THE AUTOTROPHIC OXIDATION OF IRON BY A NEW BACTERIUM: THIOBACILLUS FERROOXIDANS[1]

KENNETH L. TEMPLE AND ARTHUR R. COLMER[2]

Engineering Experiment Station, West Virginia University, Morgantown, West Virginia

The microbial oxidation of ferrous iron and particularly the claim of autotrophic growth on iron have been subjects of controversy for many years (Molisch, 1910; Winogradsky, 1922; Cholodny, 1926; Baas-Becking and Parks, 1927; Cataldi, 1939; Starkey, 1945; Pringsheim, 1949a,b). In neutral or alkaline waters a nonbiological atmospheric oxidation of ferrous iron takes place. This has made it impossible to determine the relative importance of microbial and purely chemical oxidations of iron. Atmospheric oxidation would cease to be a factor in acid media, but the classical iron bacteria do not develop under strongly acid conditions.

An environment rich in ferrous iron and highly acid is found in the mine drainage water of some of the major bituminous coal sections of the United States. Colmer and Hinkle (1947) showed that the ferrous iron oxidation occurring in this acid mine water was biological. The isolation of the organism responsible and certain of its morphological and cultural characteristics have been described (Colmer, Temple, and Hinkle, 1949). The present communication presents definitive evidence that the bacterium is an autotrophic iron oxidizer.

EXPERIMENTAL METHODS

Media, inoculation, and culture maintenance. The original medium consisted of $FeSO_4 \cdot 7H_2O$ to give 2,000 ppm ferrous iron, 0.1 per cent $MgSO_4 \cdot 7H_2O$, 0.05 per cent $(NH_4)_2SO_4$, distilled water, and enough conc H_2SO_4 to give a final pH of 2.0 to 2.5. More recently an iron level of 26,000 ppm ferrous iron has been adopted.

Sterilization of the medium may be accomplished by autoclaving, or by filtering with a Seitz, sintered glass, or porcelain type equipment. Autoclaving gives a voluminous precipitate of ferric hydrate, but the loss of ferrous iron is actually negligible. To rid the medium of the ferric hydrate the precipitate may be allowed to settle and the supernatant solution decanted, or the solution may be autoclaved in a large short-stemmed separatory funnel and the ferric hydrate drawn off at the bottom. Using the separatory funnel, the removal of 50 ml of solution from 1,050 ml gets rid of the entire precipitate. Nitrate media were prepared by substituting 0.05 per cent $NaNO_3$ for the ammonium sulfate.

Agar plates containing 26,000 ppm ferrous iron were prepared by using the basal medium with the addition of 2 per cent agar. To prevent hydrolysis of agar during sterilization, the ferrous sulfate was autoclaved separately in one-

third of the water; the other ingredients were autoclaved in the remainder of the water, and the two solutions were mixed just prior to plate pouring. With this much ferrous sulfate no H_2SO_4 was needed to give the desired pH.

Growth was satisfactory in Erlenmeyer flasks or bottles but slow in tubes. The use of a rotary shaker or of air bubblers gave faster growth. The culture was maintained by inoculating liquid media with one ml of a culture that had effected nearly complete oxidation of ferrous iron in the solution medium. Transfers were made at approximately two week intervals. Experimental flasks were inoculated with varying amounts of cultures developing in the solution medium.

Analytical methods. Ferrous iron was determined by the Zimmermann-Reinhardt (Treadwell and Hall, 1942) volumetric titration with permanganate. Determinations of ferric and total iron were abandoned when it was found that the value for ferrous iron gave an accurate picture of the changes in iron content. Carbon analyses were conducted by the Van Slyke-Folch (1940) procedure for wet combustion. The 2 ml samples used were dried overnight in a vacuum desiccator.

Growth on synthetic iron media. The bacterium was kept on the original synthetic medium for 24 months by subculturing every two weeks, but it has been noted that the culture remains viable in closed bottles stored for several months. Growth was faster on the agar or solution media containing 26,000 ppm ferrous iron, being appreciable in 3 to 5 days. Motility was most marked on young agar streaks. At the higher iron concentration the bacteria were vastly more numerous as shown by microscopic observation. No growth was detected when the content of ferrous iron was less than 125 ppm. No upper iron level was determined. Because of the tremendous weight ratio of iron oxidized to carbon fixed, very large amounts of ferrous salts can be used. The optimum level of magnesium sulfate was between 0.01 and 0.1 gram per liter. The amount of ammonium sulfate provided considerably more nitrogen than was required for growth. In fact, special methods were required to demonstrate nitrogen deficiencies even when no nitrogen salt was added to the medium. Also, since ammonia and iron were the only oxidizable components of the medium, it was deemed advisable to investigate the possibility of a relation between ammonia level and the extent of iron oxidation. When ammonium sulfate was omitted entirely, the culture could be transferred four times before iron oxidation was reduced in the solution medium containing 2,000 ppm ferrous iron. Moreover, to demonstrate this effect it was necessary to keep the flasks over sulfuric acid or to bubble acid-washed air through the culture solution. Such cultures regained the ability to oxidize iron when $NaNO_3$ was added, and could be maintained on a nitrate medium, but the rate of oxidation was slower than when ammonia was the nitrogen source. To remove the possibility that the medium contained organic materials that supported growth, in several of the foregoing experiments the flasks were cleaned with hot concentrated sulfuric acid and the water used was redistilled from acid permanganate.

In figure 1 a measure of the reciprocal of the ferrous iron concentration was plotted against time. Using this function, the curves rose with decreasing con-

centration of ferrous iron (or increasing concentration of ferric iron) analogous to the ordinary growth curve. The resemblance to the growth curve extends from the initial part of the curve through the lag phase into the logarithmic phase. Here the resemblance ends, since the value plotted is the concentration of substrate rather than the numbers of bacteria or the amount of bacterial substance. When all the ferrous iron was oxidized, another increment of 2,000 ppm of ferrous iron was added, and thus the curve returned to the initial level. The rate of oxidation of the second increment was greater than the first, and
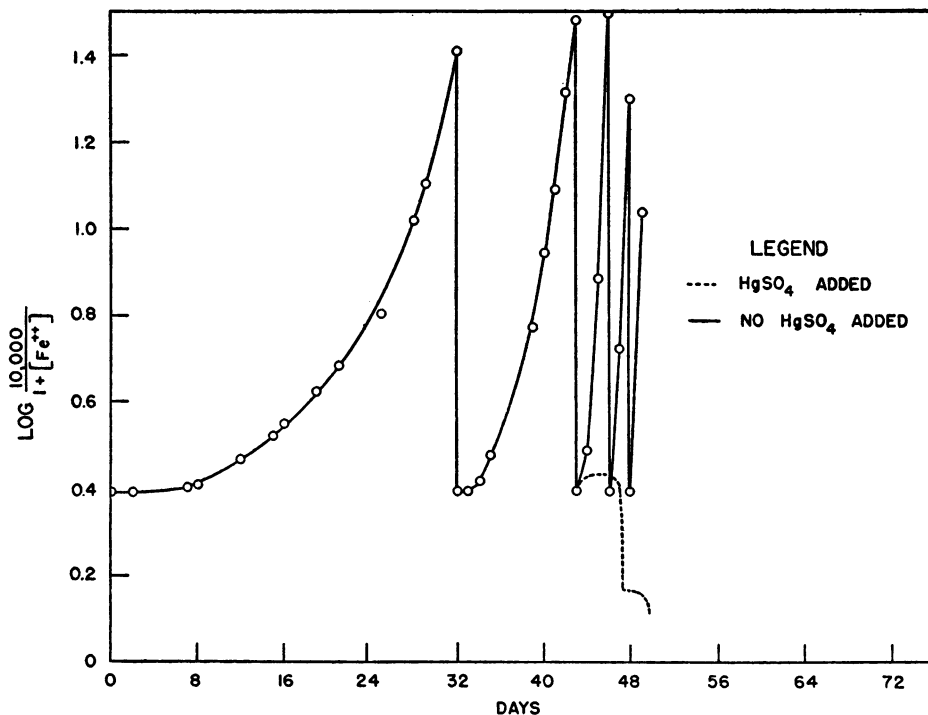

Figure 1. Changes in the rate of oxidation of successive additions of ferrous iron.

the rate of oxidation was still greater for each of the subsequent increments; for the fifth increment oxidation was complete in less than 24 hours. Eventually further additions of ferrous iron failed to be oxidized.

On the forty-third day the culture was divided in half and to one part 2 grams per liter of $HgSO_4$ were added. The dotted line of figure 1 is a representation of the iron content when microbial activity ceases. No oxidation took place in the solution containing mercury, hence the successive additions of ferrous iron caused the plotted value to drop in the manner shown.

Carbon fixation. For growth and iron oxidation carbon dioxide was needed and was supplied by the air. Alkali-washed air did not support either iron oxidation or cell growth. With the medium containing 2,000 ppm ferrous iron it was not possible to detect any increase in the carbon content after iron oxi-

dation was complete. In another experiment, by adding successive amounts of ferrous iron as just described, a measurable increase in carbon was noted (figure 2).

The flat portion of the curve was caused by the fact that sampling, analysis, and addition of fresh $FeSO_4$ were sometimes delayed after oxidation was complete. The calculations for efficiency of energy utilization were made by assuming that the free energy change of ferrous to ferric iron was 11.3 calories per gram atom (Bichowsky and Rossini, 1936) and that the fixation of one gram atom of
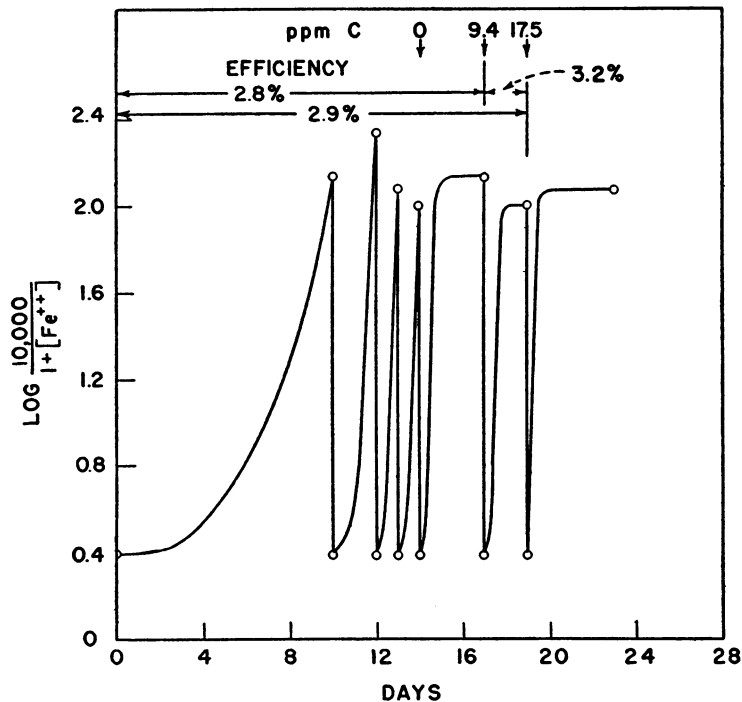

Figure 2. Carbon fixation and calculated efficiency of energy utilization in relation to the oxidation of successive additions of ferrous iron.

carbon required 115 calories (Starkey, 1945). For an interval where the amounts of carbon fixed were largest, the oxidation of 120 grams of $FeSO_4 \cdot 7H_2O$ resulted in the fixation of 16.05 mg C, an efficiency of 3.2 per cent. This figure is close to that reported for other autotrophs (Baas-Becking and Parks, 1927). The experiment was conducted three times with similar results. In all of the experiments in which several increments of iron were used, there were two liters of medium in culture bottles of three liter capacity, and the medium was aerated by a vigorous stream of filtered air. No increase in carbon took place in a control bottle containing mercuric sulfate at a level of two grams per liter.

Proof of the biological nature of oxidation. Sterile solutions of 2,000 ppm ferrous

iron at pH 2.0 to 2.5 did not oxidize in the atmosphere. Sterilizing with anti-
bacterial agents was as effective as autoclaving or filtering. Sterile solutions were
bubbled with filtered air indefinitely without appreciable loss of ferrous iron.
When NaN$_3$ or iodoacetic acid was added to the media to make a concentration
of 3×10^{-3} M, there was no oxidation of ferrous iron. Mercuric sulfate added
to the actively oxidizing solution stopped the reaction. Cell-free filtrates of the
culture solutions had no effect on the stability of ferrous solutions.

RESULTS AND DISCUSSION

The results show conclusively that the bacterium repeatedly isolated by us
from natural acid iron-rich waters continues to grow in serial transfers on a
simple synthetic medium high in iron and that the oxidation of ferrous iron in
this medium is dependent upon bacterial growth. The unique cultural condition
that distinguishes these experiments on iron oxidation from previous ones is
the high acidity at which the bacterium grows. At this pH the ferrous iron is
not oxidized by atmospheric oxygen. The recent report by Gleen (1950) involving
soil at pH 3.0 is the only other case of iron oxidation in an acid environment
that has come to our attention.

Criticisms of the claims of autotrophy of iron bacteria have called attention
to the lack of conclusive evidence and to the extremely large amounts of iron
that would have to be oxidized to provide energy for bacterial development. The
500:1 weight ratio of iron oxidized to cellular material produced, predicted by
Starkey (1945), is consistent with the results here reported. Evidence of carbon
fixation could not be obtained until extremely large amounts of iron had been
oxidized, large amounts, that is, from the viewpoint of the normal iron content
of natural waters. In calculating the energy utilization efficiency, the values for
free energy of formation of ferrous and ferric iron were taken (Bichowsky and
Rossini, 1936) instead of the values used by other authors for the reaction
involving the oxidation of ferrous carbonate. Low partial pressure of carbon
dioxide and high acidity (pH 2.0 to 2.5) preclude the existence of ferrous car-
bonate in any quantity. The efficiency value of 3.2 per cent compares with
reported values of about 5 to 10 per cent for the other autotrophs (Baas-Becking
and Parks, 1927).

Other possibilities which might explain some of the experimental results are
that the bacterium obtains its energy from the oxidation of ammonia, or traces
of organic matter, or that the oxidation of ferrous iron depends upon a secondary
reaction with some products of bacterial growth. The acid environment does
not suggest ammonia utilization for energy. The bacterium utilizes ammonia
nitrogen better than nitrate, but the concentration of ammonia has no effect
upon the rate of oxidation or the total amount of iron oxidized. Moreover, the
experiments with successive increments of iron rule out the possibility of ammonia
oxidation serving as a main source of energy. Increases in cell numbers and in
total carbon depended only on the amount of iron oxidized. When the total
energy theoretically available from the complete oxidation of ammonia to

nitrate is calculated, and the amount of carbon that could be fixed thereby is ascertained, the result is 0.033 grams carbon per liter. The energy utilization would probably not be over 10 per cent which would reduce the figure to 0.0033 grams C per liter, a value considerably below that obtained.

The possibility of traces of organic matter serving for growth was excluded by the use of reagent grade chemicals, scrupulously clean flasks, and permanganate distilled water.

The following reaction might conceivably occur in a medium containing nitrate or where all the ammoniacal nitrogen became oxidized to nitrate in some way:

$$6 \; FeSO_4 + 2 \; HNO_3 + 3 \; H_2SO_4 \rightarrow 3 \; Fe_2(SO_4)_3 + 2 \; NO + 4 \; H_2O.$$
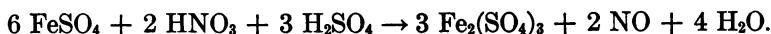
The amount of ferrous iron so oxidized would be only 3.45 grams per liter, but actually more than twenty times this amount was oxidized.

It is obvious that the composition of the media as given does not include all of the elements essential to life and metabolic activity. Presumably additional essential minerals were supplied as impurities in the reagent grade chemicals used. It was impossible to provide more than traces of soluble phosphate due to the insolubility of iron phosphate.

In view of the foregoing facts which show the mutual interdependence of bacterial growth and iron oxidation, the fixation of carbon during iron oxidation, and an efficiency of energy utilization consistent with theoretical considerations, it is concluded that the bacterium is an autotrophic iron oxidizer. It has already been shown that the iron-oxidizing bacterium develops autotrophically on thiosulfate (Colmer, Temple, and Hinkle, 1949). Morphologically there is no resemblance between this iron oxidizer and any of the sheathed or stalked iron bacteria. The iron bacterium cannot be distinguished from *Thiobacillus thiooxidans* under the microscope in stained or unstained preparations. The two organisms both produce acid in thiosulfate broth and are similar in their acid tolerance. As a gram-negative nonspore-forming motile rod living autotrophically on thiosulfate, the iron bacterium would fall into the genus *Thiobacillus*. It is sharply differentiated from *T. thiooxidans* (the member of that genus which it most resembles) by the oxidation of ferrous iron and by its failure to grow on elemental sulfur. It appears to be an obligate autotroph, failing to utilize glucose, peptone, or organic iron compounds. The ferrous iron oxidation is an outstanding characteristic of the bacterium; hence the name *Thiobacillus ferrooxidans* n. sp. is proposed for this organism.

The iron bacterium described in this report furnishes a clear cut example of an iron-oxidizing autotroph according to the concept so clearly restated by Winogradsky in 1922. In view of this fact the possibility of a mechanism for utilizing the energy of iron oxidation must not be denied in the case of other iron bacteria without conclusive evidence. Mere statements of the improbability of such a mechanism are now unsound. On the other hand the present work provides no evidence in favor of such a mechanism for any bacteria other than the one studied, and this bacterium seems to be more closely related to the genus *Thiobacillus* than to any of the previously described iron bacteria.

SUMMARY

An iron-oxidizing bacterium from acid mine water has been shown to live autotrophically upon inorganic media containing ferrous iron under conditions such that atmospheric oxidation was excluded. Iron oxidation and cell growth are mutually interdependent, and iron oxidation results in a measurable increase in cellular carbon. 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 weight ratio of iron oxidized to cellular material elaborated conforms to the requirements as stated by Starkey (1945). The bacterium, which also grows autotrophically on thiosulfate, has been assigned to the genus *Thiobacillus*, and the specific name *Thiobacillus ferrooxidans* n. sp. is suggested.

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