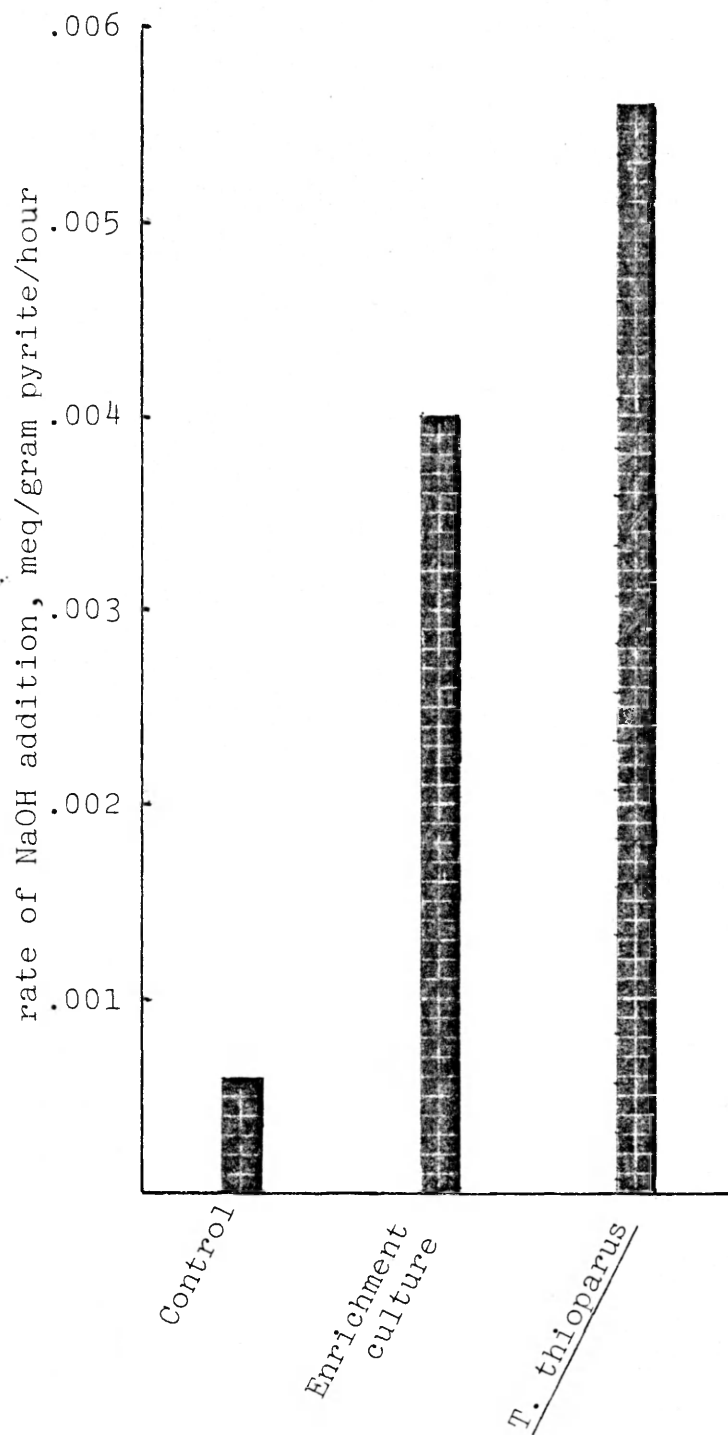


Figure 18. Results of rate of addition of NaOH at pH 6.5, 30°C, with O₂/CO₂ mixture for pyrite oxidation with a) no bacteria, b) enrichment culture, and c) T. thioparus.

in NaOH consumption could mean that the addition of bacteria increases the rate of pyrite oxidation. However, this rate is dependent upon the particular sulfur oxidation species produced, which is discussed by Goldhaber (1980). Hence, it can be concluded from the information presented in Figures 17 and 18 that both T. thioparus and enrichment cultures increase the rate of hydrogen ion production (and hence, sulfate ion production) during pyrite oxidation under controlled experimental conditions.

G-2. Thiosulfate Oxidation in Stanier Medium
Inoculated With Bacteria

The results are shown in Figure 19 and Appendix B, Table B-10.

The experiments utilizing thiosulfate as an energy medium for the bacteria consumed relatively small amounts of NaOH. The experiment inoculated with the enrichment culture consumed more than the control run for this set of experiments, while the T. thioparus-inoculated run actually required addition of acid to maintain it at pH 6.5.

H. Examination of Iron During Pyrite Oxidation

Iron plays a significant role in the complicated

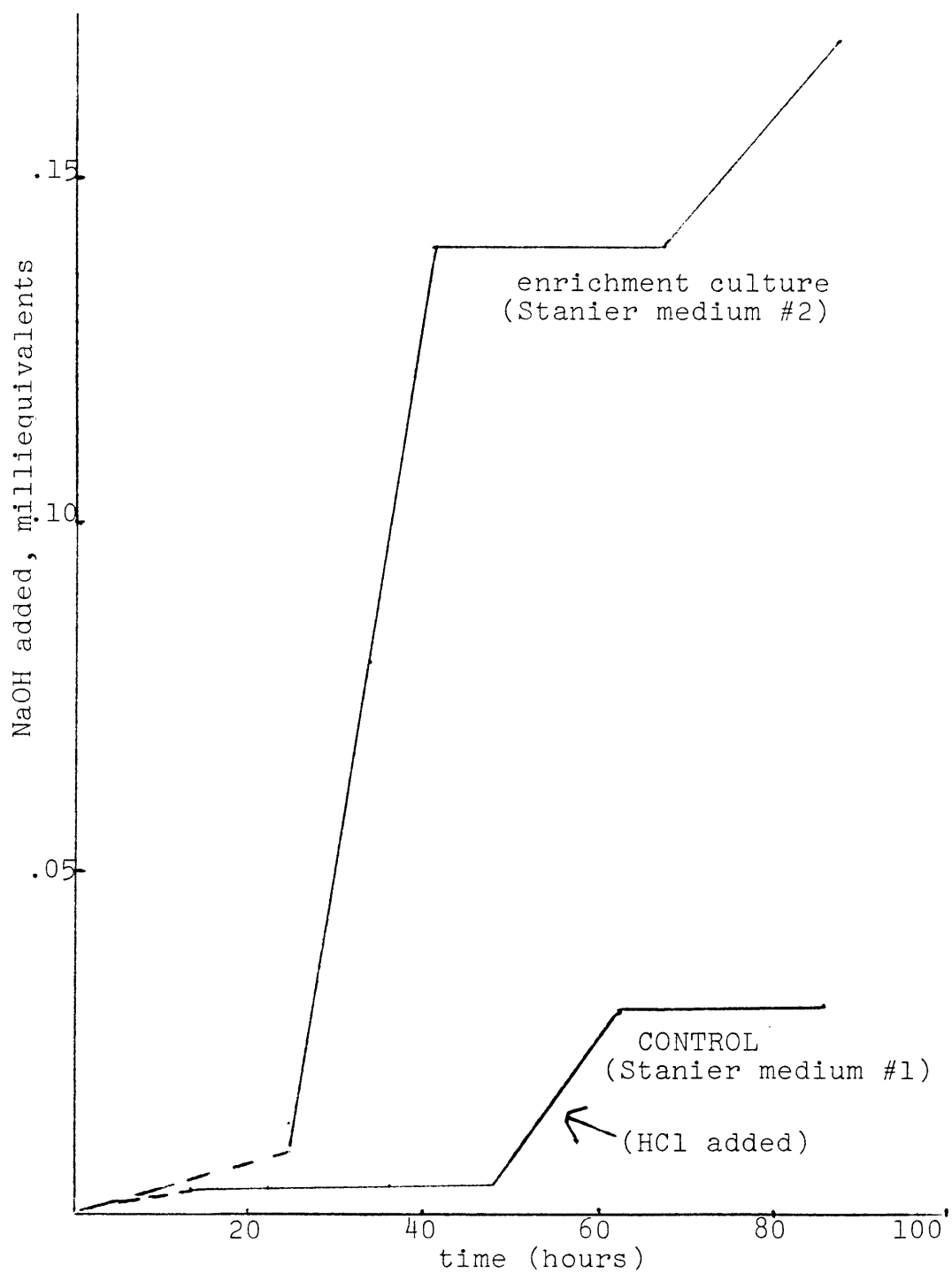
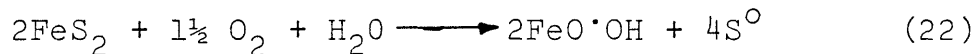


Figure 19. NaOH consumption versus time during thiosulfate oxidation in Stanier medium with O_2/CO_2 mixture at pH 6.5, $30^\circ C$.

pyrite oxidation process, as explained in the introduction. Sato (1960) states,

". . . of all the complexities of the oxidation reactions of a sulfide mineral, the most crucial and the least understood part is the first step of the heterogeneous reaction through which the solid sulfide is partially or totally dissolved into the surrounding solution upon breaking up its crystal bonds. . ."

Once this step is established (equation 2), the sulfur and iron oxidation reactions which occur can be investigated separately. Of particular concern is the fact that pyrite oxidation producing elemental sulfur (S^0) as the only sulfur species can occur according to the following equation (Goldhaber, 1980):



Consequently, pyrite oxidation could conceivably be occurring even though low rates of hydrogen ion production are observed. Hence, a qualitative study of iron was initiated to determine if there was any oxidized iron which was not predicted from the analytically determined sulfur species.

The ferrozine method (Stookey, 1970) was used to determine the iron content from the run which utilized KCl and pyrite at pH 6.0. Ferrozine reacts with di-

valent iron to form a stable magenta complex species which is very soluble in water and may be used for the direct determination of iron in water. However, results from this method indicate the experimental runs do not contain enough iron to be concerned with for the purpose of this paper (i.e. < 1 ppm Fe).

I. Examination of Bacterial Cultures Throughout Oxidation Experiments

The purpose of this section of the experiment was to establish a semi-quantitative number of bacteria determined to be present before, during and after the five inoculated runs. The cultures were examined microscopically immediately preceding the run to insure inoculation with a viable culture. A sample taken within the initial ten hours of the experiment was plated and counted as described in Appendix A. The same procedure was followed on a sample approximately halfway through some runs, and again at the end of all runs.

A typical growth curve for a bacterial culture is shown in Figure 20 (Stanier, 1963). At least four principal phases in the history of a culture are recognized. These are the lag phase, the logarithmic phase, the maximum stationary phase and the death phase. When

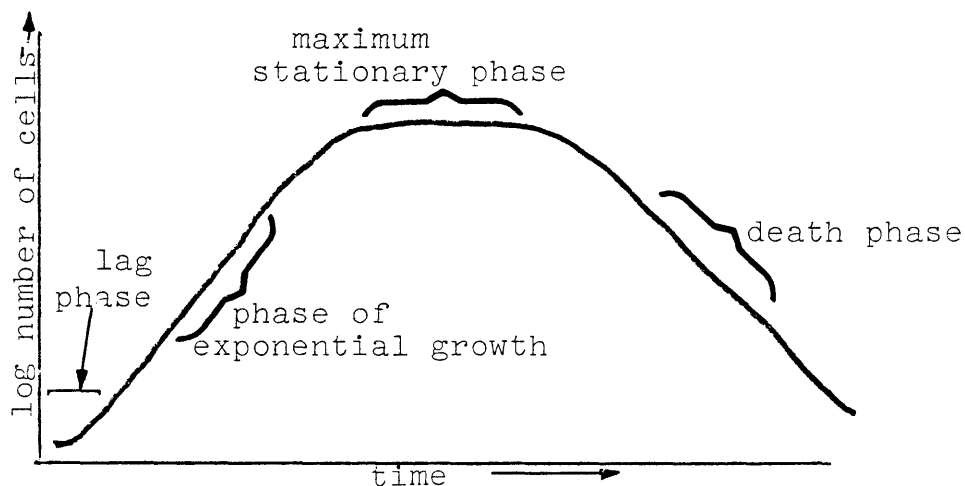


Figure 20. Generalized growth curve of a bacterial culture.

possible, the cultures were transferred into the experimental reaction vessel during the logarithmic phase, as all the cells produced during this phase are viable.

Two different types of bacteria were utilized. First the enrichment culture technique, based on free competition among different organisms in liquid media. This technique offers a means, not only of isolating known bacterial species, but also of obtaining for study undescribed organisms that are capable of flourishing in any given environment. It is for the latter benefit that an enrichment culture was studied in this series of experimentation.

In principle it is essentially an application of natural selection. A culture medium is inoculated with a mixed microbial population and factors such as the

carbon source, energy supply, temperature and pH are defined and maintained. For each particular set of conditions, a particular kind of microorganism will come to predominance. In this case, Stanier medium was inoculated with mine gangue, as described in Appendix A. Hence, the organisms which thrive in Stanier medium could be cultivated and studied further in the experimental runs.

I-1. Bacterial screening experiment: The purpose of this experiment was to determine which Thiobacillus species was the most adaptable and grew best on pyrite (FeS_2). This was determined by observing any drop in pH in an inoculum of one of the individual species considered, with pyrite available as an energy source.

Five 125-ml erlenmeyer flasks, each containing 50 ml of the autoclaved Stanier medium, were inoculated with 0.5 ml of one of the Thiobacillus species in the log growth phase*. The fifth flask was the control. The flasks were stopped with cotton and placed on a rotary shaker to provide continuous aeration.

The pH in each flask was monitored daily for six days with a drop in pH indicating the bacterial oxidation of FeS_2 to produce H_2SO_4 . At the conclusion of this ex-

* Approximately 0.2g FeS_2 which had been washed and sized was added.

periment, T. thioparus was shown to have the best growth under these conditions and it was determined that the actual pyrite runs would utilize this particular specie of Thiobacillus.

I-2. Enrichment Cultures

Three runs were made which were inoculated with enrichment culture. All runs appeared capable of sustaining a microbial population and in two of the runs, an increase was noted by the end of the experiment.

The results are shown below in Table 6.

Table 6. Number bacteria present in experiments inoculated with enrichment cultures.

Energy source added	# bacteria present (cells/ml)		
	begin	during	after
pyrite	-	5.7×10^6	-
pyrite	8×10^8	-	1.75×10^9
thiosulfate	-	3.8×10^5	5.9×10^5

I-3. Thiobacillus thioparus Cultures

Two runs were made which were inoculated with T. thioparus cultures, one supplied with thiosulfate as an energy source and the other supplied with pyrite.

Both runs illustrated an ability to sustain microbial populations. The run with pyrite showed a more noticeable increase in bacteria by the end of the run than the one with thiosulfate. The results are shown below in Table 7.

Table 7. The number of bacteria present in experiments inoculated with T. thioparus.

Energy source added	# of bacteria present (cells/ml)	
	begin	end
pyrite	2.6×10^9	1.8×10^{10}
thiosulfate	1.6×10^9	3.0×10^9

C O N C L U S I O N S

Studies on inorganic oxidation of pyrite show that metastable sulfur oxyanions can be produced in a simple solution under specific experimental conditions. This concurs with the work of Goldhaber (1980). The only difference between the present studies and those done by Goldhaber under similar conditions is that these studies reveal the presence of thiosulfate during pyrite oxidation at pH 6.5. Goldhaber detected thiosulfate only in solutions from pH 7 and 9. This apparent discrepancy could be a result of using different methods for calculating the amount of thiosulfate. However, this difference is insignificant in light of the fact that the important information gained from both studies was the detection of intermediate sulfur species.

Oxidation studies on the bacterial medium without pyrite shows that the abundance of polythionates decline after approximately 75 hours, presumably being oxidized to sulfate. This contrasts with the oxidation studies on the KCl solution which shows the amount of polythionates increase during a similar time period. This is one of the important conclusions resulting from this study, as it appears to indicate a catalytic effect

produced in the complex media which is not present in the simple KCl matrix.

These studies show that the rate of total sulfur produced during pyrite oxidation in a KCl solution (6×10^{-3} mM/hr) and in the bacterial medium (7×10^{-3} mM/hr) under similar experimental conditions is the same. The difference is that sulfate is the dominant sulfur species in the bacterial medium, whereas tetrathionate is dominant in the KCl solution. This is attributed to the initial sulfate content of the medium.

The limited iron studies indicate that pyrite oxidation in the KCl solution at pH 6.0 produces intermediate sulfur species other than elemental sulfur (S^0). This conclusion is also supported by the fact that no buildup of elemental sulfur was observed in any of the experiments done in this study.

Pyrite oxidation studies involving T. thioparus show that these bacteria influence the rate of sulfur species produced. T. thioparus appears to increase the amount of sulfate present in solution during pyrite oxidation, presumably by catalyzing the conversion of intermediate sulfur species. The eventual oxidation of all intermediate sulfur species in solution simultaneously produces sulfate and hydrogen ions. T. thioparus also

increases the rate of hydrogen ion production considerably. Hence, these results indicate T. thioparus increase the overall rate of pyrite oxidation.

Pyrite oxidation studies involving an enrichment culture show results similar to those studies utilizing T. thioparus, although to a lesser degree. From these studies it could be concluded that both T. thioparus and the enrichment culture readily influence pyrite oxidation by increasing the rate. However, it should be noted that the increased hydrogen ion concentration in the early stages of the experimental runs could be a result of a change in the distribution of intermediate sulfur species being produced.

The parallel set of experiments utilizing thiosulfate instead of pyrite as an energy source indicate that the bacteria actually catalyze the initial oxidation step of pyrite, rather than utilizing the intermediate sulfur species which would eventually appear in the solution.

In conclusion, there are three major results of this study. First, metastable sulfur oxyanions can be produced during biogenic oxidation of pyrite under specific experimental conditions. Second, that T. thioparus

and enrichment cultures appear to be capable of catalyzing the oxidation of these sulfur oxyanions. Third, T. thioparus and enrichment cultures ultimately increase the rate of pyrite oxidation.

A P P E N D I X

A

Materials and Methods

MATERIALS AND METHODS

The experimental equipment and procedures, materials, microbiological techniques, and the analytical procedures are described in the following section.

M A T E R I A L S

All reagents were stored in polyethylene bottles and used throughout the duration of the experiment, except as noted.

REAGENTS

All materials not specifically discussed below were "analytical grade" materials. The term "water" refers to water which had been distilled and deionized unless specified otherwise.

Sodium tetrachloromercurate (II) solution (0.1M):
0.05 mole (13.6 grams) reagent grade mercury(II) chloride and 0.1 mole (5.85 grams) of reagent grade sodium chloride were dissolved in water and diluted to 500 mls.

Formaldehyde (0.2%): 5 ml of 40% formaldehyde diluted to 1 liter with water.

Hydrochloric acid-basic fuchsin solution (0.04%):
0.25 gram basic fuchsin ($C_{20}H_{19}N_3 \cdot HCl$) was dissolved in 25 ml water to make a 1% solution. 4 ml of the 1% solu-

tion was mixed with 6 ml concentrated hydrochloric acid and then diluted to 100 ml with water in a volumetric flask to make the 0.04% solution. The 0.04% solution was prepared as needed due to its instability after more than 2-3 days.

Sodium sulfite standard solution: 0.9845 grams

Na_2SO_3 was dissolved in 250 ml water to produce a solution containing 1000 μg sulfite-S per ml. A 1:200 dilution produced a 5 μg sulfite-S per ml solution.

Sodium thiosulfate stock solution: 0.9678 grams

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and 0.0388 grams Na_2CO_3 dissolved in 250 ml water to produce a solution containing 1 mg of thiosulfate-S per ml. 1 ml of the above solution was diluted to 100 ml with water to produce a solution containing 10 μg of thiosulfate-S per ml. These solutions were made fresh as needed to avoid oxidation of the thiosulfate.

Ferric nitrate-nitric acid solution

(0.25M $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$:3.1M HNO_3): 50 grams of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ dissolved in 100 ml of concentrated HNO_3 (sp. gr. 1.42). This solution was diluted to 500 ml with water.

Cupric chloride solution (0.033M): 5.63 grams of $\text{CuCl}_2 \cdot 7\text{H}_2\text{O}$ was dissolved in 800 ml water and diluted to 1 liter with water.

Sodium cyanide solution (0.1M): 4.9 grams of NaCN

was dissolved in 800 ml water and diluted to 1 liter with water.

Ferrozine solution: A commercially prepared solution was used.

Iron stock solution: The 1000 μ g Fe/ml standard solution for the AA was used.

Buffer solution, pH 5.5: 400 grams ammonium acetate was dissolved in water. 350 ml concentrated ammonium hydroxide was added and then the solution was diluted to 1 liter with water.

Potassium chloride solution (0.1M KCl): 14.91 grams KCl was dissolved in 2 liters water. This was prepared fresh for each experiment.

Pyrite (FeS_2): Obtained from Climax Molybdenum Mine. All FeS_2 used in the experimental runs was ground and sized by sieving at <44 micrometer*, washed with dilute HCl under a nitrogen atmosphere, rinsed with water followed by an acetone rinse. The pyrite was stored in a vacuum desiccator between experiments. A large amount of the pyrite was initially prepared in an attempt to standardize the FeS_2 utilized throughout the experimental process. However, another batch of pyrite was prepared separately and used

* The experimental stirring rate was capable of suspending all material less than or equal to 44 micrometer. The stirring rate in all experiments was the same.

in all runs inoculated with bacteria. The pyrite used as an energy source in the bacterial media was ground but did not need to be as fine-grained as that used in the actual experimental runs. Consequently, it was sized and sieved at 44-62 micrometer.

BACTERIAL MEDIA

All media were appropriately autoclaved, cooled and refrigerated. The water used was distilled and deionized.

#290: Contained the following chemicals dissolved in 1 liter water:

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	2.26g
KH_2PO_4	1.8g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1g
$(\text{NH}_4)_2\text{SO}_4$	0.1g
CaCl_2	0.03g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.033g
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.022g
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	15.7g

#152: Contained the following chemicals dissolved in 1 liter water:

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	10.0g	Yeast extract	1.0g
NH_4Cl	1.0g	KH_2PO_4	0.4g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	1.06g	FeCl_3	0.02g
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	0.78g		

Stanier #1: Contained the following chemicals dissolved in a 20 liters water:

NH_4Cl	20g
K_2HPO_4	20g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4g
CaCl_2	1.4g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1g
Trace element solution*	20 ml

Stanier #2: Contained the following chemicals dissolved in 1 liter water:

NH_4Cl	1g
K_2HPO_4	1.3g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g
CaCl_2	0.02g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.05g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.002g
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.001g
Zn metal	(tr)
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	(tr)
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	(tr)

290B Agar: Contained the following chemicals dissolved in 1 liter water:

* Trace element solution contained 0.01g of each of the following chemicals dissolved in 500 ml water: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$.

Na_2HPO_4	1.2g
KH_2PO_4	1.8g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1g
$(\text{NH}_4)_2\text{SO}_4$	0.1g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.03g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.02g
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.02g
$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$	10.0g
agar agar	15g

The solution was heated until dissolved.

Bacterial Cultures

Thiobacillus intermedius: #15466, American Type Culture Collection. It was grown in medium #152 and stored in the refrigerator.

Thiobacillus neapolitanus: #23638, American Type Culture Collection. It was grown in medium #290 and stored in the refrigerator.

Thiobacillus thioparus: #23648, American Type Culture Collection. It was grown in medium #290 and stored in the refrigerator.

Thiobacillus novellus: #8093, American Type Culture Collection. It was grown in medium #152 and stored in the refrigerator.

Enrichment culture: Contained a mixture of acid mine

water and river water collected from Clear Creek, close to Illinois St. in Golden, Colorado. Initially inoculated into five separate media containing different combinations of Stanier #1, mine gangue, CaCO_3 , water and pyrite. The five inocula were aerated to enhance bacterial growth. The supernatant bacterial material was decanted from the separate inocula into a polyethylene bottle containing 500 ml 0.1M KCl and five grams FeS_2 as an energy source. For approximately three months, the enrichment culture was aerated continuously and titrated daily with 0.6N NaOH to maintain a pH of 6.4-6.8. Titrating ceased but aeration was continued for several more months.

Approximately one year later, the remaining residue was used to inoculate five tubes of Stanier #1, incubated and microscopically determined to be viable.

ANALYTICAL PROCEDURES

The method used for sulfite detection proved to be sensitive, reproducible and accurate. However, quantitative determination of thiosulfate and tetrathionate individually in the presence of a relatively large amount of sulfate is difficult. The precision of the analytical procedure used to detect these two sulfur species in soils was demonstrated by Nor and Tabatabai (1976).

Goldhaber (1980) discusses the consequences of higher polythionates such as pentathionate being present, and notes this would result in an underestimate of polythionates relative to thiosulfate.

As the purpose of this study was to determine if bacteria had an effect on pyrite oxidation, the accuracy of the analytical procedures was initially not a major concern. However, as the data were being analyzed more closely, the accuracy and reproducibility of these techniques came under scrutiny.

Erratic values were obtained, for unknown reasons, for the total sulfur values obtained by ion chromatography. When this occurred, the total sulfur data were interpolated from the remaining values. When pentathionate was considered the predominant polythionate present, the values were calculated from tetrathionate values and can only be considered as semiquantitative. In several samples, the sum of the intermediate sulfur species was larger than the value obtained for total sulfur. This made it impossible to accurately calculate the sulfate content by difference and lends a note of discredit to other values obtained using the same analytical techniques.

The fact that no reproducibility studies were done

and discrepancies in values were noted are weak points in the work presented here. Consequently, conclusions based on results from these semiquantitative techniques should be viewed conservatively and be considered preliminary in nature.

Sulfite determination: Sulfite (SO_3^{2-}) was determined by the colorimetric procedure of West and Gaeke (1956).

Thiosulfate and tetrathionate determination: Thiosulfate ($\text{S}_2\text{O}_3^{2-}$) and tetrathionate ($\text{S}_4\text{O}_6^{2-}$) were determined by cyanolysis followed by spectrophotometric determination of the resulting thiocyanate (Nor and Tabatabai, 1976). This method assumed tetrathionate was the only polythionate present and used an empirical factor of 1.75 to estimate the amount of tetrathionate in samples which contained thiosulfate. This factor was used in all calculations for tetrathionate within the scope of this research.

Thiosulfate determination #2: In addition to the method outlined above, $\text{S}_2\text{O}_3^{2-}$ determinations were made on the ion chromatograph (IC) for three experiments to verify the thiosulfate data from the method outlined above. A 1:1000 dilution was made of a small aliquot of the original sample extracted during the experiment. Two ml

was injected into the IC and the results were compared to a standard curve. This procedure produced values for $S_2O_3^{2-}$ which were consistently smaller than those obtained with the chemical procedure (see Appendix B, Table B-6).

Pentathionate determination: See Appendix C.

Total-S determination: The total sulfur as sulfate (SO_4^{2-}) determination was made using the IC. A 1 ml sample, diluted when necessary, was placed in a 25-ml erlenmeyer flask with 4 ml H_2O_2 . This was covered with a watch glass, heated to just below the boiling point and maintained at that temperature for approximately eight hours. 2 ml was injected into the IC and the results were compared to a standard curve.

In addition, the IC results were compared with results obtained by the Johnson and Nishita (1952) method* used by Goldhaber (1980). Goldhaber reported that results from this technique were in generally reasonable agreement (± 10 percent) with values obtained by oxidation of the sample and subsequent gravimetric determination of sulfate as $BaSO_4$. The IC values for total sulfur were consistently larger than those obtained by the Johnson

*This method, initially used in this research, consisted of reducing all sulfur oxyanions to H_2S and determining the amount of H_2S by methylene-blue colorimetry. It proved to be too time-consuming (approximately 2 hrs/sample) and erratic values were obtained for unknown reasons.

and Nishita procedure. Consequently, an empirical factor of 1.8 was used to estimate the amount of total sulfur in each sample.

Sulfate determination: Initially, a routine turbidimetric determination of sulfate as BaSO_4 was planned. However this method provided inconsistent results, presumably due to suspension interferences inherent in the bacterial medium. To isolate the specific interference within the medium, five possible agents were identified and an experiment run with each agent dissolved in distilled water. Erratic values were still obtained and the basic turbidimetric method was abandoned in favor of determining sulfate as the difference between total-S and the sum of the analyzed constituents. Hence the sulfate values depend on the accuracy of the total-S values.

Iron determination: Oxidized iron was determined by leaching pyrite previously oxidized in the pH-stat in boiling 6N HCl for ten minutes (Goldhaber, 1980). Pyrite is insoluble under these conditions, whereas iron oxides are soluble (Berner, 1970; Goldhaber, 1980). Iron was then determined on the supernatant by colorimetry utilizing the ferrozine method (Stookey, 1970).

MICROBIOLOGICAL TECHNIQUES

Autoclave: When reference to autoclaving is made, this indicates a steam pressure system of 15-20 lbs/in² at 121°C was maintained for 15-20 minutes.

Stock cultures: As the bacterial cultures were used over a period of two years, it was important to ensure availability of viable cultures by maintaining both reserve and working stock. The reserve stock cultures of all species were not used for making routine inoculations; instead they were stored in the refrigerator after incubation until a transfer was needed for another reserve stock or working stock culture. The working stock cultures were used for routine inoculations and were replaced approximately monthly by a fresh culture from the reserve stock.

Inoculation of bacteria: The generally-accepted microbiological methods outlined in Seeley and VanDemark (1972) were followed.

Bacterial plating: The spread plate technique utilized throughout the duration of experiments is also outlined in Seeley and VanDemark (1972).

Bacterial counting methods: There are many methods for enumerating microbes and the standard plate count

was used in this research. Dilutions of $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$ were made. Commercially prepared nutrient agar was used for enumerating the enrichment cultures and Stanier medium agar was used for T. thio-
parus.

A P P E N D I X

B

Data Tables

Table B-1. Results of Pyrite Oxidation in 0.1M KCl at pH 6.5, O₂ Saturation

SO ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₄ O ₆ ²⁻ sulfur (mM)	SO ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
---	---	.020	---	(.19) ^a	0.5	.007
---	.005	.039	---	---	3	.071
---	.017	.056	.16	.23	6	.15
*	.056	.16	.09	(.31) ^a	19	.53
---	.068	.21	.08	.35	24	.63
*	.107	.29	.01	.41	34	.85
---	.090	.32	.03	(.44)	44	1.1
---	.120	.35	.02	.49	49	1.2

a sample unavailable

() interpolated value

--- not detected

* < 0.01

Table B-2. Results of Pyrite Oxidation in 0.1M KCl at pH 6.0, O₂ Saturation

S ₂ O ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₄ O ₆ ²⁻ sulfur (mM)	SO ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequi- valents)
---	*	.031	.38	.41	0.5	.19
---	.076	.24	---	---	11	.79
*	.081	.38	.10	.56	17	1.2
*	.105	.52	.57	1.19	23	1.6
*	.201	.89	.29	1.38	35	2.4
.016	.192	.84	.39	(1.43)	41	2.7
.018	.198	.99	.36	(1.57)	47	3.1
.022	.46	1.36	---	1.78	59	3.7
.052	.18	.98	.61	(1.82)	65	4.1
.038	.25	.95	.65	(1.89)	71	4.5

() interpolated value

--- not detected

* < 0.01

Table B-3. Results of Stanier Medium #1 Oxidation at pH 6.5, O₂ Saturation

S ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
---	.018	.045	.84	.90	4.5	---
---	.040	.061	2.19	2.29	23	.25
*	.058	.061	1.69	1.81	31	.25
*	.022	.051	---	---	46.5	.53
---	.011	.047	.35	.41	74.5	.65

--- not detected

* < 0.01

Table B-4. Results of Stanier Medium #1 Oxidation, pH 6.5, O₂ Saturation, Yeast Extract

SO ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	SO ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequi- valents)
*	.011	.033	1.77	1.81	2.5	.10
*	---	.134	1.67	1.81	18	.42
*	*	.042	1.83	(1.87)	26	.42
---	.013	.056	1.83	(1.90)	47	.42
*	*	.036	1.90	1.94	53.5	.42
*	*	.036	1.92	(1.96)	66.5	.42
*	.011	.031	1.95	(1.99)	72.5	.42

--- not detected

* < .01

() interpolated value

Table B-5. Results of Pyrite Oxidation in Stanier Medium #1, pH 6.0 and O₂ Saturation.

S ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequi- valents)
---	---	.028	.32	.35	3.3	.08
---	---	.038	.31	.35	22.6	.14
---	---	.065	.39	.45	32.2	.15
---	.015	.067	.21	.29	46.8	.16
---	---	.050	.49	.54	54.8	.17
---	---	.040	.39	.43	60.5	.18
---	*	.028	.99	1.02	70.8	.19
---	---	(.005)	.96	1.02	79.2	.19

--- not detected

* < 0.01

() interpolated value

Table B-6. Results of Pyrite Oxidation in Stanier Medium #2 at pH 6.5 with O₂/CO₂ Mixture (A Control Run).

S ₂ O ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM) (Method A)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ O ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
*	.011	.008	.008	7.27	1.5	.59
*	.009	.010	.018	2.54	16	.59
---	.021	.006	.022	2.69	27.5	.59
---	.016	.007	.022	2.88	45.75	.69
---	.008	.004	.046	2.97	71.5	.92
---	.012	.003	.025	3.08	87.75	1.24

--- not detected

() interpolated value

* < .002

Method A is chemical procedure

Method B is ion chromatography

Table B-7. Results of Thiosulfate Oxidation in Stanier Medium #1 at pH 6.5 with O₂/CO₂ Mixture.

S ₂ O ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ O ₄ ²⁻ sulfur (mM)	Total ^a sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
.94	50.9	1.5	---	53.3	0.5	0
3.59	58.6	1.7	---	63.9	13.75	.003
.94	60.1	1.6	---	62.6	22.5	.003
1.56	57.6	2.1	---	61.3	37.5	.003
1.87	56.4	1.7	---	60.0	47	.004
1.87	56.5	2.8	---	61.2	62.5	.03
.90	61.2	1.5	---	63.6	73	.03
.94	50.7	1.6	---	53.2	88	.03

--- not detected

^a calculated as summation of S₃O₃²⁻, S₂O₃²⁻ and S₅O₆²⁻

Table B-8. Results of Pyrite Oxidation in Stanier Medium #2 with Enrichment Culture, pH 6.5 and O₂/CO₂ Mixture.

S ₂ O ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ O ₄ ²⁻ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequi- valents)
*	.061	.035	1.57	1.67	14.5	.001
*	.029	.052	.17	(.25)	27.5	.24
*	.028	.061	.43	.52	39.5	.51
*	.074	.123	.63	.83	51.5	.51
*	.03	.059	1.17	(1.26)	62.5	1.0
*	.027	.056	1.59	1.67	72.5	1.2

* < 0.003

() interpolated value

Table B-9. Results of Pyrite Oxidation in Stanier Medium #2 with T. thiooparus and O_2/CO_2 Mixture at pH 6.5.

SO_3^{2-} sulfur (mM)	$S_2O_3^{2-}$ sulfur (mM)	$S_5O_6^{2-}$ sulfur (mM)	SO_4^{2-} sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
*	*	.014	4.33	4.34	7.5	1.3
*	.014	.028	4.40	(4.44)	24.75	1.9
*	.014	.027	4.47	4.51	33.75	2.1
*	.015	.033	4.51	(4.56)	50.5	2.3
*	*	.085	4.54	4.63	60.75	2.9
*	.*	*	1.91	1.91	65.75	2.9

* < 0.004

() interpolated value

Table B-10. Results of Thiosulfate Oxidation in Stanier Medium #2 with Enrichment Culture, pH 6.5, O₂/CO₂ Mixture.*

S ₂ O ₃ ²⁻ sulfur (mM)	S ₂ O ₃ ²⁻ sulfur (mM)	S ₅ O ₆ ²⁻ sulfur (mM)	S ₄ O ₆ ²⁻ sulfur (mM)	Elapsed time (hrs)	NaOH added (milliequivalents)
2.34	59.5	1.72	---	1	0
2.03	55.6	1.85	---	24	.008
1.56	56.6	1.52	---	33.25	.08
1.50	41.8	1.95	1.7	42.5	.14
1.56	53.3	1.56	---	50.75	.14
1.65	52.8	2.26	---	69	.14
1.25	49.3	2.34	1.81	87.5	.17

--- not detected

* total sulfur values obtained via IC were smaller than thiosulfate values and hence, not included.

Table B-11. Results of Thiosulfate Oxidation in Stanier Medium #2 with T. thioparus and O_2/CO_2 Mixture at pH 6.5.

$S_2O_3^{2-}$ sulfur (mM)	$S_2O_3^{2-}$ sulfur (mM)	$S_5O_6^{2-}$ sulfur (mM)	$S_4O_6^{2-}$ sulfur (mM)	Total sulfur (mM)	Elapsed time (hrs)
3.90	54.2	1.68	1.82	61.6	15
3.90	45.4	1.72	---	46.3	32
3.12	57.1	2.38	---	47.4	52.5
3.59	51.3	2.22	---	50.5	62.25
2.34	51.6	2.22	---	54.1	72

--- not detected

A P P E N D I X

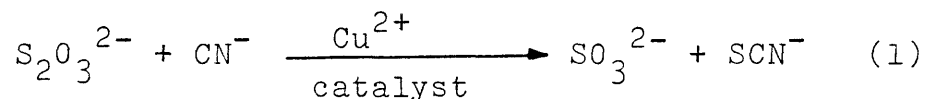
C

Basis for Calculations

BASIS FOR CALCULATIONS

In the cyanolysis procedure described by Nor and Tabatabai (1976) for the detection of thiosulfate and tetrathionate, tetrathionate is assumed to be the only metastable polythionate present in solution. Their assumption also appears to agree with the data obtained in this study in which a KCl solution was used as a matrix for abiological pyrite oxidation. This section describes and discusses the detailed calculations, assumptions and technical basis which lead to the conclusion that pentathionate ($S_5O_6^{2-}$) is the polythionate present in the oxidation experiments using Stanier medium.

A standard curve used for determining $S_2O_3^{2-}$ and $S_4O_6^{2-}$ is shown in Figure C-1. However, theoretically when one mole of thiosulfate undergoes cyanolysis in the presence of Cu^{2+} , one mole of thiocyanate is produced which contains 50% of the amount of sulfur present in the thiosulfate according to equation (1).



Hence, to obtain a standard curve to describe the $\mu\text{g S}$ as SCN^- (rather than as $S_2O_3^{2-}$) the μg values on the

x-axis must be halved, as shown in Figure C-2.

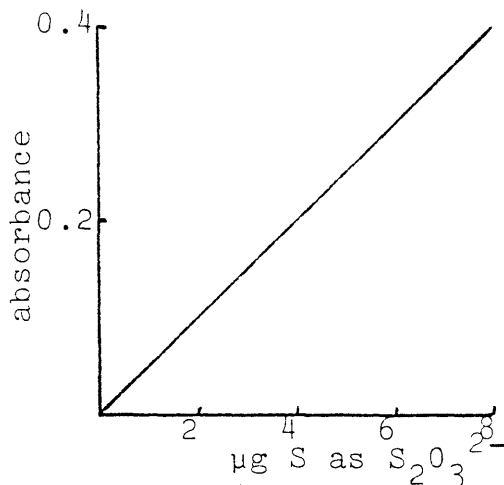


Fig. C-1. Standard curve for thiosulfate.

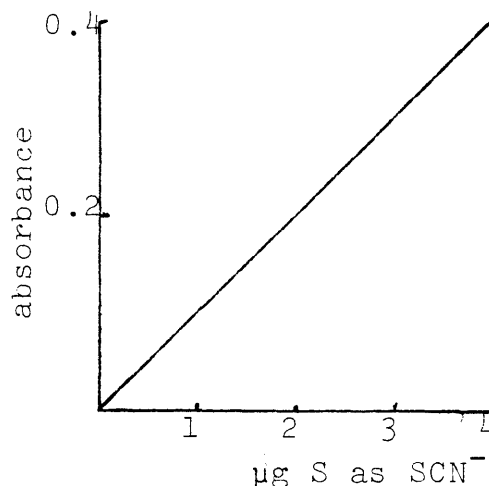


Fig. C-2. Standard curve for SCN^- .

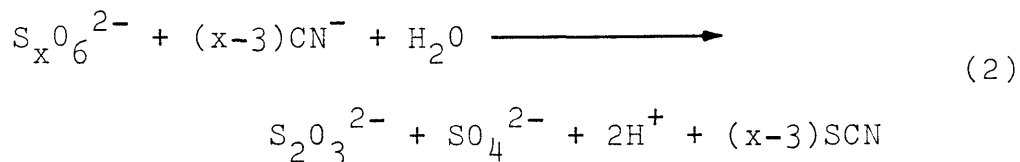
The values used in the following example were determined experimentally in a preliminary study similar to Run B-1. The results from one sample are shown below in Table C-1.

Analytical method used*	$\mu\text{g S}_2\text{O}_3^{2-}\text{-S}$ (Figure C-1)	$\mu\text{g SCN}^-\text{-S}$ (Figure C-2)
$\text{S}_2\text{O}_3^{2-}\text{-Cu}$	14.5	7.25
$\text{S}_2\text{O}_3^{2-}\text{+Cu}$	22.0	11.0

Table C-1. Amount of sulfur produced in Stanier medium at pH 6.5, 30°C, and O_2 saturation.

* See Appendix A

In the presence of a copper catalyst, one mole of thiocyanate is formed from thiosulfate, two from tetrathionate, three from pentathionate and four from hexathionate. In the absence of a copper catalyst, the reaction proceeds as follows:



At room temperature, reaction (2) only occurs with polythionates of $x \geq 4$ (Nor and Tabatabai, 1976). The thiocyanate equivalents formed without a copper catalyst from tetra-, penta-, and hexathionate are one, two, and three, respectively.

Consequently, the 7.25 μ g SCN⁻-S in Table C-1 could be from either S₄O₆²⁻, S₅O₆²⁻, S₆O₆²⁻ or any combination thereof. The 11 μ g SCN⁻-S in Table C-1 includes sulfur from S₂O₃²⁻ in addition to sulfur from either S₄O₆²⁻, S₅O₆²⁻, S₆O₆²⁻ or any combination. If the 11 μ g SCN⁻-S was exclusively from S₂O₃²⁻, then the method without the copper catalyst would also have produced 11 μ g SCN⁻-S, so this possibility was eliminated.

To determine the amount of SCN⁻-S which was from S₂O₃²⁻ only, the following procedure was used:

$$(\text{Total SCN}^- \text{-S}) - (\text{SCN}^- \text{-S not from S}_2\text{O}_3^{2-}) =$$

$$11\mu\text{g} - 7.25\mu\text{g} = 3.75\mu\text{g SCN}^- \text{-S from S}_2\text{O}_3^{2-} \text{ only}$$

Theoretically, the ratio of SCN⁻-S from tetra-, penta- and hexathionate, to the SCN⁻-S from thiosulfate-only is one, two, and three, respectively. This relationship could be expressed as

$$\frac{\text{SCN}^- \text{-S from S}_x\text{O}_6^{2-}}{\text{SCN}^- \text{-S from S}_2\text{O}_3^{2-}} = \begin{array}{l} 1 \text{ if } x=4, \\ 2 \text{ if } x=5 \text{ and} \\ 3 \text{ if } x=6. \end{array} \quad (3)$$

The appropriate values from the example above yield an approximate ratio of 2:1, according to equation (4).

$$\frac{7.25\mu\text{g SCN}^- \text{-S}}{3.75\mu\text{g SCN}^- \text{-S}} = 1.93 \quad (4)$$

This indicates the SCN⁻-S is from pentathionate rather than tetrathionate. Similar calculations on random samples from other runs also produced this ratio.

There are other indications that S₅O₆²⁻ is the major polythionate present. In the absence of the copper catalyst, the ratio of sulfur atoms present to sulfur atoms detected is 4:1 for tetrathionate, 5:2 for pentathionate and 6:3 for hexathionate. Hence, the 7.25μg SCN⁻-S de-

tected in this particular sample would correspond to 29 μ g S, 18.1 μ g S and 14.5 μ g S for tetra-, penta-, and hexathionate, respectively.

In the presence of a copper catalyst, the ratio of sulfur atoms present to sulfur atoms detected as thiocyanate is 2:1 for thiosulfate, 4:2 for tetrathionate, 5:3 for pentathionate and 6:4 for hexathionate. Hence, the 11 μ g S actually detected would correspond to 22 μ g S for both thiosulfate and tetrathionate, 18.3 μ g S for pentathionate, and 16.5 μ g S for hexathionate. Results from analysis with and without the copper catalyst for pentathionate (18.1 μ g S vs. 18.3 μ g S) are more similar than the results for the other polythionates.

For the purpose of discussion within this paper it is concluded that pentathionate is the major polythionate present in the experimental solutions. The theoretical factor of 5/2 was used to estimate the amount of pentathionate in samples which also contain thiosulfate. However, this conclusion must be viewed with caution. First it is based on the assumption that only one polythionate is present, which is not necessarily correct. Second, the data from these few random samples may be artifacts of the experimental or analytical technique.

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