A STUDY OF BACTERIAL ACTIVITY AND ECOLOGY OF BINGHAM CANYON MINE DUMPS

by

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ABSTRACT

There were at least two types of chemoautotrophic thiobacilli found in the leaching streams of Bingham Canyon mine dump. One of these organisms oxidizes free sulfur to sulfuric acid which reaction is its energy reaction. This organism is very similar to *Thiobacillus thiooxidans*. The other organism oxidizes ferrous to ferric ion as its initial energy reaction and is similar to *Thiobacillus ferrooxidans*. These two bacterial strains complement each other in that *T. thiooxidans* produces acid which keeps the ferric ion produced by *T. ferrooxidans* in solution, thereby forming an effective acidic ferric sulfate leaching solution for copper minerals.

In addition to these two types of chemoautotrophs, a yeast-like organism was found in every location of the Bingham mine dump waters. The cell biology and physiology of these yeast-type organisms were studied. It was found not to be a chemoautotrophic, but an acidophilic heterotroph.

The microbial population in the mine water samples suggests the coexistence of a balanced ecological system in which carbon fixation is dependent on chemoautotrophic bacteria. Acidophilic heterotrophs, utilizing organic matter as their energy and carbon source, are acting to keep the organic content of leach waters at a low concentration favorable to the development of chemoautotrophs.

Three different counting techniques, such as the Petroff-Hausser counting technique, the silica gel plate counting technique and the radioisotope labeling technique for the enumeration of the chemoautotrophic thiobacilli were studied. Of these methods, the direct microscopic counts were the only ones found to be satisfactory as the most rapid and relatively reliable method of estimating this type of cell numbers. Statistical evaluation of Petroff-Hausser counting technique was also made to prove the reliability of this method.

The correlation between the conversion of ferrous to ferric ion and bacterial growth were studied. It was shown that a given amount of conversion of ferrous to ferric ion over a given time period corresponded to a given increase in the bacterial population.

The distribution of *Thiobacillus ferrooxidans* and their ecological environment in the Bingham mine dump were investigated. It was noted that there was a relationship between the numbers of *T. ferrooxidans* in leaching systems and the chemical reactions which were known to occur in this process.

INTRODUCTION

Leaching of copper from mine overburden by utilizing bacterial oxidation has been of great importance from an economic standpoint. In open pit mines, large tonnages of low grade waste materials must be removed to gain access to the ore. While the waste contains only a trace amount of copper, the huge tonnages represent a substantial amount of copper.

Heretofore, leaching of copper from mine overburden has been more of an art than a science. For example, leached copper was obtained by the natural oxidation of copper sulfide minerals after meterologic (rain and snow) and other water had percolated through the dumps. To recover the copper, metallic iron was introduced into the copper-bearing water that had been collected at a central point. The iron displaced the copper from solution to form cement copper. The copper-free water was then recycled to the dumps for percolation leaching.

A study of the mine dump waters revealed the presence of bacteria that actually thrive in the warm strongly acid dump water solutions. The bacteria not only thrived but accelerated the oxidation of sulfide minerals to form acid and convert the sulfide minerals to soluble sulfates.

One strain of bacteria, *Thiobacillus thiooxidans*, utilizes the oxidation of the sulfur as its energy source. The other strain,

Thiobacillus ferrooxidans, oxidizes ferrous ion to ferric ion as its energy source. These bacterial strains complement each other in that one produces acid which keeps the ferric ion, produced by the other, in solution, thereby forming an effective acidic ferric sulfate leaching solution for copper minerals.

It is well known that the presence of organic matter tends to inhibit the growth of chemolithotrophic bacteria. It is proposed that bacterial cellular debris may accumulate in the recirculated dump waters and thus slow down the leaching process. Therefore, the effect of dead thiobacillus on the oxidation process may become an important factor. The presence of increased organic matter may give rise to other ecological relationships. There is a possibility that acidophilic heterotrophs, utilizing the organic matter as their carbon and energy source, are acting to keep the organic content of leach waters at a low concentration.

The purposes of the work to be presented in this thesis include: (1) An attempt to study the cell biology of these microorganisms and their ecology in the mine dumps. This involves the development of counting methods and studies of samples from the dump to learn the basic bioecological relationships of the dump. (2) An attempt to correlate the conversion of ferrous to ferric ion and bacterial growth. This correlation was studied for a means of bioassay. Does a specific amount or rate of conversion of ferrous to ferric ion represent a specific number of bacteria or a specific increase in numbers over a

given time period? (3) An attempt to determine the distribution pattern of iron-oxidizing bacteria in leaching waters. This distribution pattern would serve as an indicator of the intensity of the oxidizing activity of this bacteria in any given area of the dump.

LITERATURE REVIEW

I. MICROORGANISMS INVOLVED IN THE OXIDATION OF IRON AND SULFUR

Breed et. al. (1957), listed nine species of the genus Thiobacillus, which are of the family Nitrobacteriaceae, and are described as small gram negative, rod shaped cells. They grow under acid or alkaline conditions deriving their energy from the oxidation of sulfur compounds and carbon from carbon dioxide or from bicarbonates in solution. Some are obligate and some facultatively autotrophic. One species is facultatively anaerobic. Six species have a pH optimum for growth close to neutrality while the growth optimum for the three remaining members is between pH 2.0 and 5.8. Only two species of the genus will be considered in this thesis.

Thiobacillus thiooxidans was isolated and characterized in 1922 by Waksman and Joffe, using dilution of composts of sulfur, rock phosphate and soil to inoculate culture media, and was named for its ability to utilize sulfur to sulfuric acid. The cells are short rods with rounded ends, usually occurring singly, occasionally in pairs, and rarely in triplets. The majority are less than 1 micron long and about 0.5 micron in diameter. They are non-spore forming; the majority are non-motile although some motility was observed in young cultures. The organism is Gram negative and stains well with gentian violet and methylene blue.

Optimum temperature for growth and sulfur oxidation is 28 to 30°C. Optimum hydrogen-ion concentration for growth is a pH of 2.0 to 2.8, although the organism continues to live at a pH as low as 0.6 or as high as 6.0. Sulfur is the important energy source and is oxidized to sulfuric acid. Nitrogen aids growth and is best supplied by the addition of inorganic ammonium salts. Carbon and oxygen are both derived from the atmosphere. Thiosulfate is utilized to a small extent. Growth was not obtained on agar or other solid media.

Thiobacillus ferrooxidans was not isolated and characterized until 1950 (Colmer et. al., 1950). Classification was reserved until 1951 (Temple and Colmer, 1951). This organism, first found in the acid drainage from bituminous coal mines, resembles *Thiobacillus thiooxidans* and cannot be morphologically distinguished in either stained or unstained preparations. Like *T. thiooxidans*, it oxidizes thiosulfate but is sharply differentiated by its ability to oxidize ferrous ion and by its failure to grow on elemental sulfur. Its optimal pH for growth is between 2.5 to 5.8 with no growth above pH 6.0. Photomacrographs show lobed colonies, rounded colonies and iron precipitation on mine water agar, and "frosty" colonies on sodium thiosulfate agar. Cells are Gram negative rods occurring singly or in pairs.

Temple and Colmer (1951) presented evidence to show the mutual interdependence of bacterial growth and iron oxidation. The iron oxidation resulted in a measurable increase in cellular carbon and

the efficiency of the utilization of the energy available from iron oxidation for cellular growth was found to be similar to that of other autotrophs. The medium used consisted of 0.1% $MgSO_4.7H_2O$, 0.05% $(NH_4)_2SO_4$, distilled water and enough $FeSO_4.7H_2O$ to give 26,000 ppm ferrous ion. Agar plates containing 26,000 ppm ferrous ion were also used successfully although no description of the colonies was given. The bacterium, which also grows on thiosulfate, has been assigned to the genus Thiobacillus and since the oxidation of ferrous ion is an outstanding characteristic of this bacterium, the specific name _Thiobacillus ferrooxidans_ n. sp. was proposed.

A new iron-oxidizing bacterium, _Ferrobacillus ferrooxidans_, was isolated and characterized by Leathen and Braley (1954). The optimal pH for growth was 3.5 and the optimal growth temperature was 20 to 25°C. This organism is distinguished from the genus Thiobacillus by its failure to oxidize either sulfur or thiosulfate. The cells are Gram negative, actively motile, rod shaped, and 0.6 to 1.0 microns in diameter by 1.0 to 1.6 microns in length. This iron-oxidizing bacterium was given the generic name Ferrobacillus to indicate the transfer of iron from the ferro to the ferric state, and the specific designation, _Ferrobacillus ferrooxidans_, was given.

An improved medium for the obligate chemoautotrophic iron bacterium _F. ferrooxidans_ that would support the growth of 2 to 4 x 10^8 cells per ml was devised by Silverman and Lundgren (1959a) together with a method of harvesting cells relatively free from the accompanying

precipitate of oxidized iron. The demonstration of the coincidence of ferrous ion oxidation and growth further confirms the autotrophy of this organism. Some of the physiological properties of this organism were further studied manometrically using intact cells (Silverman and Lundgren, 1959b). Iron was oxidized at an unusually rapid rate ($Qo_2(N)$ of from 2027 to 5131). Oxygen uptake was over 92 per cent of the theoretical amount required by the reaction:

$$4\ FeSO_4 + O_2 + 2\ H_2SO_4 \longrightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O$$

The optimal pH and temperature for the iron-oxidizing system were 3.0 to 3.6 and 37°C respectively. There was no evidence of iron toxicity to the intact cells at concentrations as high as 500 u moles Fe^{++} per Warburg Vessel. During the oxidation of 50 u moles of iron, *F. ferrooxidans* assimilated CO_2 with an average efficiency of 20.5 ± 4.3 per cent. Phosphate moderately inhibited iron oxidation; citrate was strongly inhibitory. Resting cells were unable to oxidize ammonium and thiosulfate. However, a low but significant oxidation of elemental sulfur was observed.

In 1960, Kinsel isolated a sulfur oxidizing iron bacterium from mine water in Western Pennsylvania. The cells were rods, usually occurring singly, and rarely in pairs, 0.5 micron by 1.0 to 1.5 microns, motile, Gram negative, non-spore forming, an obligate aerobe, optimal temperature 32°C, and optimal pH 2.85. On iron agar, colonies were circular in form with a red area of oxidized iron eventually appearing in the center. On the sulfur agar, colonies were clean and irregular in form.

The organism exhibited many characteristics similar to those of T. thiooxidans, T. ferrooxidans, and F. ferrooxidans; however, its ability to oxidize sulfur distinguished it from T. ferrooxidans and F. ferrooxidans; whereas, its ability to oxidize iron differentiated it from T. thiooxidans. The designation Ferrobacillus sulfooxidans sp. n. was proposed.

The classification of microorganisms is very difficult and it is not unreasonable to suggest that Ferrobacillus ferrooxidans, Ferrobacillus sulfooxidans, and Thiobacillus ferroxidans may be identical. The validity of the species T. ferrooxidans has been questioned by Leathen et al. (1953), while Breed et al. (1957) questioned F. ferroxidans. More recent investigators (Jones, 1963; Razzell and Trussell, 1963a) have reported bacterial oxidation of sulfur by F. ferrooxidans thus conflicting with earlier investigators (Colmer et al., 1950; and Temple and Colmer, 1951) who reported the failure of T. ferrooxidans to oxidize sulfur. In the future it is likely that we will see changes in the classification of these iron-oxidizing bacteria.

II. OXIDIZING BACTERIA PERTAINING TO KENNECOTT COPPER MINE OPERATIONS

The oxidation of sulfide minerals in the waste dumps at Kennecott's Bingham Canyon mine have been known for years. This oxidation and the resulting solubilization were shown to be in part a biological process involving autotrophic bacteria found in the leaching streams (Bryner et al., 1954). Tests using pyrite (FeS_2) as

the oxidizable substrate indicated that the organisms present in the leaching streams were similar to T. *thiooxidans* and T. *ferrooxidans*. Oxygen and carbon from the air were necessary for the growth. An increase in hydrogen ion concentrations increased activity. Lowering the temperature decreased activity. Tests conducted in total darkness showed increased activity. Bingham Canyon float concentrate and reagent grade CuS were used in the study as well as the minerals, chalcopyrite ($CuFeS_2$), covellite (CuS), chalcocite (Cu_2S), bornite (Cu_5FeS_4) and tetrahedrite ($Cu_8Sb_2S_7$). Inoculated samples in every case yielded more soluble copper than the sterile controls and it was found that the bacteria oxidized copper sulfide in the presence or absence of iron.

Zimmerley et al. (1955) applied for a patent on a cyclic leaching process involving the use and the continual regeneration of a ferric sulfate sulfuric acid lixiviant which has been inoculated with iron oxidizing autotrophic bacteria tolerant to relatively high concentration of the particular metal or metals to be extracted.

Further studies by Bryner and Anderson (1957) showed that molybdenite was also oxidized by bacteria. In the presence of copper sulfide, preferential oxidation of copper sulfide occurred. Later studies supported these findings and resulted in the isolation and characterization of bacteria. Bryner and Jameson (1958) succeeded in isolating two morphologically similar organisms. One of these bacteria is able to oxidize free sulfur ferrous ion, iron pyrites,

molybdemite as well as several copper sulfide minerals. Thus, this organism exhibited somewhat different characteristics than either *T. thiooxidans* or *T. ferrooxidans*, although nearly the same as the latter. The other organism oxidized free sulfur but did not oxidize the sulfide mineral. This one was similar to *T. thiooxidans*.

Beck (1960) isolated an iron-oxidizing bacterium from the acidic leaching water at Kennecott's Bingham Canyon mine by using conventional methods with both liquid and solid media. It grew on completely inorganic media at pH 2.0 to 3.0, obtaining its carbon from carbon dioxide and its energy by oxidation of either ferrous to ferric ion or sulfur to sulfate. Its utilization of thiosulfate, although detectable in manometric experiments, was slow compared to its action on sulfur.

Colonies were isolated on both silica gel and agar plates with incubation of 10 to 14 days at 30°C. Colonies on agar were typically larger and showed less tendency to be entirely darkened than those on silica gel. The bacterium was morphologically similar to *T. thiooxidans* except for reduced motility. A small ovoid or short rod, it occurred singly or more frequently in pairs. Cells (0.5 to 0.7 by 0.8 to 1.0 micron) were gram negative, staining poorly with basic stains, but strong with acidic stain. Difference in physiological properties from those ascribed to either *F. ferroxidans* or *Thiobacillus ferroxidans*. Cell counts were made by noting the highest dilution of inoculum at which growth would occur in the liquid medium. The cell

count in the acidic leach water was found to be between 10^4 and 10^5 cells per ml, while laboratory cultures in the stationary phase showed counts between 10^7 and 10^8 cells per ml.

Cell suspensions of the bacterium oxidized ferrous ion rapidly with stoichiometric oxygen uptake. The $Qo_2(N)$ values ranged from 2400 to 4200, becoming considerably less with increased cell density. The $Qo_2(N)$ values for sulfur oxidation ranged from 120 to 320, being dependent on the amount of sulfur used and, interestingly, are usually increased by use of mixed ferrous ion and sulfur substrate. Endogenous activity as measured by oxygen uptake in air or CO_2-free atmosphere at 30°C in Warburg manometers was not detectable even after 10 hours, nor was fixation of CO_2 by cell suspensions in the absence of oxidizable substrate as measured by uptake of $C^{14}O_2$.

Carbon dioxide fixation by growing cultures or cell suspensions was determined by measuring cellular radioactivity after contact with $C^{14}O_2$. During growth about 1 u mole of CO_2 was fixed for each 100 u moles of ferrous ion oxidized; whereas, cell suspensions fixed about 1 u mole CO_2 for each 40 u moles oxygen absorbed while oxidizing either ferrous ion or sulfur. With mixed ferrous ion-sulfur substrate the efficiency of carbon dioxide fixation was greatly increased to values as high as 1 u mole CO_2 for each 13 u moles oxygen uptake.

A laboratory investigation was made to determine the effectiveness of the leaching streams at Kennecott's Bingham Canyon Mine as a nutrient solution for biological oxidation and solubilization of sulfide minerals by Decker (1959).

Using precipitation plant tailing solution as the nutrient, it was found that a pH of 3.0 and a temperature of 35°C were near the optimum for both oxidation of ferrous ion and for the solubilization of chalcopyrite. Nonbiological oxidation does not become appreciable below 50°C. Added nitrogen had little or no effect on oxidation rates and is assumed to be present in adequately available form in the solution. Oxidation rates increased greatly by an increase in aeration, while the presence of pyrite retards the solubilization of chalcopyrite due to preferential oxidation of pyrite.

It was concluded that the reduced form of the leaching stream (tailing solution) was an effective leaching fluid for the biological solubilization of chalcopyrite due to its relatively high pH (3.7 as compared to 2.7 for heading solution) and its nutrient content.

Reporting on the role of bacteria in the alteration of sulfide minerals, Malouf and Prater (1961) showed that it was possible to increase the tolerance of the bacteria to high acid and salt concentrations and to greatly increase the rate of bacterial oxidation above that found in nature. Active cultures were established in solutions containing as much as 15 grams per liter (gpl) of copper, as much as 40 gpl of iron, and in sulfuric acid concentrations high enough to yield a pH of 0.5. Iron oxidation rates were obtained as high as 20 grams iron per liter of solution per 24 hours and pyrite dissolution rates as high as 1.2 per cent of total present per 24 hours. This is approximately 1,000 times faster than the chemical oxidation of ferrous ion under similar conditions without the acid of bacteria.

The control of pH by the addition of small amounts of sulfuric acid to the waste dump leaching solution has been reported (Malouf and Prater, 1962) to increase the copper concentration of the solution by 30 per cent. This was brought about mainly by the dissolution of precipitates on and near the surface of the dumps, resulting in increased dump permeability and aeration. This in turn resulted in a higher bacterial oxidation rate, which increased solution temperature, further increasing mineral oxidation and dissolution. Field tests indicated that dump permeability was increased from two gallons per square foot per hour to 15 gallons per square foot per hour. With the increased rate of solution penetration shallow leaching channels are now replacing the expensive leaching ponds which were formerly necessary.

III. THE ROLE OF BACTERIA IN THE OXIDATION OF SULFIDE MINERALS

Investigations of the role of bacteria in the oxidation of sulfide minerals have been very scanty until recently. The effect of bacteria in the oxidation of pyrite was studied by Rudolfs (1922), who, with Helbronner, also studied the effect of bacteria on the leaching of zinc from zinc blends (Helbronner and Rudolfs, 1922). As a result of the work of Rudolfs and Helbronner, it has been ascertained that the oxidation process is biogenic, but it is not yet clear which bacteria act on pyrite and zinc sulfide or to what degree the bacteria accelerate the oxidation of these minerals.

In studying the biological oxidation of sulfides, an important agent was brought to light by the discovery of a new autotrophic organism, *T. ferrooxidans*, which was first isolated by Colmer and Hinkle (1947) in the acid waters draining coal mines.

In many coal beds there are inclusions of iron sulfide, such as pyrite or marcasite. As a result of oxidation of these inclusions, the waters in the mine become acid. In regions of bituminous coal in the U. S. A. acid drainage waters form in great quantities. They contaminate streams and cause corrosion of mine equipment. In this connection, the oxidation of coal inclusions is of great practical importance; and, in investigating the effect of bacteria on sulfides, special attention has been directed toward it.

Laboratory experiments of Temple and Delchamps (1953), and also of Leathen, Braley, and McIntyre (1953), on the influence of cultures of *T. ferrooxidans* and *T. thiooxidans* on samples of pyrite and marcasite extracted from coal demonstrated a considerable acceleration of the oxidation of these minerals over the rate occurring in a sterile control. The laboratory experiments have been supported by observations of Temple and Delchamps, who, in studying only exposed coal beds, found the reaction of the water in this zone to be neutral and detected no *T. ferrooxidans* or *T. thiooxidans*. But after a few days the water acquired high acidity and both species of bacteria were present in large numbers. Ashmeed (1955), studying the formation of acid in the coal mines of Scotland, concluded that four-fifths of

all the sulfuric acid that formed was due to the oxidation activity of bacteria.

In studying the oxidation of sulfur contained in coal inclusions, it has already been discovered that _T. ferrooxidans_ had the chief role in the oxidation of sulfide. This organism grew exclusively in the acidic environment, at a pH below 4.5. Colmer, Temple, and Hinkle (1950) have shown that this species oxidizes the lower oxides of sulfur as well as of iron. Although the capacity to oxidize sulfur compounds is denied by Leathen and others (Leathen, Kinsel, and Braley, 1956), experiments by a whole series of workers (Bryner, Beck, Davis, and Wilson, 1954; Bryner and Anderson, 1957; Bryner and Jameson, 1958), conducted on sulfides containing no iron, confirm the view that the organism does possess this capacity. In these experiments, the investigators used cultures isolated from the copper mine waters of Bingham Canyon, Utah. The experiments were carried out in percolators, in which ore was placed, mixed with sand, and a culture of _T. ferrooxidans_. In these percolators, the oxidation was effected except the control. In the experiments of Bryner, Beck, and others, in which bacteria acted on chalcopyrite, ten times as much copper went into solution as in the sterile control.

The mechanism of oxidation was obviously not the same for the various sulfides. Concerning the oxidation of pyrite (FeS_2), Temple and Delchamps (1953) believe this process to occur in nature according to the following scheme:

$$(1) \quad FeS_2 + H_2O + 3\tfrac{1}{2}O_2 \longrightarrow FeSO_4 + H_2SO_4$$

The first stage of oxidation is entirely chemical. Then the ferrous sulfate is converted to ferric sulfate.

(2) $2FeSO_4 + \frac{1}{2}O_2 + H_2SO_4 \xrightarrow{\text{T. ferrooxidans}} Fe_2(SO_4)_3 + H_2O$

Since this reaction is almost impossible chemically in an acid environment, the oxidation takes place through the influence of T. ferrooxidans. The ferric sulfate reacts with available pyrite, and the sulfur forming from this reaction is oxidized by T. thiooxidans.

(3) $FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3\ FeSO_4 + 2S$

(4) $S + 1\frac{1}{2}O_2 + H_2O \longrightarrow 2H^{+} + SO_4^{=}$

The merit of this scheme is that it includes both the purely chemical oxidation of pyrite and the role of bacteria in the process. It is clear that the indirect role of T. ferrooxidans, which involves the formation of ferric sulfate, is very large.

It is possible that T. ferrooxidans is able to act directly on the pyrite molecule (Silverman and Ehrlich, 1964). The direct contact mechanism is independent of the action of ferric ions, requiring only intimate physical contact between the bacteria and sulfide mineral under aerobic conditions. Operation of the direct contact mechanism is suggested by observation of bacterial acceleration of the rate of oxidation of iron-free sulfide minerals such as covellite (CuS), chalcolite (Cu_2S), tetrahedrite ($Cu_8Sb_2S_7$), and synthetic CuS and Cu_2S in essential iron free systems (Bryner and Anderson, 1957; Bryner et al., 1957; Ehrlich, 1962, 1963; Razzell and Trussell, 1963a).

However, this mechanism becomes difficult to demonstrate with iron-containing sulfide minerals such as arsenopyrite ($FeS_2 . FeAs_2$), bornite (Cu_5FeS_4), and chalcopyrite ($CuFeS_2$), owing to the release of soluble iron during oxidation.

The oxidation by F. ferrooxidans of untreated pyrite (FeS_2) as well as HCl pretreated pyrite (from which most of the acid-soluble iron species were removed) was studied manometrically by Silverman (1967). Oxygen uptake was linear during bacterial oxidation of untreated pyrite; whereas, with HCl-pretreated pyrite both a decrease in oxygen uptake and nonlinear oxygen consumption were observed. Ferric sulfate added to HCl-pretreated pyrite restored approximately two-thirds of the decrease in total bacterial oxygen uptake and caused oxygen uptake to revert to nearly linear kinetics.

Ferric sulfate also oxidized pyrite in the absence of bacteria and oxygen; recovery of ferric and ferrous ions was in excellent agreement with the reaction $Fe_2(SO_4)_3 + FeS_2 \longrightarrow 3FeSO_4 + 2S$, but the elemental sulfur produced was neglible. Neither H_2S nor $S_2O_3^{=}$ was a product of the reaction. It was proposed by Silverman that two mechanisms of bacteria pyrite oxidation operate concurrently: the direct contact mechanism which requires physical contact between bacteria and pyrite particles for biological pyrite oxidation, and the indirect contact mechanism according to which the bacteria oxidize ferrous ions to the ferric state, thereby regenerating the ferric ions required for chemical oxidation of pyrite.

IV. FACTORS INFLUENCING THE BIOLOGICAL OXIDATION OF SULFIDE MINERALS

Microbial oxidation involving the solubilization of copper sulfide ores, first demonstrated by Bryner et al. (1954) has been adequately confirmed by many investigators. The chief oxidizing agent was oxygen from the air which also supplied carbon dioxide as a source of carbon. These bacteria were thus similar to the green plants in making all cell materials from carbon dioxide and water, but differ from green plants in using iron, sulfur or metallic sulfides as the energy source rather than light. Such simple requirements depended on a very complex pattern of growth processes, few of which have yet been examined.

Razzell (1960) studied the bacterial leaching of metallic sulfides and indicated that stationary leaches (bottles) were easier to control and the reaction proceeded further than those in air-lift percolators. He also listed five conditions required for practical leaching:

(1) a pH of 2.0 to 4.0

(2) a low but definite concentration of soluble magnesium, ammonium and phosphate ions in the water

(3) an adequate supply of air

(4) a temperature above $40^{o}F$ and below $98^{o}F$

(5) a large bacterial population.

Of all these conditions, the control of pH was considered the most important and critical factor.

The bacterial oxidation of copper sulfide minerals was studied in the laboratory by Malouf and Prater (1961) to determine more effective methods of copper recovery from mine waste dumps. Malouf and Prater (1961) indicated that bacterial cultures became inactive, but were not destroyed, when exposed to direct sunlight. The bacteria were extremely sensitive to ultra-violet light. Only a short time exposure to this radiation would completely sterilize a bacterial culture. Temperature was found to exert a very pronounced effect on the rate of bacterial conversion of ferrous to ferric ions with maximum bacterial activity occurring at approximately 35°C. Temperatures above 50°C were found to destroy the bacteria. Below 35°C the rate of bacterial action decreased non-linearly as the temperature was reduced. The oxidizing bacteria were active in acid media with a pH of between 2.0 and 3.5. Both above and below this pH range the rate of bacterial oxidation decreased, and at pH values above 6.0 bacterial action was almost completely inhibited. Control of pH was necessary to prevent precipitation of ferric ion salts from solution. Such pH control did not affect bacterial oxidation rates adversely.

One of the most interesting findings from the laboratory of Malouf and Prater (1961) was that bacterial oxidation rates depended upon the extent of the surface area of solid material present in the oxidation columns. These observations indicated that the bacteria concentrates at the surface of solids in contact with the solution, and builds up a large bacterial colony there. It was also indicated

that the iron oxidation rate was increased by increasing the aeration rate or the solution circulation rate.

The effect of some factors on iron oxidation by T. ferrooxidans also has been extensively studied by Ivanov (1962) who concluded that T. ferrooxidans greatly accelerated the oxidation of ferrous sulfate to ferric sulfate at relatively low iron ion concentrations in the solution. In the oxidation of ferrous sulfate by T. ferrooxidans in the presence of sulfide minerals, a dynamic equilibrium between ferrous and ferric ion was established. The point of balance depended on the relative rates of oxidation of the ferrous ion by T. ferrooxidans and reduction of the ferric ion by sulfides.

Of the two forms of iron, ferrous and ferric, only ferric ion was a sulfide oxidant; and an excess of ferrous ion hindered oxidation. In the oxidation of ferrous sulfate by T. ferrooxidans in the presence of easily oxidized sulfides, which were most promising for industrial leaching, the balance between ferrous and ferric ion in the solution was considerably shifted in favor of ferrous ion. Hence, the acceleration of bacterial leaching of small sulfides required the oxidation of ferrous ion by T. ferrooxidans both directly in the percolator with the material for leaching and in an additional separate apparatus with the subsequent return of the regenerated solvent into the sulfide leaching cycle.

Laboratory studies were made and evaluated by Jones (1963) on the biological oxidation of sulfur to sulfuric acid. An optimal

temperature was found to be about 30°C. The biological oxidation of sulfur to sulfuric acid continued until the acidity reached a pH of almost 1.0. The highest biological activity was in the range of a pH of 2.0 to 3.0. Optimal nutrient solutions were developed using either ammonium nitrate or ammonium chloride as a nitrogen source. Both disodium hydrogen phosphate and dipotassium hydrogen phosphate were effective phosphate sources. It was demonstrated that metal cations had an initial toxicity to the biological oxidation process. As the concentration of the cations was increased, the oxidation rate of the sulfur decreased. An optimum concentration of sodium chloride was found to be 2000 ppm in the biological oxidation of sulfur to sulfuric acid. The oxidation rate was nearly doubled when the oxidation was run in the dark. The highest biological oxidation rates were obtained from samples with the largest surface area.

Razzell and Trussell (1963a) showed that the biological leaching rate of chalcopyrite was more rapid with fine particles than with coarse ones; however, the leaching rate was independent of the quantity of mineral present and it is said that the most critical factor in leaching is the alkalinity of the gangue about mineral particles since bacterial action will not proceed appreciably above pH 3.0. Furthermore, alkaline gangues favor surface precipitation of iron which carries down some copper from solution and may further slow leaching by presenting a physical barrier preventing access of bacteria to the mineral surface.

Some of the factors affecting the air oxidation of sulfide minerals was further studied by Bryner et al. (1966). The effect of temperature on the biological oxidation of pyrite and copper (++) sulfide was studied over the range from 25°C to 75°C. The optimal temperature for biological oxidation of pyrite was near 35°C and activity dropped off to a minimum near 65°C with a slight increase at 75°C. The optimal temperature for the biological oxidation of copper (++) sulfide was 35°C and a minimum at 55°C. Ferric ion was known to be effective in the oxidation of many sulfide minerals. Activated carbon was used to increase the oxidation of ferrous ion in the presence of the mineral, and it was found that the effect of activated carbon on the copper (++) sulfide was marked. This indicated that activated carbon had a surface action effect on the oxidation of the mineral in addition to its effect on the oxidation of ferrous ion.

In the oxidation of copper (++) sulfide the bacteria required an optimal ferrous ion concentration of about 0.5 g/l. Studies with carbon also indicated that only a small amount of ferrous ion was needed for efficient oxidation of copper (++) sulfide.

The air oxidation of pyrite in 0.1 N sulfuric acid, with ferrous ion and activated carbon or ferric ion was 15 times faster than when the iron salts were absent. The optimal pH for the biological oxidation was around 3.0 and the activated carbon was more effective at pH 1.0.

MATERIALS AND METHODS

I. MEDIA

A. Liquid Media Used

1. Natural mine waters were used as media in this study and was obtained from the tailings pond of Bingham Canyon dump. Sterilization of the natural mine water was accomplished by filtration through a Seitz filter.

2. Medium 9K (Leathen et al., 1958). The final composition of the medium was as follows:

Solution A:	$(NH_4)_2SO_4$	3.0 grams
	KCl	0.1
	K_2HPO_4	0.5
	$MgSO_4 \cdot 7H_2O$	0.5
	$Cu(NO_3)_2$	0.01
	Distilled water	700.0 ml
	10 N H_2SO_4	1.0 ml
Solution B:	$FeSO_4.7H_2O$, 300 ml of 14.74% (w/v) solution	

The two solutions above were autoclaved separately at 121° for 15 minutes, and were mixed together immediately before use.

3. Medium TC (Temple and Colmer, 1951).

$FeSO_4.7H_2O$	10.0 grams
$MgSO_4.7H_2O$	0.1

$(NH_4)_2SO_4$	0.5 grams
Distilled water	1,000.0 ml

Sterilization was accomplished by autoclaving for 15 minutes at 121°C. The final pH was adjusted to 2.5 with concentrated sulfuric acid.

4. Sodium thiosulfate medium (Colmer et al., 1950).

$Na_2S_2O_3.5H_2O$	5.0 grams
KH_2PO_4	3.0
$(NH_4)_2SO_4$	0.2
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.2
Distilled water	1,000.0 ml

The medium was autoclaved at 17 pounds pressure for 15 minutes. The final pH was adjusted to 4.7 to 5.0.

5. Waksman medium (Waksman, 1921). This medium was used for isolation of free sulfur oxidizing bacteria. The Waksman medium consisted of the following:

$(NH_4)_2SO_4$	2.0 grams
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.5
KCl	0.5
$FeSO_4.7H_2O$	0.01
Sulfur	10.00
$Ca_3(PO_4)_2$	10.00
Distilled water	1,000.0 ml

The medium was distributed in 25 cc portions into 125 cc Erlenmeyer flasks and sterilized in flowing stream for 30 minutes on three consecutive days.

B. Solid Media Used

1. Medium 9K Silica gel. The liquid medium 9K was solidified by adding treated Ludox (as described in section VI. B.) in a ratio of 1.4 to 1.0 and autoclaved for 10 minutes at 121°C.

2. Medium T. C. Silica gel. The liquid medium T. C. was solidified by adding treated Ludox in a ratio of 1.5 to 1.0 and autoclaved for 10 minutes at 121°C.

3. Sodium thiosulfate agar. The liquid medium containing 0.5 per cent of $Na_2S_2O_3$ was solidified by the use of 1.5 per cent agar.

4. Mycophil agar. Formula in grams per liter.

Phyton	10
Dextrose	10
Agar	16

Thirty-six grams of the powder were suspended in a liter of distilled water and sterilized at 118°C for 15 minutes in the autoclave. The pH was adjusted to 4.0 by adding 15 ml of sterile 10 per cent lactic acid prior to pouring plates.

5. Sabouraud dextrose agar. Formula in grams per liter.

Neopeptone	10
Dextrose	40
Agar	15

Sixty-five grams of dehydrated medium were suspended in a liter of distilled water, sterilized in the autoclave for 15 minutes at 121°C, and poured in petri dish plates.

II. METHODS OF ANALYSES

A. Determination of Soluble Iron

The total soluble iron (Fe^{++} and Fe^{+++}) of a solution was determined spectrophotometrically with potassium thiocyanate (Sandell, 1959). To a sample containing 2 to 9 ug of total iron in 4 ml of water were added 0.5 ml of 6 N HCl and 0.05 ml of 0.6% hydrogen peroxide. After the solution had been thoroughly mixed, 0.5 ml of 2% potassium thiocyanate was added. The solution was remixed and the absorption at 440 mu determined with a Beckman Model DU Spectrophotometer immediately. Soluble ferric ion was determined as above, without hydrogen peroxide, and the ferrous ion determined by difference between total iron and ferric ion. A standard curve was prepared by use of reagent grade ferrous ammonium sulfate ($Fe(SO_4)_2(NH_4)_2.6H_2O$).

Table 1 shows the relation between various concentrations of ferric ion and its optical density at 440 mu.

A linear relationship between optical density and ferric ion concentration in the range of 2 ug/ml to 9 ug/ml is obtained as shown in Figure 1. From the curve in Figure 1, the following relationship may be derived: Fe^{+++} (ug/ml) = 21.88 x A_{440}.

Table 1. Relation between the optical density and ferric ion concentration.

Concentration of ferric ion (ug/ml)	Optical density at 440 mu
1.0	0.031
2.0	0.093
3.0	0.121
4.0	0.189
5.0	0.227
6.0	0.285
7.0	0.331
8.0	0.365
9.0	0.402
10.0	0.395

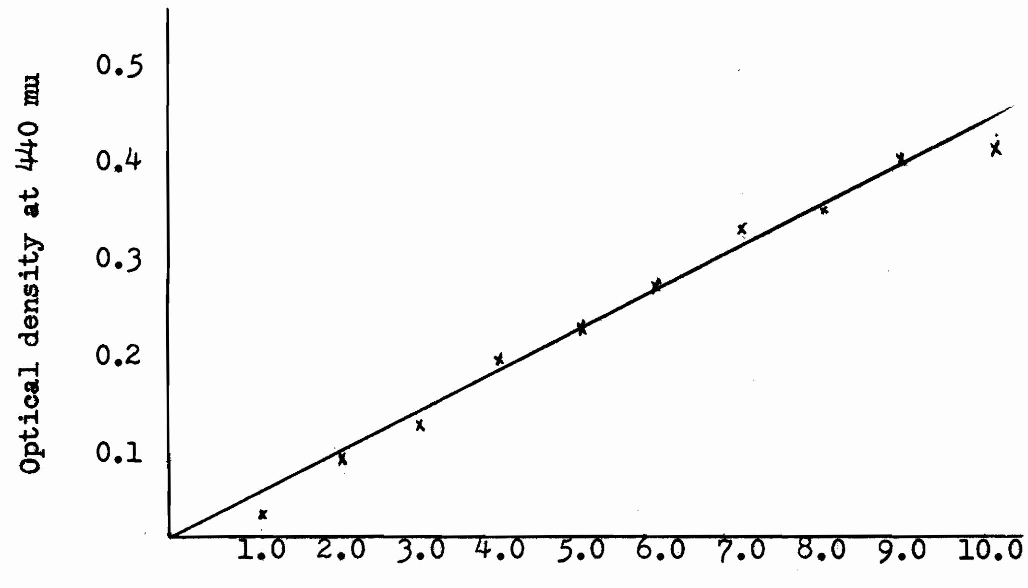

Figure 1. Standard curve of ferric ion solution.

B. Determination of Soluble Copper

Soluble copper salts were measured spectrophotometrically after reacting with 1,10-phenanthroline (Wilkins and Smith, 1953). One ml of 0.002 M 1,10-phenanthroline in 95 per cent ethyl alcohol, 2 ml of 5 per cent iron and copper free hydroxyl-ammonium chloride and 5 ml of phosphate buffer sufficient to bring the pH to 7.0 to 8.0 were added to a sample solution containing soluble copper salts contained in a 60 ml separatory funnel. Four ml of n-octyl alcohol were added and the mixture shaken for 2 minutes. This was allowed to stand for 5 minutes to facilitate the separation of the liquid layers. The lower, aqueous layer containing the red ferrous-1,10-phenanthroline compound was then drown off and the n-octyl alcohol layer, containing the yellow cuprous-1,10-phenanthroline compound was transferred to a 25 ml volumetric flask. The funnel was washed with a little 95 per cent ethyl alcohol and the washings added to the volumetric flask after which the mixture was diluted to the mark with 95 per cent ethyl alcohol. The absorbancy of the n-octyl alcohol layer was measured at 435 mu and the amount of copper present determined by reference to a standard curve which had been prepared with known concentrations of reagent grade copper sulfate ($CuSO_4.5H_2O$). The Beckman Model D. U. Spectrophotometer was used for the determination of copper.

Table 2 shows the relation between copper concentration and optical density at 435 mu.

Table 2. Relation between optical density and copper concentration.

Copper concentration (ug/ml)	Optical density at 435 mu
1.0	0.071
2.0	0.315
3.0	0.291
4.0	0.302
5.0	0.412
6.0	0.340
7.0	0.578
8.0	0.485
9.0	0.720
10.0	0.684

A linear relationship between optical density and copper concentration in a range of 1.0 ppm to 7.0 ppm was obtained as shown in Figure 2. From the curve in Figure 2, the following relation may be derived: Cu (ug/ml) = 11.72 x A_{435}.

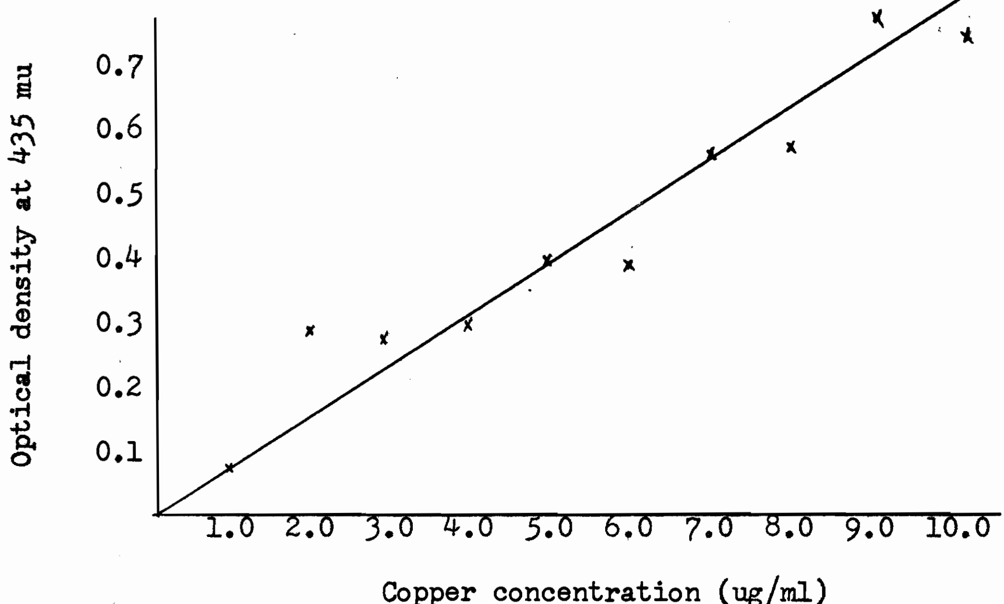

Figure 2. Standard curve of copper solution.

C. Determination of pH Values

The pH values of the various solutions were determined with a Beckman Model H Glass Electrode pH meter.

III. ISOLATION OF MICROORGANISMS

A. Enrichment Dilution Technique

Mine dump water from the Bingham Canyon dump served as the source of microorganisms. Twenty-five ml of sterile 9K medium or TC medium were placed in a sterile 125 ml Erlenmeyer flask. Each flask was inoculated with 1 ml of mine dump water, closed with a cotton stopper and incubated at room temperature. The flasks were shaken on an oscillating type shaking machine during incubation.

After the media had become turbid and there was a precipitate on the sides of the flask, 1 ml was taken to serve as the inoculum for another 25 ml of 9K or TC medium in a similar flask. Successive transfers were made as soon as the characteristic precipitate was formed, for a total of three transfers.

Streak plates of 9K medium silica gel, TC medium silica gel, and sodium thiosulfate agar were made from the successively transferred flasks. Although the fresh mine waters might be plated, the successive transfer method proved to be the easier technique to gain pure cultures.

B. Membrane Filter Technique

A fresh sample of mine dump water from the tailings pond located immediately below the precipitation plant was obtained. A 10 ml sample

was filtered through a 0.4 micron pore diameter membrane filter, and the filter then placed on a paper pad soaked with the salt solution medium 9K, described by Leathen et al. (1958). The plate was then incubated at room temperature for 48 hours.

IV. MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES OF THE ISOLATED ORGANISMS

A. Organisms Obtained by the Enrichment Dilution Technique

Morphology of the bacteria were studied by the conventional Gram staining technique and by the use of wet mounts from the successively transferred flasks.

In addition to the two iron media described above, the liquid sodium thiosulfate medium of Colmer et al. (1950) was also used to test whether or not there were chemolithotrophic bacteria which could utilize $Na_2S_2O_3$ as an energy source. Twenty-five ml of the sterile 0.5 per cent sodium thiosulfate medium were placed in a sterile 125 ml Erlenmeyer flask and the flasks inoculated with 1 ml of mine dump water and incubated at room temperature. As soon as the media became turbid and the pH decreased to 2.5, one ml was taken to serve as the inoculum for another 25 ml of sodium thiosulfate broth in a similar flask. A total of three transfers were made. Streak plates on 0.5 per cent $Na_2S_2O_3$ agar and 9K medium silica gel were made from the successively transferred flasks.

The oxidation of elemental sulfur was determined by attempting to grow the microorganism in Waksman's medium (1922). The flasks containing 25 ml of Waksman's medium were inoculated with 1 ml of mine

dump water and incubated at room temperature. After the medium became cloudy and the pH decreased to 2.0, one ml was taken as inoculum for another 25 ml of this media in a similar flask. A total of three transfers were made. Streak plates of sodium thiosulfate agar and 9K medium silica gel were made from the successively transferred flasks.

Four air lift percolators (Bryner et al., 1954) were prepared and charged with 200 ml of 9K medium. Three of these were inoculated with 1 ml of culture isolated on sulfur, sodium thiosulfate and ferrous ion (9K medium) respectively; the other was left as a control to be aerated but uninoculated. All percolators had approximately two inches of coarse glass wool in the bottom. The air passing through the percolators was saturated with water and then sterilized by passage through a column packed with sterilized nonabsorbent cotton. The four percolators were aerated for a period of ten days. Each percolator was sampled every 24 hours and the oxidation of ferrous ion was determined by the Sandell method (1959).

B. Organisms Obtained by the Millipore Filter Technique

Microscopic examination of the colonies growing on the membrane filters showed these organisms not to be *Thiobacillus ferrooxidans* but a yeast-like organism. In a search of the literature dealing with the autotrophic organisms associated with mine water, only one article by Razzell et al. (1963b) mentioned a yeast-like organism presented growing in conjunction with *Thiobacillus ferrooxidans*. These authors have

apparently identified this organism as belonging to the genus Candida. The obvious question was whether or not this was an autotrophic yeast. A test for the autotrophism of this microorganism was made by seeding it to both acidified mycophil agar and Sabouraud dextrose agar. An experiment was designed to determine if a yeast-like organism isolated from the Bingham mine dumps would grow on a completely inorganic medium. This was done by seeding a single colony to each of six percolators (Table 3) which contained 200 ml of 9K medium as a nutrient. Three of these had approximately two inches of coarse glass wool in the bottom; whereas, the other three did not contain the glass wool.

Table 3. Tabulated description of air-lift percolators for autotrophism test of yeast-like organism.

Percolator No.	Percolators with glass wool in the bottom (9K medium) 200 ml	Percolator No.	Percolators without glass wool in the bottom (200 ml 9K medium)
1	Control (without inoculum)	4	Control (without inoculum)
2	Seeded one single colony obtained from membrane filter	5	Seeded one single colony obtained from membrane filter
3	Seeded one single colony obtained from mycophil agar	6	Seeded one single colony obtained from mycophil agar

The six percolators were aerated for a period of eight days. Each percolator was sampled four times over this period and cultured

on membrane filters, silica gel and agar plates in which the 9K medium was used as a nutrient.

V. DISTRIBUTION OF YEAST-TYPE ORGANISMS

A membrane filter technique was used to count yeast-type organisms in the mine dump waters eminating from various areas of the dump. Fresh samples of mine dump waters from different locations of Bingham Dumps were obtained as shown in Table 4.

Table 4. Location of water samples obtained from Bingham Canyon Mine Dump for yeast counts.

Sample No.	Location of sampling
J-S-1	Stream from east dump
J-S-2	Upper small pond - east dump
J-S-3	Precipitate plant tailings pond
J-S-4	West dumps - upper pond
J-S-5	West dumps stream
J-S-6	Head of precipitation plant
J-S-7	Middle of precipitation plant
J-S-8	End of precipitation plant

One hundred fifty ml of each sample was allowed to stand unstoppered at room temperature (22°C) for the period of 14 days. Ten ml of each sample were taken daily over this period and filtered through a 0.4 micron pore diameter membrane filter and the filter then placed on a paper pad soaked with the inorganic iron salts medium 9K described by Leathen et al. (1958).

A large volume (30 ml) of mine dump water samples were poured through millipore membrane filters in order to pick up yeast-type colonies from those samples which were not found in small (10 ml) volumes as tested above.

VI. METHODS OF BACTERIAL COUNTS

A. Petroff-Hausser Technique

A phenol crystal-violet dye was prepared for staining the bacteria for the Petroff-Hausser counting chamber. This was done by mixing 25 ml of a 0.5 per cent phenol solution with 0.5 ml of a saturated alcoholic crystal violet solution. The resulting mixture was filtered twice through double thick Whatman No. 1 filter paper to remove undissolved particles. This dye preparation was then added in the amount of 0.2 ml to a 1.0 ml culture sample. After vigorous mixing, the Petroff-Hausser counting chamber was filled with this stained preparation and counts were made.

In making counts 50 squares were randomly selected and the number of organisms per square was recorded by focusing up and down through the cube formed between the bottom of the cover slip and the top of the counting chamber and the four sides of the etched square. No trouble was encountered in differentiating the blue stained organisms and odd shaped yellowish precipitate particles which were sometimes present. No square was counted which contained a large piece of this material. A square of the counting chamber is 1/20 mm by 1/20 mm and the chamber is 1/50 mm deep. Therefore, if the average

number of bacteria per square is multiplied by 20 by 20 by 50 by 1,000 by a dilution factor of 1.2, one arrives at the number of organism per ml of the original culture sample. This final factor turns out to be 2.40×10^7. Statistical evaluation of Petroff-Hausser count for estimating bacterial populations was studied by counting the same mine water sample 20 times. The mean counts, standard deviation, coefficient of variation and confidence limits of the sample were then calculated.

B. Surface Plating Technique

Silica gel plates were used in an attempt to develop colonies of *Thiobacillus ferrooxidans* rapidly on the surface of plates. Silicic acid was prepared by using a sodium silicate compound called "Ludox." Ludox is an opalescent aqueous colloidal solution, containing approximately 40 per cent of SiO_2. This material was treated by passing through an ion exchange column. This column was constructed of Pyrex glass tubing 2 inches in diameter and 24 inches long, with a stopcock at the bottom. The column was packed with 500 grams of analytical grade "Dowex" 50 (Na^+) resin, which had been treated with HCl to change it to the H^+ from. By replacement of the Na^+ ion with H^+ ion, the Ludox then became silicic acid (H_2SiO_3). The acid collected at the bottom of the column had a pH of 2.0 ± 0.2. The operating efficiency of the column was followed by the use of wide range pH indicator paper. All fractions were discarded until a pH of 2.0 was reached. Exhaustion of the resin was indicated by a rapid rise in pH

to that of the sodium silicate solution. Passage of 1,000 ml of 10 per cent HCl through the resin completely regenerated the column.

Silica gel plates were prepared by adding silicic acid to medium 9K or natural mine water in a ratio of 1.4 to 1.0 and then heating the plates in the autoclave for 10 minutes at 121°C which sterilized and solidified the medium. Five-hundredth ml to 0.1 ml of culture samples obtained from the successively transferred flasks, percolators, and fresh mine water were put on the surface of medium 9K silica gel and mine water silica gel plates and spread with a glass rod over the surface of the plate. These plates were incubated at room temperature for 10 days to 14 days. In order to check the effect of a larger inoculum, a drop of the culture was put onto these plates and allowed to incubate without spreading.

C. Radioactive Isotope Technique

An attempt to determine the viable cells of iron oxidizing bacterium in the mine waters, radioactive isotope P^{32} was considered for labeling to detect viable bacterial cells. Mine dump waters from the Bingham Canyon dump served as the sources of the iron-oxidizing bacterium. Carrier free radioactive phosphorus as $H_3P^{32}O_4$ (in 0.02 N HCl) was obtained from New England Nuclear Corp., Boston. The volume received was diluted with sterile distilled water to give a concentration of 1 uc of P^{32} per ml.

Incubation medium and labeling procedure: The composition of the incubation medium is presented in Table 5. This solution was sterilized

in the autoclave at 121°C for 15 minutes. To 8 ml of this solution, in a sterile 25-ml Erlenmeyer flask, were added 1 ml of P^{32} solution (1 uc/ml) and 1 ml of culture solution. A blank without cells was prepared by substituting 1 ml of sterile glass-distilled water (GDW) for the culture solution. The flasks were incubated at room temperature in a shaker for one hour.

Table 5. Compositions of incubation medium.

Component	Concentration
KCl	0.05 G/l
$(NH_4)_2SO_4$	0.15 g/l
P^{32}	1 uc/ml

The P^{32} retained by the cells was detected as follows: A 1 ml portion of the incubation mixture was filtered through a sterile millipore filter. The cells on the filter paper were then washed by passing different volumes of sterile GDW, 0.01 NNH_4OH, or 0.01 NHCl through the filter.

Each incubation mixture was sampled five times. One ml portions of the incubation medium prepared without cells were treated in the same manner as those containing cells to serve as blanks. Since only a limited number of filter holders were available, the funnel of the filter apparatus was rinsed in sterile GDW between samplings.

After the filtrations and washings, each filter was air dried and then placed on a clean planchet. The radioactivity retained on the filters, both in the case of the samples containing cells and the blanks, was determined with a thin end window Geiger-Muller tube attached to a Picker scaler. The scaler was preset to record 2,560 counts and the time to accumulate this number of counts in the case of each sample was determined. Results were recorded as counts per minute per milliliter of incubation mixture filtered. The operating voltage for G-M counter was selected at 1350 volts.

VII. DISTRIBUTION OF IRON OXIDIZING BACTERIUM IN MINE DUMP WATERS

In an attempt to find if there is a relationship between the numbers of Thiobacillus ferrooxidans in leaching systems and the chemical change which are known to occur, bacterial population in water samples collected from different locations of the Bingham Canyon dump were counted.

Fresh samples of mine dump waters from different locations of Bingham Mine dumps were obtained as shown in Table 6.

The temperature of water samples was measured immediately after sampling from each location. The pH, soluble copper and soluble iron (Fe^{++} and Fe^{+++}) were determined as soon as the samples reached the laboratory. The bacteria population in each water sample was estimated by the use of the Petroff-Hausser technique.

Table 6. Description of the water samples obtained from Bingham Canyon Mine dump for bacteria counts.

Sample No.	Description of sample
1	Midas
2	Markham
3	Blue water #1
4	Blue water #2
5	Ppt. plant, heading
6	Ppt. plant, tailing
7	Small pond
8	Large pond

VIII. CORRELATION BETWEEN THE CONVERSION OF FERROUS TO FERRIC ION AND BACTERIAL GROWTH

Two air lift percolators were prepared and charged with 200 ml of medium 9K (pH 3.5). One percolator was inoculated with 2 ml of mine dump water obtained from precipitation tailing pond, the other was left as a control to be aerated but uninoculated. Sterile air saturated with water was used to circulate the solutions. These two percolators were aerated for a period of 12 days at room temperature. Each percolator was sampled every second day for 12 days, and the number of bacteria and the conversion of ferrous to ferric ion ($Fe^{++} \rightarrow Fe^{+++}$) were determined by use of the Petroff-Hausser technique and the Sandell method (1959) respectively.

EXPERIMENTAL RESULTS

I. CHEMOSYNTHETIC AUTOTROPHS

A. Morphological Characteristics

The bacteria found in the leaching streams in Bingham Canyon, Utah, were short rod, usually appearing separately, but sometimes grouped in pairs. They were motile and about 0.5 to 0.8 u by 1.0 to 1.3 u. The cells were Gram negative, non-sporeforming and were difficult to stain. Smears of cell suspensions either did not stain, or stained weakly with carbol fuchsin, safranin, malachite green, crystal violet and methylene blue. Smears were stained, however, either by steaming for 10 minutes in carbol fuchsin, or by applying safranin for 10 minutes in the Gram staining technique.

The bacteria formed extremely small colonies on silica gel with ferrous ion as substrate. In dense cultures where the colonies were located close to each other, an amber-colored iron hydroxide sediment appeared surrounding the colonies. The sediment usually began to form at the center of the colonies and spread outward, sometimes forming lobes.

It was also observed that the growth of iron-oxidizing bacteria on sodium thiosulfate agar frequently showed colonies with a covering of white, needle-like frosty material as shown in Figure 3 (see at end of the Results).

B. Physiological Characteristics

Ferrous ion, sodium thiosulfate, and sulfur were used as oxidizable substrates in the enrichment-dilution technique. After three transfers, it was assumed that the isolation of the organism had been accomplished. This was substantiated by observing that stained preparations showed uniform morphological characteristics.

Growth in sterile acid mine water and ferrous ion broth was indicated by a murky appearance and an amber-colored pellicle which formed across the surface of the liquid and on the sides of the flasks. A microscopic examination of a wet mount of the surface pellicle showed flakes of the precipitated iron compound with entrapped microorganisms.

On sodium thiosulfate medium, the liquid became turbid, and the pH of medium decreased from 5.0 to 2.5. A wet mount showed globules of sulfur and organism when studied under the microscope. Crystals of undetermined composition were formed in the medium.

Waksman medium containing sulfur as a source of energy became uniformly cloudy, without surface growth or sediment formation. The original reaction of the medium was pH 4.5. As the substrate was oxidized, the medium became gradually acid and the pH was reduced to 2.0 by the seventh day of incubation as shown in Table 7.

Upon examination of the culture under the microscope, it was found to contain an abundance of very minute, active motile bacteria. It was also found that the culture isolated on sulfur and sodium thiosulfate failed to oxidize ferrous ion as shown in Figure 4.

Table 7. Age of culture and change of pH in Waksman medium.

Age of culture days	pH
zero time	4.5
1	4.5
2	4.2
3	3.8
4	2.9
5	2.3
6	2.1
7	2.0

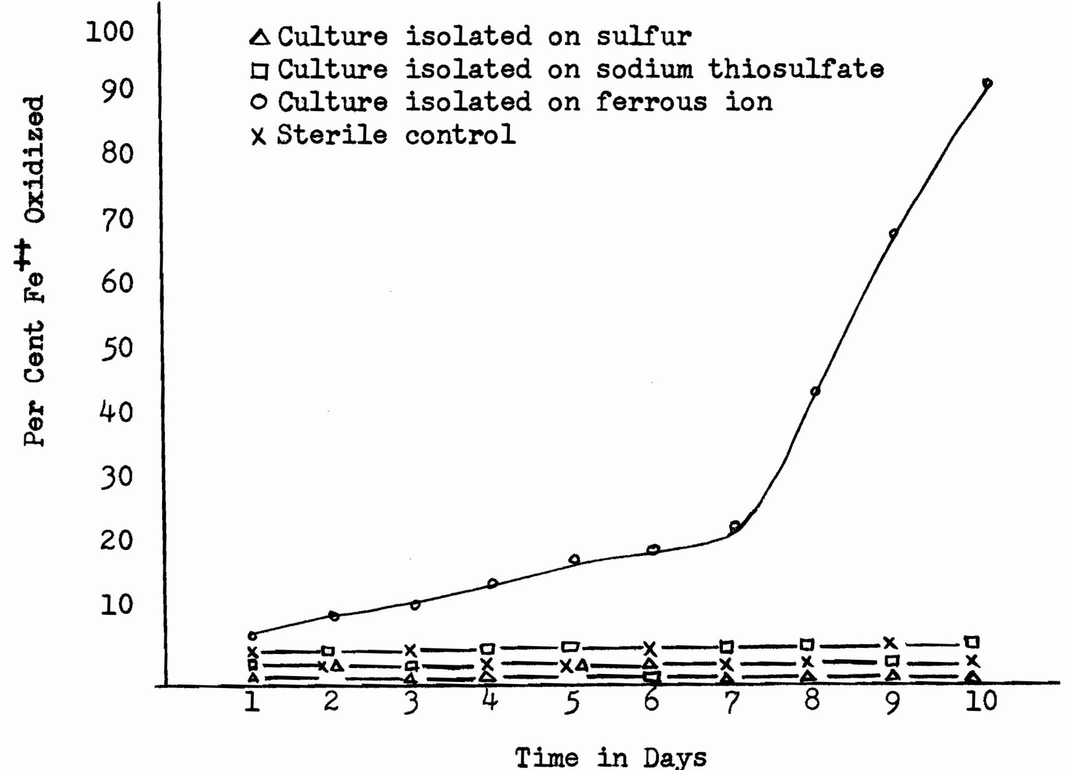

Figure 4. A comparison of the oxidation of ferrous ion by cultures isolated on sulfur, sodium thiosulfate and ferrous ion.

The bacteria isolated on ferrous ion could, however, grow on sodium thiosulfate agar, exhibiting a typical amber color about the growing colonies. Microscopic examination showed that the culture was morphologically similar to that found on silica gel with ferrous ion as the energy substrate.

It was found that the culture isolated on sulfur would also grow on sodium thiosulfate agar but would not grow on the ferrous ion silica gel plate.

II. YEAST-TYPE ORGANISM

A. Morphology and Cytology

A microscopic study of the colonies which were picked up on the Millipore membrane filter showed these organisms to be yeast-like organisms but not bacteria. Figure 5 (see at end of Results) presents a polaroid photograph of a cluster of yeast-like organisms stained by the Gram staining method. A deep green filter was used to accentuate the contrast of these cells with the background.

Examination of several of these colonies by the Gram stain presented an interesting microscopic growth pattern:

1. When lightly stained, the organism presented deeply Gram positive internal granules with a Gram negative cytoplasm. The larger, and apparently older cells, were filled with a greater amount of the Gram positive staining material, while the small, apparently younger cells, stained almost entirely Gram negative.

2. The cells regularly appeared in interesting clusters most often with a large deeply staining Gram positive cell surrounded by smaller cells. The Gram positiveness stained with varying intensity. This was interpreted possibly to be a state of aging. The picture suggested a parent cell with smaller younger cells remaining near and about the parent cell. These younger cells apparently had not grown to the size of the parent spheres. This is probably due to competition in the rather unfavorable inorganic environment. The two photographs of Figure 5 illustrate this grouping. The variation in staining intensity from light through dark illustrates the different intensity of Gram positive staining.

B. Physiology

The yeast grew very well on both acidified mycophil agar and Sabouraud dextrose agar, much better, in fact, than on the filter membranes. Most of the colonies were deep orange in color on both media, but some white mutants were interspersed among the amber colonies. Both macro and micro colonies were seen on both media as shown in Figure 6 (see at end of the Results). The microcolonies were in every instance colorless. Some were so small as to be hardly visible when viewed without the aid of a microscope.

Growth on the organic substrate changed the microscopic appearance of these cells. They had changed from the almost round spherical cells to an elongated form to become large ellipsoidal cells. Budding appeared to be terminal with plasmodesma joining the buds to the parent

cells. There were no groupings of the cells around a central large sphere as had been observed in cells from colonies off membrane filters. Figure 7 (see at end of the Results) presents polaroid photographs of the cell as grown on the organic media. These may be compared with the picture of cells in Figure 5. For comparative purposes Figure 8 (see at end of the Results) presents a photograph of the yeast-like organism interspersed with cells of *Staphylococcus aureus*.

The results of an experiment to determine whether or not the yeast-like organism was a strict autotroph showed that it was not strictly autotrophic. No growth was observed on any of the membrane filters, silica gel or agar plates with 9K medium as nutrient which were seeded from the percolators.

C. Distribution of Yeast-Like Organisms in Mine Dump Waters

Table 8 shows a definite increase in the yeast colony count of the mine water sample (J-S-3) obtained from precipitation plant tailings pond over a period of two weeks. Figure 9 graphically presents the data of Table 8. Except for J-S-3, no yeast were found in the 10 ml samples by this method of examination. However, the yeast-type colonies could be found on membrane filters from each location at the Bingham mine dump when a large volume (30 ml) of mine dump water was used as shown in Table 9.

Table 8. Growth of yeast-like cells.

Days	No. of yeast per ml of mine water (Medium 9K)	No. of yeast per ml of mine water (Medium TC)
1	0	0
2	0	0
3	9	0
4	75	0
5	192	112
6	996	640
7	7,000	4,500
8	8,300	5,300
9	11,600	7,000
10	13,000	9,000
11	15,000	11,500
12	16,200	13,000
13	24,000	20,000
14	32,400	21,600

Table 9. The number of yeast colonies found on the membrane filters when these were seeded with large volume (30 ml) of mine dump waters.

Mine dump water	Ml of mine dump water seeded	No. of colonies on membrane filter
J-S-1	30 ml	4
J-S-2	30 ml	7
J-S-4	30 ml	5
J-S-5	30 ml	13
J-S-6	30 ml	10
J-S-7	30 ml	3
J-S-8	30 ml	3

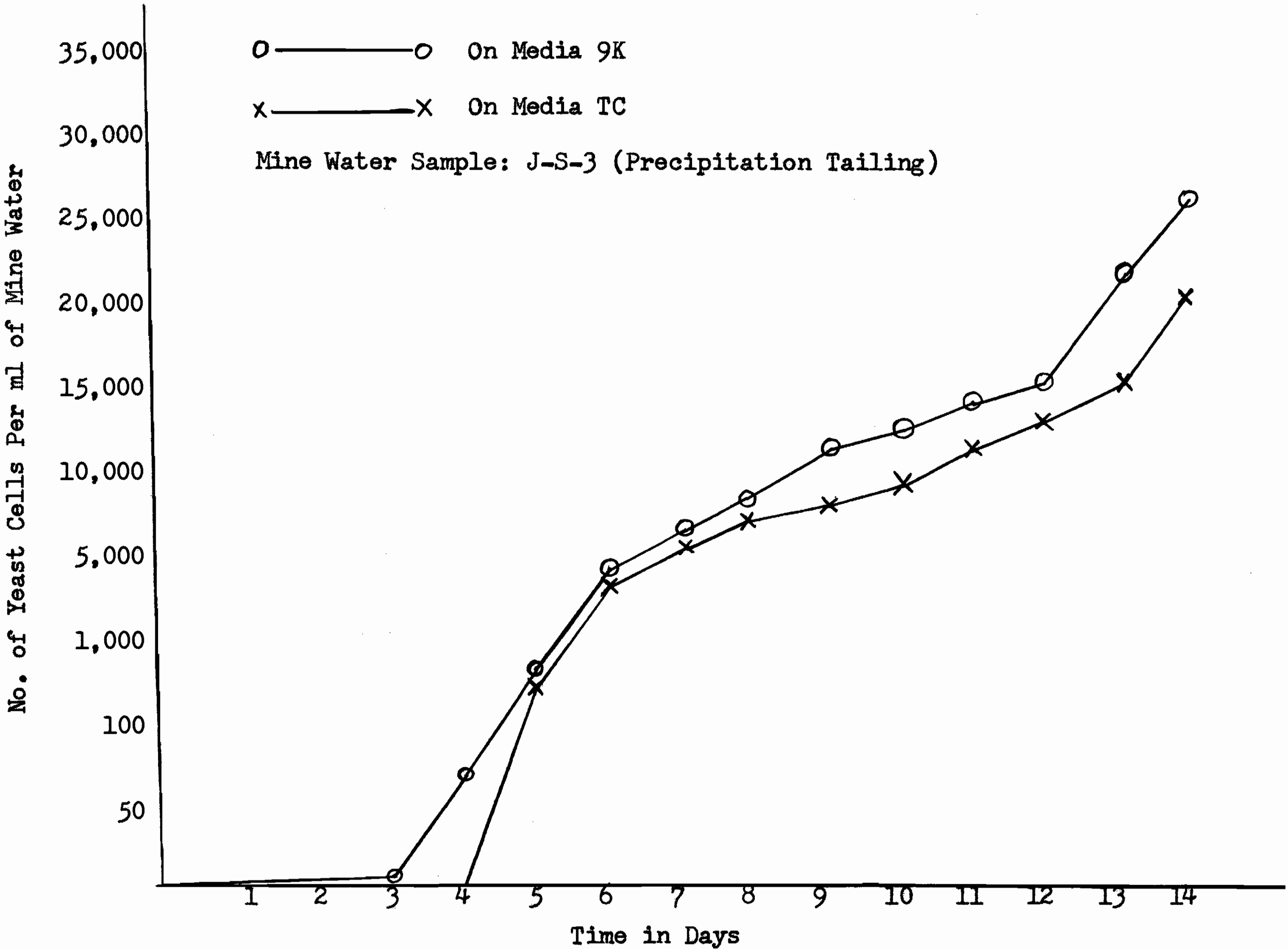

Figure 9. Increase in the number of yeast cells per ml of mine water.

III. STATISTICAL EVALUATION OF THE PETROFF-HAUSSER TECHNIQUE FOR ESTIMATION OF BACTERIAL POPULATION

The purpose of presenting statistical data was to check the accuracy of the counting chamber and to evaluate the reliability of the obtained results.

Bacteria counts of a sample of mine water was repeated 20 times by use of a Petroff-Hausser counting chamber. In making counts, 50 squares were randomly selected and the number of organisms in these 50 squares was recorded. The results, individual counts on a single culture of bacteria, are shown in Table 10.

Table 11 presents the distribution by tabulating the frequency associated with certain class intervals. Figure 10 is a frequency histogram and polygon plotted from Table 11.

Table 10. Results of direct microscopic counts on a single culture sample (in 50 squares of counting chambers).

No.	Count	No.	Count
1.	95	11.	105
2.	90	12.	120
3.	100	13.	125
4.	85	14.	114
5.	110	15.	110
6.	75	16.	95
7.	110	17.	89
8.	140	18.	108
9.	125	19.	102
10.	105	20.	120

Table 11. Frequency distribution (using class interval) for data of Table 10.

Class interval	No. of results in specified class intervals = frequency (f)	Relative frequency (r.f.)
70-80	1	0.05
80-90	3	0.15
90-100	3	0.15
100-110	7	0.35
110-120	3	0.15
120-130	2	0.10
130-140	1	0.05

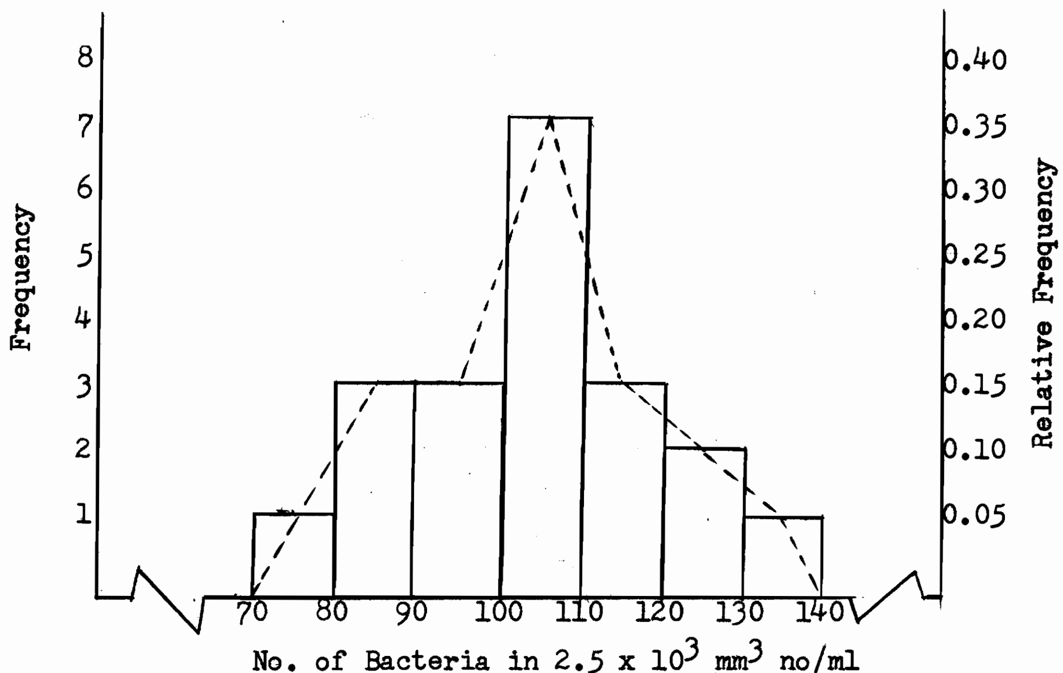

Figure 10. Frequency histogram and polygon plotted from Table 11.

The mean counts, standard deviation, coefficient of variation, standard error and confidence limits for the sample counts are shown in Table 12.

Table 12. Mean counts, standard deviation, coefficient of variation, standard error and confidence limits for sample counts.

Mean count $\bar{X} = \frac{\sum X_i}{N}$	Standard deviation $S = \sqrt{S^2} = \sqrt{\bar{X}}$	Coefficient of variation $C.V. = \frac{S}{\bar{X}} \times 100$	Standard error $S_{\bar{X}} = \sqrt{\frac{S^2}{N}}$	Confidence limits (95%) $U = \bar{X} \pm t_{0.975(n-1)}S$
106.15	10.31	9.71	2.32	$U = 106.15 \pm (2.093 \times 10.31)$ = 84.57 to 127.73

IV. ENUMERATION OF VIABLE BACTERIAL CELLS

A. Plate Counting Technique

Attempts to estimate cell numbers employing silica gel plates were unsuccessful. Any colonies that developed after 10 to 14 days' incubation were so small as to be indistinguishable from air bubbles or other minute surface irregularities when examined by both transmitted and reflected light using 40x magnification. In addition, considerable atmospheric oxidation of surface ferrous ion caused an accumulation of ferric salts to precipitate on the surface of silica gel and further obscured the recognition of colonies. This is illustrated in Figure 11. In any cases, colonies were extremely

small. Gram staining revealed that those colonies present contained no more than 30 cells per colony. However, excellent growth has been obtained if drops of fluid containing many organisms were put on the surface of silica gel plates and allowed to incubate without spreading. Figure 12 shows the appearance of a plate incubated in this manner.

B. Radioactive Isotope (P^{32}) Technique

1. Time required for labeling cells.

One ml of P^{32} solution (1 uc/ml) and 1 ml of culture solution were added to 8 ml of incubation medium in a sterile 25-ml Erlenmeyer flask. The flasks were incubated at room temperature in a shaker. One ml portion of the incubation mixture was sampled every 15 minutes for 5 times. After the filtration through a Millipore membrane filter, the radioactivity retained on the filters was determined.

The time required for maximal uptake of P^{32} was found to be 60 minutes (Figure 13). The incubation period selected for further study was 1 hour.

2. Effect of temperature.

This experiment was conducted to establish a suitable incubation temperature on the time required for maximal uptake of P^{32} by *T. ferrooxidans*. It was noted that the most rapid labeling occurred when the incubation temperature was 27°C. The results in Table 13 show that cells of *T. ferrooxidans* became extensively labeled at 27°C rather than at 37°C.

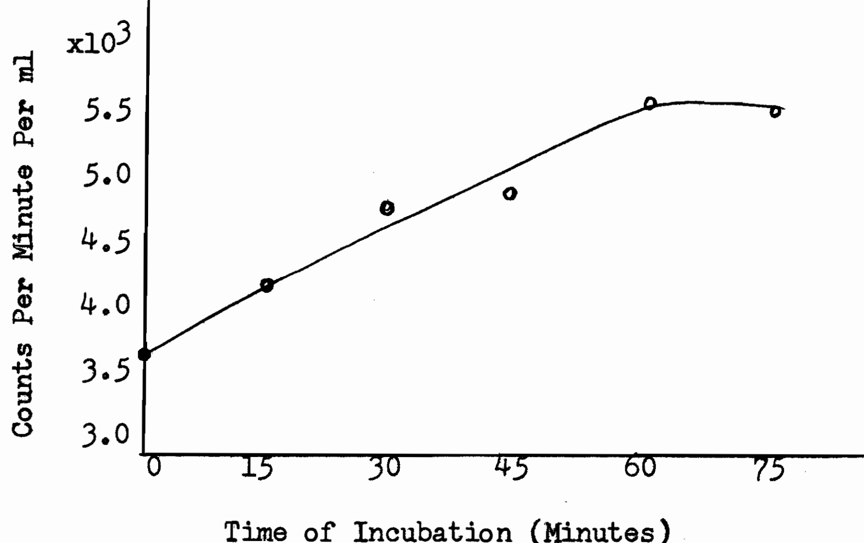

Figure 13. Effect of incubation time on the extent of labeling of cells of *T. ferrooxidans*.

Table 13. Effect of incubation temperature on the time required for maximal uptake of P^{32} by *T. ferrooxidans*.

Incubation time	Incubation temperature 27°C counts/min./ml	Incubation temperature 37°C counts/min./ml
minutes		
0	3656	3352
15	4246	3516
30	4893	4118
45	4925	3647
60	5587	3900
75	5524	4014

3. Effect of type and volume of washing fluid on the retention of P^{32} by labeled cells of _T. ferrooxidans_.

Since the composition of the washing solution is known to affect the extent of retention of intracellular solutes in the case of marine bacteria (Drapeau and MacLeod, 1965), the effect of washing on the retention of P^{32} was examined. Washing cells of this organism with increasing volumes of 0.01N NH_4OH caused a progressive decrease of P^{32}; washing with distilled water or 0.01N HCl, however, did not remove the isotope. The data in Table 14 show that there is no significant difference of the radioactivity retained on the filters between the case of the samples containing cells and the blanks prepared without cells.

V. DISTRIBUTION OF IRON-OXIDIZING BACTERIA IN MINE WATER

The results of the chemical and microbiological analyses of the mine waters obtained from different locations in the Bingham Canyon mine dump are shown in Table 15.

In this data, it should be noted that the large increase in bacterial numbers in the oxidation ponds from 4.8×10^6 per ml in the precipitation plant tailing to 4.8×10^7 per ml in the small pond and to 4.8×10^9 per ml in the large pond. This is accompanied by a decrease in ferrous ion content, the oxidized iron (Fe^{+++}) formed apparently being lost by precipitation. Also, the low bacterial numbers in the dump effluent solutions compared with the count in the feed water were observed. It is very interesting finding that the

Table 14. Effect of type and volume of washing fluid on the retention of P^{32}.

Volume	Washing fluid: Glass-distilled water, Filter + cells counts/min/ml	Glass-distilled water, Blank counts/min/ml	0.01N HCl, Filter + cells counts/min/ml	0.01N HCl, Blank counts/min/ml	0.01N NH_4OH, Filter + cells counts/min/ml	0.01N NH_4OH, Blank counts/min/ml
ml						
0	6420	6010	6244	5835	6244	5835
5	6250	5916	6128	6020	6140	5320
10	5960	6115	5926	5766	5520	5016
15	6125	5720	6418	6241	5280	4560
20	6205	5866	6520	5880	4720	3624

Table 15. Occurrence of *Thiobacillus ferrooxidans* in Bingham Canyon mine dump waters.

Sampling points: Sample No.	Sampling points: Description	pH	Cu (g/l)	Fe^{++} (g/l)	Fe^{+++} (g/l)	*T. ferrooxidans* per ml
1	Midas	2.30	1.97	0.05	1.36	2.8×10^8
2	Markham	2.40	1.25	0.65	0.63	2.4×10^6
3	Blue water #1	2.49	1.59	0.30	1.17	4.8×10^6
4	Blue water #2	2.35	1.60	1.70	0.58	4.8×10^6
5	Ppt. plant heading	2.72	1.54	0.60	0.85	4.8×10^5
6	Ppt. plant tailing	3.69	0.01	1.50	0.04	4.8×10^6
7	Small pond	3.41	0.007	0.78	0.20	4.8×10^7
8	Large pond	3.29	0.003	0.42	0.10	4.8×10^9

copper laden water with the highest bacterial count also has the highest copper content (Sample No. 1, Midas).

A relationship between the bacterial count and the temperature of the leaching effluent water was also observed as shown in Table 16. Water temperature well above 30°C was noted and other observations showed that temperatures of 70°-90°C may be found in some sites of the ore dump. Laboratory experiments have shown that *T. ferrooxidans* has an optimal growth temperature of about 30°C. It was found that leaching effluent water with the highest bacterial count also had a temperature about 30°C.

VI. CORRELATION BETWEEN THE CONVERSION OF FERROUS TO FERRIC ION AND BACTERIAL GROWTH

In this experiment, each percolator solution was sampled every second day for a period of 12 days. The number of bacteria and the conversion of ferrous to ferric ion were determined in duplicate and the average value for each sample was obtained.

The relationship between the conversion of ferrous to ferric ion and the growth of bacteria in the percolator solution is illustrated in Figure 14. The curve for the conversion of ferrous to ferric ion was obtained merely by plotting the average value of the duplicate determination in each sample. However, the bacterial growth curve was formed by joining the midpoints of the 95 per cent confidence intervals for the bacterial counts per ml by use of Petroff-Hausser technique.

Table 16. Relationship between the numbers of _T. ferrooxidans_ and the temperature of effluent leaching water.

Sample No.	Description	Temperature °C	_T. ferrooxidans_ per ml
1	Midas	30.5	2.4×10^8
2	Markham	38.0	2.4×10^6
3	Blue water #1	40.0	4.8×10^6
4	Blue water #2	42.0	4.8×10^6

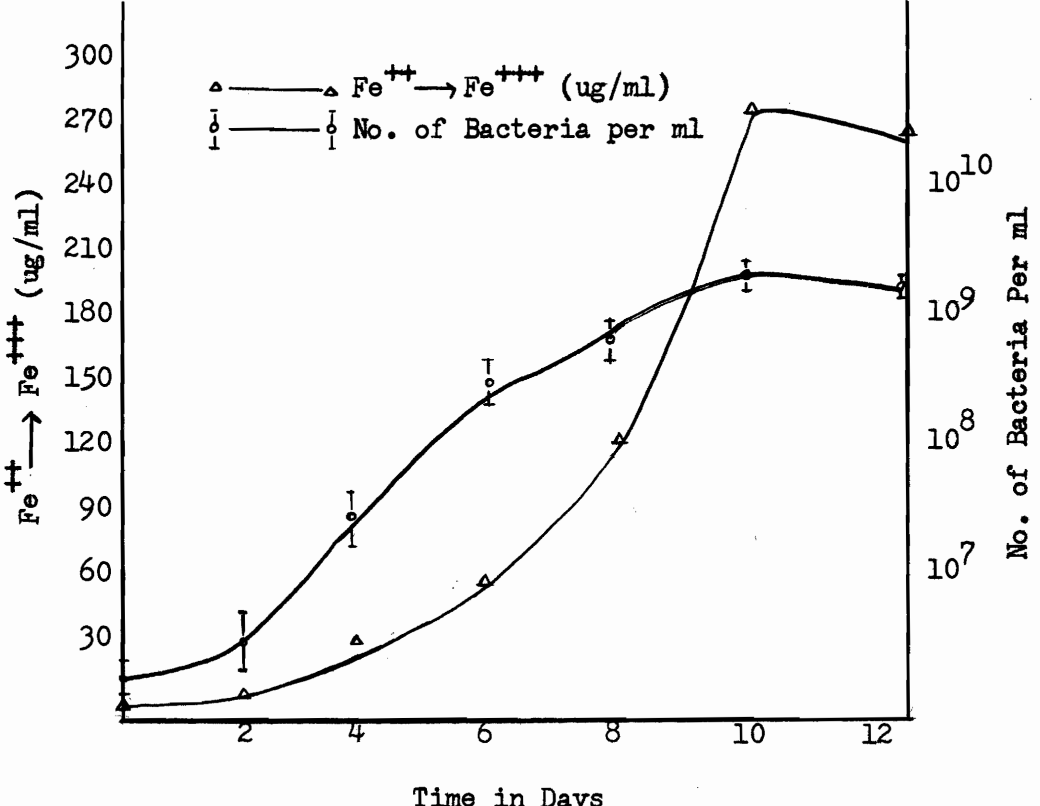

Figure 14. Correlation between the conversion of ferrous to ferric ion and bacterial growth.

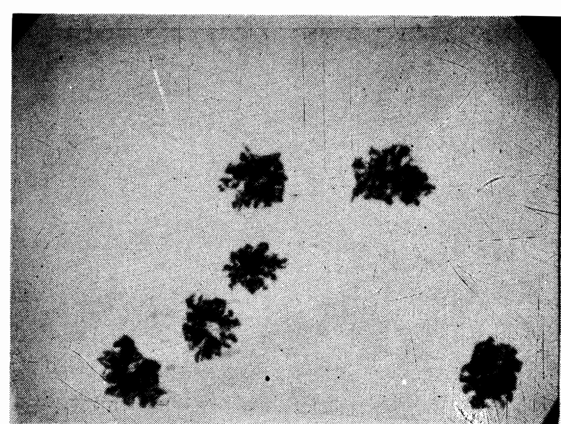

FIGURE 3. Colonies of *Thiobacillas ferrooxidans* on sodium thiosulfate agar. Magnification 40x

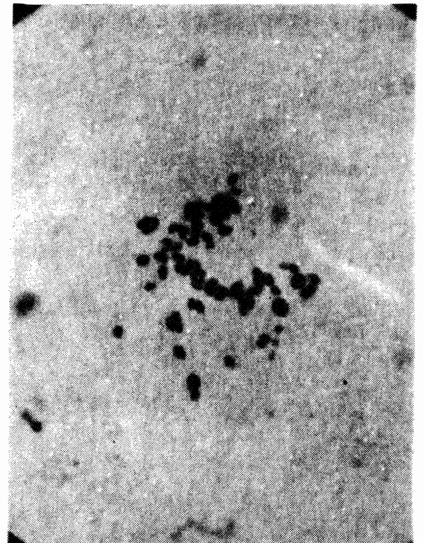

FIGURE 5. Photomicrographs of yeast-like organisms as found on membrane filters. Magnification 40x

FIGURE 6. Colonies of yeast-like organism grown on acidified mycophil agar. Several microcolonies suround the larger colonies. Mignification 60x

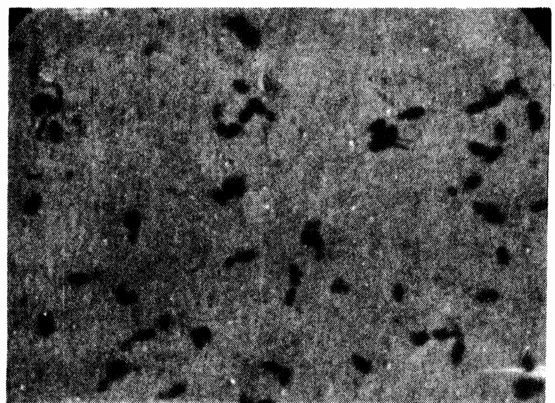

FIGURE 7. Photomicrograph of yeast-like cells grown on organic medium (acidified mycophil agar). Magnification 40x

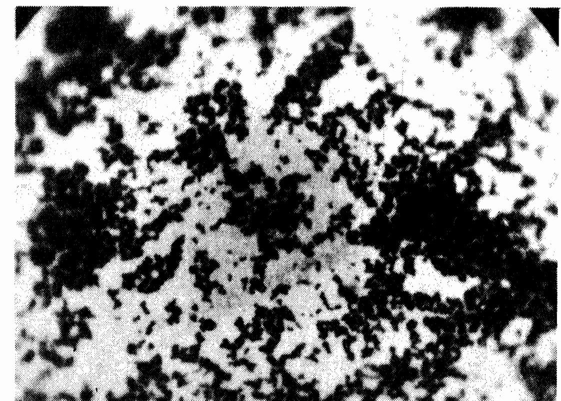

FIGURE 8. Photomicrograph of yeast-like organism interspersed with cells of *Staphylococcus aureus*. Magnification 40x

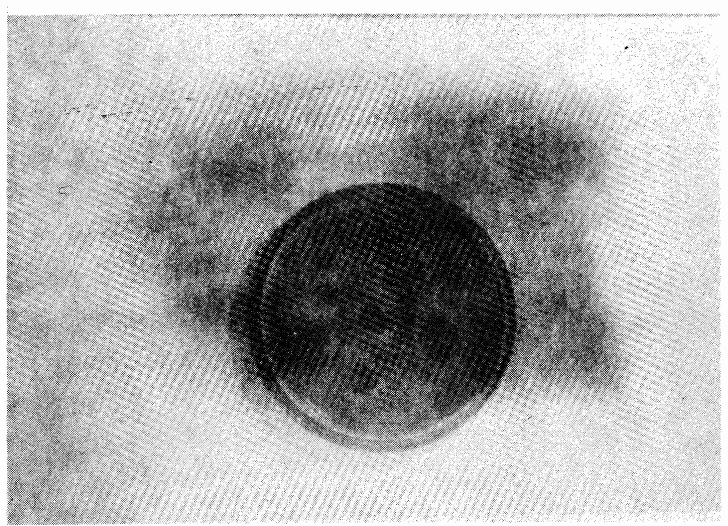

FIGURE 12. Illustration of "spot" growth of *Thiobacillus ferrooxidans* on silica gel plates. Magnification 40x

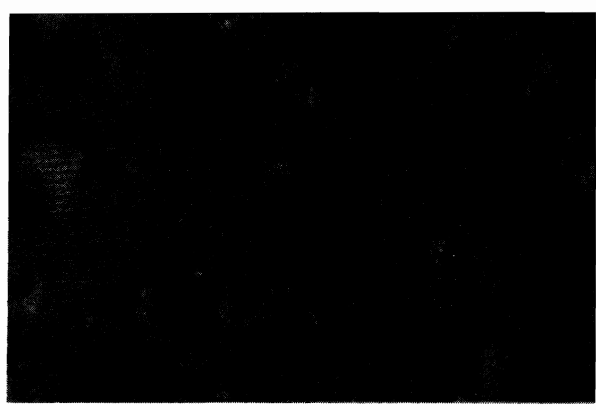

FIGURE 11. Small colonies of *Thiobacillus ferrooxidans* and other surface irregularities shown on the silica gel plates. Magnification 60x

DISCUSSION

The microbial oxidation of ferrous ion and particularly the proof of autotrophic growth with iron as an energy source have been subjects of controversy for many years (Molisch, 1910; Winogradsky, 1922; Cholodny, 1926; Baas-Becking and Parks, 1927; Starkey, 1945; and Pringsheim, 1949a, b).

An environment rich in ferrous ion and highly acid is found in the mine drainage water of some of the major bituminous coal sections of the United States and in the leaching streams of Bingham Canyon, Utah. It has been shown by Colmer and Hinkle (1947) and Leathen et al. (1953) that the ferrous ion oxidation occurring in these acid mine waters was biological. It has also been shown by Bryner et al. (1954) and Bryner and Anderson (1957) that iron pyrite (FeS_2) chalcocite (Cu_2S), chalcopyrite ($CuFeS_2$) and molybdenite ($MoCuFeS_2$) are oxidized biologically in exposed ore bodies to sulfates, sulfuric acid, and other soluble products.

The primary studies of this thesis were carried out to isolate chemosynthetic bacteria responsible for this oxidation in the Bingham Canyon mine dump. It was found that there were at least two types of chemolithotrophic bacteria in the Bingham mine waters. One of these organisms oxidized ferrous ion to ferric ion as its energy source. This organism was very similar to *Thiobacillus ferrooxidans*. The

other type of organism oxidized free sulfur to sulfuric acid as its energy source. This one was similar to *Thiobacillus thiooxidans*.

It is also very interesting that *Thiobacillus ferrooxidans* occurred with *Thiobacillus thiooxidans* in the Bingham mine dump waters. *Thiobacillus ferrooxidans* is obviously involved in the oxidation of sulfide ores; whereas, *Thiobacillus thiooxidans* may take part in the oxidation of free sulfur formed during oxidation of pyrite (FeS_2) or chalcopyrite ($CuFeS_2$). A large quantity of acid would be formed thus keeping the environment at a pH of 2.0-3.5, an ecological condition favorable to the development of these two types of acid-tolerant chemoautotrophs present in the mine dump.

In addition to the two types of chemoautotrophic bacteria, a yeast-like organism was found in the Bingham mine dump. It was noted that there is a significantly larger number of yeast-like organisms in the precipitation plant tailings pond than any other sampling point. These yeast-like organisms could be counted by the membrane filter technique. It was also found that the yeast-type organisms could be found in every location of Bingham mine dump when membrane filters were seeded with a large volume (30 ml) of mine dump water.

The precipitation plant tailings pond is located immediately below the precipitation plant, and the yeast-like organisms were far more numerous in this tailing pond than in the precipitation plant waters. One would immediately wonder why this area, which is but a few yards from the precipitation plant trenches, would have so many

more yeast-like organisms per ml than samples obtained from the precipitation plant or other sources throughout the mine and mine dump.

These organisms have not been shown to utilize ferrous ion as a source of energy, and other experiments in this laboratory indicated that this yeast-like organism was not in any sense autotrophic but must have carbon from an organic source. The presence of this organism in greater abundance at the tailings pond suggested that some toxic agent may have been removed or that a source of organic carbon has been provided. The source of energy and carbon for this yeast-like organism must be from either scavenger activity by the yeast-like organism on dead thiobacilli or through the contamination of the dump waters of humus carbon from soil. The latter is certainly minimal. Another source might be food contaminants left on the scrap metal or possibly materials from sewage from Bingham. The fall off in numbers of organisms from the tailings pond to the ponds at the top of the dump could well be due to dilution of the dump water from the holding reservoir. The toxic substance might well be the copper removed by the precipitation process. Copper sulfate in concentrations found in the precipitation trenches is known to be inhibitory to most forms of life.

The presence of yeasts and other fungi in acid mine waters has also been reported by various investigators (Ehrlich, 1962, 1963; Marchlewitz et al., 1961; Razzell and Trussell, 1963b). The consensus

of opinion has been that the life process of these organisms was not important in influencing the transformation of mineral sulfides. However, these organisms displayed an unusual tolerance to conditions of high acidity and high metal ion concentration normally considered too toxic for related microorganisms to exist. They must be specialized physiologically in view of their high tolerance of copper salts, iron salts and acid.

The microbial population in the mine water samples suggests the existence of a balanced ecological system in which carbon fixation is dependent on chemosynthetic and not photosynthetic autotrophy.

Hydrogen sulfide formation by reduction of elemental sulfur has been reported to be a widespread activity of fungi as well as other microorganisms (Starkey, 1956). Copper sulfide precipitation as a result of sulfate reduction by copper-resistant *Saccharomyces ellipsoides* was also reported by Ashida et al. (1963). In the experiments by Ashida et al. (1963) the copper sulfide was deposited inside the cell wall of the yeasts but not externally. Extracellular precipitation of copper sulfide, as a result of elemental sulfur reduction by yeast in an acid-glucose mineral salts medium, has been reported by Ehrlich and Fox (1966). Therefore, the presence of yeasts in the acid mine waters may be of geomicrobial importance. In nature, or in field operations involving copper sulfide leaching, elemental sulfur reduction by yeast could be important in redepositing solubilized copper in reducing zones of copper sulfide ore bodies and

waste dumps. In reducing zones, where because of a lack of oxygen, bacterial oxidation or autooxidation of copper sulfides cannot occur, ferric ion introduced by waters from the oxidation zone would oxidize the copper sulfides to copper sulfate and sulfur: $CuS + 2Fe^{+++}$ $Cu^{++} + S^{o} + 2Fe^{++}$, as was shown by Sullivan (1930, 1931). Thus, if surface waters, percolating into the reducing zone of copper sulfide ore bodies, carried enough organic matter and nitrogen, yeasts in the reducing zone could reprecipitate some of the dissolved copper with hydrogen sulfide they produce by reducing any sulfur there utilizing some of the organic matter as a sulfur and hydrogen donor. There is another possibility that the yeast-like organism, an acidophilic heterotroph present in the acid mine waters, is acting to keep the organic content of leach waters at a low concentration, an ecological environment favorable to the development of iron- and sulfur-oxidizing chemoautotrophs which play an important role in the solubilization of copper sulfides.

The dependence of rapid solubilization of copper sulfide minerals on the presence of iron-oxidizing bacteria as described in laboratory studies by various investigators (Silverman et al., 1959a; Beck, 1960; Lyalikova, 1961; Lyalikova et al., 1965) suggested that it may be possible to demonstrate some sort of correlation between the effectiveness of the leaching operations in the field and the numbers of bacteria present. Therefore, the availability of a method for reasonably accurate estimations of the bacteria in mine

water and in washings from drill ore samples from the dump was considered to be of great importance.

Three different counting techniques were used in this study for the enumeration of the chemoautotrophic thiobacilli: the Petroff-Hausser chamber counting technique, the silica gel plate counting technique, and the radioisotope labeling technique. Of these methods, the direct count of cells in the Petroff-Hausser chamber was the only one found to be satisfactory. It was the most rapid and relatively reliable method of estimating cell numbers, although it included both living and dead cells.

Statistical analysis of the results obtained by the Petroff-Hausser counts shows that though the results are not sufficient to insure that the distribution curve will have the "normal" shape, the tendency of the data to approximate the normal distribution is clearly shown in Figure 10. To the extent that the results of twenty counts do indeed cluster about the mean of average value, the data show the existence of central tendency.

The standard deviation of the results of twenty counts is 10.31. It might be noted that the mean of these results $\pm$ standard deviation, that is 106.15 $\pm$ 10.31, includes slightly less than 50 per cent of the results, i.e. nine out of twenty.

It should be noted that the 95 per cent confidence interval for the true mean from the data of twenty counts is given by $U = \bar{X} \pm t_{0.95(n-1)}S = 106.15 \pm 2.093\ (10.31) = 84.87$ to 127.73 (95% confidence interval).

Upon the examination of the twenty individual counts, only two out of twenty (10%) lie outside these confidence limits; this suggested that if a large number of estimations were made, there is a probability of only 5 per cent that the true mean would lie outside these confidence intervals.

The counting of chemoautotrophic thiobacilli by the Petroff-Hausser technique is subject to certain errors and limitations; therefore, this method cannot be used as a standard of comparison to determine accuracy or the degree of agreement with the true population mean. It appears that this method can be used for specific counting tasks, if one takes into consideration the limitation of the method. Direct microscopic counts, since they measure both living and dead cells, are probably not suitable for measurement of growth curves; however, it might be better for comparative counts at a particular stage of growth, especially during the early stages of the growth period when conditions such as cloudiness or the presence of inorganic precipitate prevent the use of optical density as a method of estimation of increase in numbers of bacteria. Before the development of any media suitable for the estimation of viable autotrophic thiobacilli, direct microscopic counts are still of great value in estimating numbers of these bacteria.

Attempts to estimate the number of autotrophic bacteria found in mine water met with very poor results when silica gel plates were employed. As yet, the reason for the failure of the thiobacillus to

show well developed colonies from a single cell on the silica gel plate when the plates were seeded with a few organisms has not been learned. The following explanations have been postulated:

1. Silica gel plates were prepared using a sodium silicate compound called Ludox. This material was treated by ion exchange through a column of Dowex-50 resin in the hydrogen form. By replacement of the Na^+ ion with H^+ the Ludox then became silicic acid. This was used to prepare the silica gel plates. The silicic acid was added to "nutrient salts" in a proper ratio, then heating the plates in the autoclave sterilized and solidified the medium.

It was not known but it was suspected that other anions, cations and organic matter present in the Ludox may have exerted an inhibitory effect on the growth of autotrophic thiobacilli when very small inocula were used.

2. Small inocula _per se_ may also have been the reason for failure of the organisms to grow. The medium which was used may require enrichment with trace elements in order to support the multiplication of single cells on the surface of the silica gel plates.

3. Autotrophic thiobacilli on silica gel probably require a low oxidation-reduction potential (Eh) to initiate growth. During the process of autoclaving iron salts with silicic acid, a considerable amount of ferrous ion is oxidized to ferric ion. In addition, a precipitate of ferric salts rapidly accumulated by atmospheric

oxidation of surface ferrous ion; and, thus, the oxidation-reduction potential of the medium rose. It is possible, therefore, that the medium must be poised at a lower Eh value for growth to occur with certainty on silica gel plates.

Up to now, no data have been published on the quantitative correspondence between total counts of autotrophic thiobacilli and plate counts on silica gel. Beck (1960) used a dilution procedure, based on the highest dilution to show precipitation of ferric ion, to estimate these bacteria.

An experiment conducted in this laboratory to check the inoculum theory indicated that if a drop of the culture were put onto the surface of silica gel plates and allowed to incubate without spreading, excellent growth occurred as shown in Figure 12. This result suggested that this type of chemoautotrophic thiobacillus required a large inoculum to initiate growth. The dilution method, however, assumes that there was only a single cell at the highest dilution at which growth would occur in the medium. Therefore, cell counts made by the highest dilution of inoculum only determine the approximate number of cells.

A study of the general question of viable plate counting for autotrophic thiobacilli is urgently required. For a group of microbes so economically important as these, it is surprising that such a study remains to be done.

Efforts to ascertain viable counts of chemoautotrophic thiobacilli to be found in the mine dump waters by use of silica gel nutrient plate were unsuccessful. For this reason, alternative methods of measuring viable cells were explored to find a better means of estimation of the number of autotrophic cells to be found in their natural habitats.

There have been many studies conducted on the labeling of bacteria with P^{32} during growth (Stonier, 1956; Kuska et al., 1964; Robson, 1964). Only a limited amount of information is available concerning the uptake of P^{32} by resting cells (Mallin and Kaplan, 1959; MacLeod et al., 1966). So far, no attempt has been made to use P^{32} labeling as a technique to detect viable cells of the chemoautotrophic thiobacilli.

It is evident from the results presented in Table 14 that there is no significant difference in the radioactivity retained on the filters between samples containing cells and the blanks prepared without cells. This suggested that radioactive isotope P^{32} may have precipitated with those compounds present in the mine water or in the incubation medium; for example, phosphate combines with Mg^{++}, Ca^{++} and Fe^{++} to form an insoluble precipitate.

Different types of solvents were used in attempting to wash cells and dissolve the precipitate. It is of interest to note that the decrease in radioactivity caused by washing with 0.01N NH_4OH might be due to both lysis of cells and disolution of precipitate,

since a progressive decrease in radioactivity was found both in the case of the samples containing cells and blanks prepared without cells when these were washed with increasing volumes of 0.01N NH_4OH.

The critical problem regarding detection of viable autotrophic thiobacilli by use of P^{32} labeling technique is that the precipitation of metal ions with P^{32} must be prevented. A suitable solvent should be found to merely dissolve the precipitate without any lysis of bacteria and also to keep P^{32} free in the solution so that it could be utilized by the bacteria. It is proposed that metal chelating agents may be used to form complexes with metal ions in the solutions and thus prevent the precipitation of metal ions with P^{32}.

It is also evident that certain factors can affect the uptake of P^{32}, such as the incubation medium, temperature, time, unlabeled phosphorus and P^{32} level. If this method of P^{32} labeling technique were carried out under appropriate conditions, it would be both rapid and sensitive and could have wide application in the detection of viable cells of microorganisms.

The correlation between the conversion of ferrous to ferric ion and bacterial growth was determined in the percolator experiment. It was found that a relationship does exist between the Petroff-Hausser counts on the samples and the conversion of ferrous to ferric ion. There was no significant increase in bacterial numbers and ferric ion during the lag phase of induction period. The ferric ion increased linearly and proportionally with the number of bacteria

during the logarithmic phase. The amount of ferric ion in solution was maximal (270 ug/ml) on the tenth day of percolation. The number of bacteria also reached a maximum of 3.98×10^9/ml at this time. This indicated that a given amount of conversion of ferrous to ferric ion over a given time period corresponded to a given increase in the bacterial population.

In attempting to compare the data (Table 15) concerning the presence of *T. ferrooxidans* with the ecological conditions of the Bingham mine dump, it was found that these ecological conditions are very favorable for the development of *T. ferrooxidans*. There is considerable ferrous ion content and high acidity (pH 2.30-3.69) in the mine waters. The mine water temperatures were noted to be about 30-40°C. The results showed in every instance typical *T. ferrooxidans* to be present in the leaching waters.

It was noted that there was a marked decrease in the numbers of bacteria in the passage of water through the precipitation plant. At the precipitation plant the copper-bearing solution passed over metallic iron scrap and the precipitated copper was recovered ($Fe + CuSO_4 \longrightarrow FeSO_4 + Cu$), resulting in the formation of ferrous ion, which lowered the oxidation-reduction potential.

In the precipitation reservoir, the oxidation-reduction potential rose where considerable bacterial activity occurred, as was evidenced by the decrease in pH and increase in ferric ion. The massive growth of *T. ferrooxidans* occurred in the precipitation

reservoir and may have served as a large inoculum to active sites in the ore body.

Interestingly, copper laden water with the highest bacterial count also had the highest copper content. The significance of this relationship can probably be attributed to the ecological conditions, such as temperature, spaces and oxygen in the mine waste dump which were favorable to the development of *T. ferrooxidans* and thus showed a maximal bacterial activity to cause a rapid solubilization of copper sulfide ores.

It was observed that the leaching effluent water with the highest bacterial count had a temperature of about 30°C. Most studies described in the literature also indicated the optimal temperature for bacterial oxidation by chemoautotrophic thiobacilli to be 30°C. Much higher temperatures of 70-90°C in the ore dumps have been recorded. High temperature levels encountered in some sites of the mine dump suggested that there is a change of oxidizing activity from biological to chemical which is caused by a sufficient heat accumulation in these areas and the biological oxidation was probably only active in the top and bottom of the mine dump.

There is another possibility that certain strains of bacteria may have adapted to these higher temperatures. Therefore, it is proposed that the adaptation of the *T. ferrooxidans* bacteria to various temperatures with maximal oxidation of copper sulfide should be studied. Also, a relationship between temperature and bacterial flora in the mine dump urgently requires study.

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