# STUDIES ON THE CHEMOAUTOTROPHIC IRON BACTERIUM *FERROBACILLUS FERROOXIDANS*

## II. Manometric Studies

MELVIN P. SILVERMAN[1, 2] AND DONALD G. LUNDGREN

*Department of Bacteriology and Botany, Syracuse University, Syracuse, New York*

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Studies of the obligate chemoautotrophic iron bacteria have been largely descriptive in character. This situation is not due to any lack of interest in the physiology and biochemistry of these bacteria, but rather to the inability to grow them in large numbers, and the attendant problem of harvesting cells free from the voluminous precipitate of iron that accompanies growth. Silverman and Lundgren (1959) described an improved medium for the propagation of large numbers of physiologically active cells of *Ferrobacillus ferrooxidans*, and a method of harvesting cells virtually free from precipitated iron. The present report concerns manometric studies of some of the characteristics of this chemoautotroph and its iron-oxidizing system using intact cells.

### MATERIALS AND METHODS

*F. ferrooxidans* strain TM was used. The inorganic iron-salts medium (9K) and the methods for growing and harvesting cells were the same as previously described (Silverman and Lundgren, 1959). Cells were harvested near the end of the logarithmic phase, suspended in distilled water acidified to pH 3.5 with $H_2SO_4$, and stored at refrigerator temperature.

Conventional manometric techniques were used in most of the studies. All attempts to find a buffer system that had no effect on the metabolic activity of cell suspensions were unsuccessful. Therefore, all pH values represent the initial pH in the main compartment after the contents of the side arm were tipped in. Each Warburg vessel contained a total volume of 1.7 ml distributed in the following manner. The main compartment contained 0.5 ml of cell suspension, and 0.5 ml made up of various combinations of weak $H_2SO_4$, water, or weak KOH necessary for the desired initial pH value. The center well contained 0.2 ml of 20 per cent KOH. The gas phase was air. After equilibration at 31 C, 0.5 ml of substrate was tipped in from the side arm and the oxygen uptake measured. Autoxidation of $Fe^{++}$ was retarded by dissolving the appropriate quantities of $FeSO_4 \cdot 7H_2O$ in distilled water acidified to pH 2.6 with $H_2SO_4$. The total nitrogen of cell suspensions was determined by the micro-Kjeldahl method. The total nitrogen of logarithmic phase cells was found to be constant from harvest to harvest. This permitted the use of a standard curve relating total nitrogen to cell numbers (direct counts, Petroff-Hausser counting chamber).

Carbon dioxide assimilation was measured by a modification of the methods of Schatz (1952) and Baalsrud and Baalsrud (1952). Vessels with two side arms were employed. Both side arms were equipped with vented stoppers. For control vessels the main compartment contained 0.5 ml of cell suspension and 1.0 ml of distilled water acidified to pH 2.6 with $H_2SO_4$. One side arm (designated side arm A) contained 12.5 $\mu$moles of $Na_2CO_3$ in a volume of 0.1 ml, whereas the other (designated side arm B) was left empty. Experimental vessels contained 0.5 ml of cell suspension and 0.5 ml of acidified distilled water (pH 2.6) in the main compartment. Side arm A contained 0.1 ml of $Na_2CO_3$ (12.5 $\mu$moles) whereas side arm B contained 50 $\mu$moles $Fe^{++}$ in a volume of 0.5 ml. The gas phase was air. After equilibration at 31 C, 0.200 ml of 0.2 per cent $HgCl_2$ in 2 N $H_2SO_4$ was admitted to side arm A of all vessels through the vented stoppers employing the technique of Schatz (1952). After 30 min the generation of carbon dioxide was complete. Substrate was then tipped into the main compart-

[1] Portion of a thesis to be presented to the Graduate School of Syracuse University in partial fulfillment of the requirements for the Ph.D. degree in Microbiology.

[2] Predoctoral Fellow of the National Cancer Institute of the National Institutes of Health.

ment of the experimental vessels. After 90 min iron oxidation appeared to be complete. The acid solution in side arm A was tipped into the main compartment of all vessels thereby halting any further metabolism and releasing any loosely bound carbon dioxide. Finally, 0.200 ml of 40 per cent KOH was admitted, via the vented stopper, to side arm B of all vessels. The absorption of free carbon dioxide was complete in 60 min. For the most accurate results, all flask constants were adjusted to reflect the two separate additions of 0.200 ml acid and base.

## RESULTS

The conditions of harvesting and storage resulted in cell suspensions whose activity (in terms of the rate of $Fe^{++}$ oxidation) consistently remained uniform for at least 8 days after harvesting. Thereafter, the activity slowly fell off with detectable activity still present after 23 days of storage. As an example, in one experiment, a 4-day-old cell suspension, in the presence of 250 $\mu$moles $Fe^{++}$, had a $Q_{O_2}(N)$ ($\mu$L $O_2$ uptake per mg total cell N per hr) of 4516 whereas at ages 15 and 23 days, the $Q_{O_2}(N)$ had decreased to 3919 and 3524, respectively.

The results of numerous experiments with 5 different harvests of *F. ferrooxidans* showed that $O_2$ uptake consistently approached the theoretical amount required by the equation:

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

Oxygen uptake ranged from 92.7 to 101.8 per cent of theoretical using substrate concentrations of from 50 to 500 $\mu$moles $Fe^{++}$. These results are in agreement with the findings of Beck and Elsden (1958). Controls were run in all experiments to determine both the magnitude of the autoxidation of $Fe^{++}$ under the acidic conditions employed, and the endogenous metabolism of cell suspensions. At no time did autoxidation ever exceed 2.0 per cent even when concentrations as high as 500 $\mu$moles $Fe^{++}$ per vessel were present. Endogenous metabolism has never been observed.

The activity of cell suspensions was not inhibited by increasing the $Fe^{++}$ concentration. Rather, increasing substrate concentrations resulted in increased rates of iron oxidation as measured by $O_2$ uptake. Figure 1 illustrates the uptake of $O_2$ in the presence of 250 and 500 $\mu$moles $Fe^{++}$. From the linear portions of these curves it can be calculated that the $Q_{O_2}(N)$ was

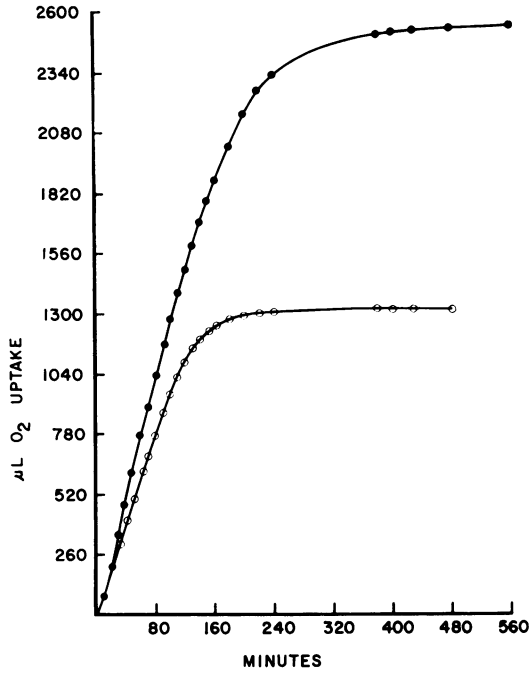


*Figure 1.* The effect of substrate concentration on the rate of iron oxidation by *Ferrobacillus ferrooxidans*. All vessels contained 15-day-old cell suspension (0.161 mg total N). *Solid circles*, 500 $\mu$moles $Fe^{++}$ (initial pH 3.3); *open circles*, 250 $\mu$moles $Fe^{++}$ (initial pH 3.0). All values corrected for autoxidation; endogenous metabolism absent.

5131 for the 500 $\mu$moles substrate level and 3919 for the 250 $\mu$moles level. The results of additional experiments with different cell harvests revealed that $Q_{O_2}(N)$ values ranged from 2027 to 4516 in the presence of 250 $\mu$moles $Fe^{++}$. The presence of 500 and 250 $\mu$moles $Fe^{++}$ in a volume of 1.5 ml are equivalent to ferrous iron concentrations of 18,598 and 9,299 ppm, respectively. Previous growth experiments (Silverman and Lundgren, 1959) had established that 9,000 ppm $Fe^{++}$ was optimum for the most rapid rate of growth whereas 18,000 ppm $Fe^{++}$ resulted in a decreased growth rate. Thus, manometric and growth experiments do not agree in this respect. Whatever the cause for decreased growth rates may be, it probably is not due to substrate inhibition of the iron-oxidizing system.

An investigation of the effect of initial pH on iron oxidation showed that optimal activity occurred over the range 3.0 to 3.6 with over 80 per cent of the maximal oxidation occurring within

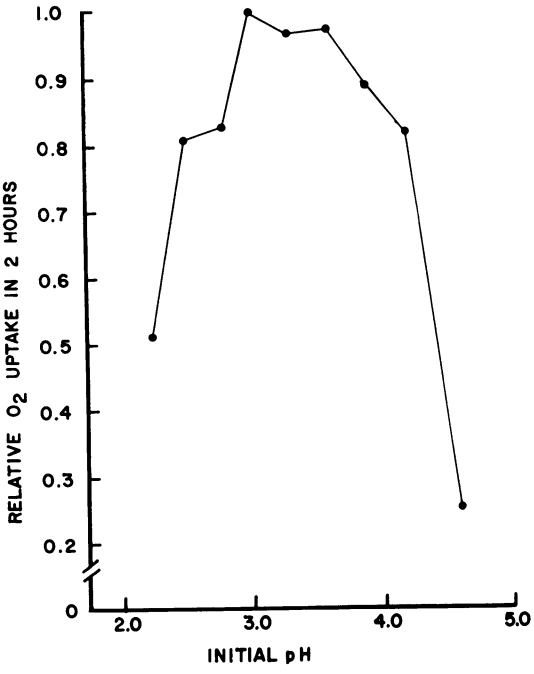


*Figure 2.* The effect of initial pH on the rate of iron oxidation by *Ferrobacillus ferrooxidans*. All vessels contained 12-day-old cell suspension (0.074 mg total N), 250 μmoles $Fe^{++}$. The relative value 1.0 was equivalent to an absolute value of 348 μL, corrected for autoxidation. Endogenous metabolism was absent.

the pH range 2.5 to 4.2 (figure 2). Thus, *F. ferrooxidans* possesses an acidic optimum for the oxidation of iron, a fact which agrees with its growth characteristics. On the other hand, a study of the effect of temperature on iron oxidation revealed that the optimal temperature did not agree with the growth characteristics of *F. ferrooxidans*. The organism grows well at 28 C but not at all at 37 C. As shown in figure 3, the most rapid oxidation of $Fe^{++}$ took place at 37 C with over 80 per cent of the maximum occurring within the range 28 to 40 C. Presumably, some other heat labile constituents(s) of the cell is responsible for the lack of growth at 37 C.

*F. ferrooxidans* assimilated $CO_2$ during the oxidation of 50 μmoles $Fe^{++}$. These data, together with the standard errors associated with the measurement of $CO_2$ by the present method, are summarized in table 1. The free energy efficiency of substrate utilization was calculated by assuming that the reaction

$$Fe^{++} \rightarrow Fe^{+++} + e$$

yielded 11.3 kcal per g-atom (Temple and Colmer, 1951), and that the fixation of 1 g-atom of carbon required 120 kcal. The average efficiency of 20.5 ± 4.3 per cent agrees well with the values reported for other chemoautotrophs. As a comparison, the data of Beck and Elsden (1958) yield a calculated range of from 4.8 to 10.6 per cent efficiency for an iron bacterium "probably

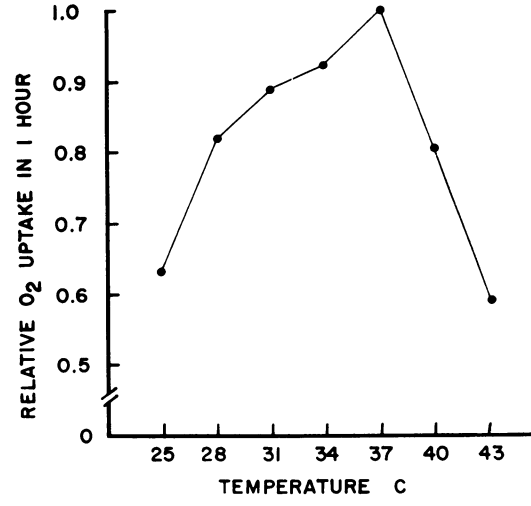


*Figure 3.* The effect of temperature on the rate of iron oxidation by *Ferrobacillus ferrooxidans*. All vessels contained 10-day-old cell suspension (0.148 mg total N), 250 μmoles $Fe^{++}$. Initial pH 3.9. The relative value 1.0 was equivalent to an absolute value of 350 μL, corrected for autoxidation. Endogenous metabolism was absent.

TABLE 1

*Assimilation of carbon dioxide by Ferrobacillus ferrooxidans during the oxidation of 50 μmoles $Fe^{++}$*

| Flask No. | $CO_2$ Assimilated | | Free Energy Efficiency |
|---|---|---|---|
| | *μL* | *μmoles* | % |
| 1 | 14.7 | 0.66 | 13.8 |
| 2 | 30.3 | 1.35 | 28.6 |
| 3 | 20.0 | 0.89 | 18.9 |
| $Mean_{1,2,3...}$ | 21.7 ± 4.6* | 0.97 ± 0.20 | 20.5 ± 4.3 |

* Standard error of the mean. Age of cells, 8 days; 0.288 mg total N per vessel; initial pH 3.0.

identical to *Ferrobacillus ferrooxidans*," and Temple and Colmer (1951) have reported a maximal efficiency of 3.2 per cent for *Thiobacillus ferrooxidans* when grown on iron.

Phosphate depressed the rate of iron oxidation even when present at 0.002 M concentration (figure 4). This effect was not due to the influence of initial pH as shown by the rates of oxidation in the two controls. It may be that phosphate acts by tying up ferrous iron as slightly soluble ferrous phosphate, thereby making it less readily available to the cells. But if this were so, then 0.01 M phosphate should have decreased the rate of oxidation even further than did 0.006 M phosphate. Since this did not occur some other explanation must be sought. Whatever the mechanism of phosphate inhibition may be, it appears that the sensitive site(s) became saturated when the external phosphate concentration reached 0.006 M.

Previous studies had demonstrated that citrate inhibited growth (Silverman and Lundgren, 1959). Manometric experiments showed that citrate inhibited iron oxidation. Progressive inhibitions of 5, 42, and 71 per cent were observed with citrate concentrations of $5.0 \times 10^{-3}$ M, $7.5 \times 10^{-3}$ M, and $1.0 \times 10^{-2}$ M, respectively. Further increases in the citrate concentration presented the curious picture of an apparent reversal of inhibition, for inhibitions of 56, 44, 40, and 25 per cent were observed with citrate concentrations of $2.5 \times 10^{-2}$ M, $5.0 \times 10^{-2}$ M, $7.5 \times 10^{-2}$ M, and $1.0 \times 10^{-1}$ M, respectively. Suitable controls showed that *F. ferrooxidans* did not oxidize citrate. This anomalous reversal was traced to a strictly chemical interaction between ferrous iron and citrate resulting in an apparent uptake of gas under the acidic conditions employed.

Iron ores in nature are usually associated with some manganese, cobalt, and nickel. In addition, as reviewed by Pringsheim (1949*a*, *b*), some of the iron bacteria have been reported as being able to utilize iron and manganese as an energy source. Therefore, it was deemed worthwhile to investigate the possibility of *F. ferrooxidans* utilizing these metals. The results of manometric experiments showed that in concentrations of 50 μmoles each, $Mn^{++}$ (as $MnSO_4 \cdot 4H_2O$, pH 3.8), $Co^{++}$ (as $CoSO_4 \cdot 7H_2O$, pH 3.7), and $Ni^{++}$ (as $NiCl_2 \cdot 6H_2O$, pH 3.7) were not oxidized. Similarly, tetravalent manganese (as $MnO_2$, 51.8

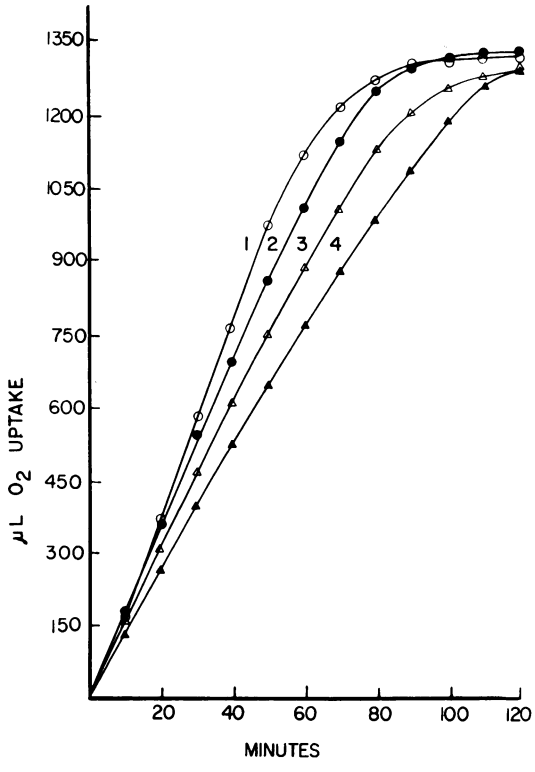


*Figure 4.* The effect of phosphate on the rate of iron oxidation by *Ferrobacillus ferrooxidans*. All vessels contained 4-day-old suspension (0.269 mg total N), 250 μmoles $Fe^{++}$. Phosphate added as $KH_2PO_4$. *Curve 1*, no phosphate, initial pH 3.0; *curve 2*, no phosphate, initial pH 3.25; *curve 3*, $2.0 \times 10^{-3}$ M phosphate, initial pH 3.15; *curve 4*, $6.0 \times 10^{-3}$ M phosphate (initial pH 3.20) and $1.0 \times 10^{-2}$ M phosphate (initial pH 3.25). All values corrected for autoxidation; endogenous metabolism absent.

μmoles, pH 3.8) was not utilized as an energy source.

Leathen *et al.* (1956) reported that *F. ferrooxidans* was unable to grow in the acid thiosulfate medium of Colmer *et al.* (1950) or in the elemental sulfur medium of Waksman (1922). In addition, our inorganic iron-salts medium (9K) contains an oxidizable constituent other than ferrous iron, namely ammonium sulfate. As a further check on the restriction of this organism to iron oxidation as its sole energy source, the action of cell suspensions on ammonium, thiosulfate, and elemental sulfur was assayed manometrically. No oxidation of ammonium (as $(NH_4)_2SO_4$, 10

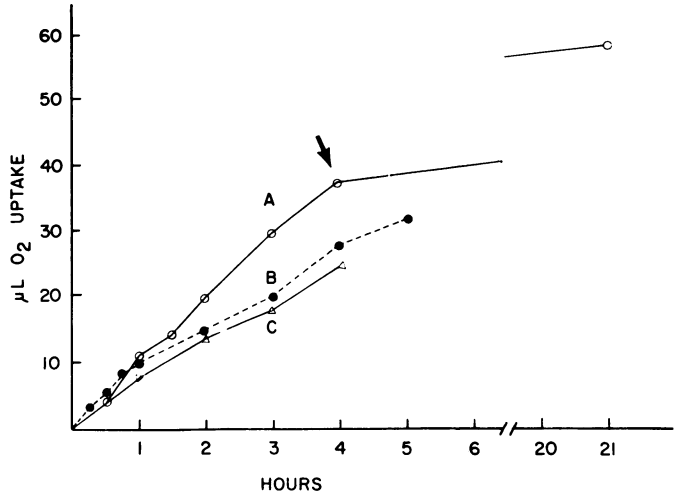


*Figure 5.* The oxidation of elemental sulfur by *Ferrobacillus ferrooxidans*. *Curve A*, 8-day-old cell suspension (0.269 mg total N), 5 μmoles colloidal S, initial pH 2.5; *curve B*, 16-day-old cell suspension (0.538 mg total N), 1000 μmoles powdered S, initial pH 3.5; *curve C*, 14-day-old cell suspension (0.269 mg total N), 1000 μmoles powdered S, initial pH 3.5. At the time indicated by the *arrow* shaking of the respirometer was halted for the duration of the experiment.

μmoles, pH 3.7) or thiosulfate (as $Na_2S_2O_3 \cdot 5H_2O$, 5 μmoles, pH 3.7) was observed. However, a slow but significant oxidation of sulfur occurred (figure 5). Controls containing sulfur in the absence of cell suspensions, or boiled cell suspensions in the presence of sulfur, showed no oxygen uptake, thereby eliminating the possibility of nonbiological sulfur oxidation. The data do not permit an evaluation of the extent of sulfur oxidation with regard to the end product. It is of interest to note that when shaking was halted the rate of sulfur oxidation was markedly decreased. This last observation agrees with the results of Starkey *et al.* (1956) and Newburgh (1954). These authors, using *Thiobacillus thiooxidans*, found an increased oxidation of sulfur during agitation.

## DISCUSSION

In view of the low energy yield of ferrous iron oxidation (11.3 kcal per g-atom) certain predictions can be made concerning the growth of bacteria at the expense of iron. First, the oxidation of relatively enormous quantities of iron should be a requisite for growth. Assuming an 8 per cent efficiency for the utilization of the energy from the oxidation of ferrous carbonate, Starkey (1945) predicted a 500:1 weight ratio of oxidized iron (as $Fe(OH)_3$) to cellular material assimilated (as $CH_2O$). From the data for the mean $CO_2$ assimilation in table 1, a 182:1 weight ratio can be calculated of $Fe(OH)_3$ produced to carbon assimilated as $CH_2O$. This lower ratio, compared to the predicted ratio of Starkey, is probably due to a greater efficiency of energy utilization for *F. ferrooxidans* (20.5 ± 4.3 per cent) than had previously been thought possible for the iron bacteria. Nevertheless, a ratio of 182:1 still justifies predictions that voluminous precipitation of iron would occur. Secondly, one would expect a rapid rate of iron oxidation to accompany a reasonably rapid rate of growth. *F. ferrooxidans* has a generation time of about 7.0 hr (Silverman and Lundgren, 1959). The present demonstration of $Q_{O_2}(N)$ values of from 2027 to 5131 tends to support this last prediction.

The oxidation of both sulfuritic and ferrous iron compounds has been reported for two other species of iron-oxidizing bacteria. *T. ferrooxidans* oxidizes thiosulfate, pyrite, and ferrous iron for growth but not free sulfur to any appreciable extent (Temple and Colmer, 1951; Temple and Delchamps, 1953). Bryner and Jameson (1958) described a chemoautotroph that oxidized ferrous iron, sulfur, and pyrite as well as other sulfide minerals. These authors concluded that this organism exhibited characteristics that were "nearly the same as *T. ferrooxidans*." The present

demonstration of the slow oxidation of elemental sulfur by resting cells of *F. ferrooxidans* raises the question of whether this bacterium can utilize this energy source for growth. Preliminary growth studies indicate that this may be the case. Further investigations along these lines are being actively pursued in this laboratory.

SUMMARY

Some of the physiological properties of the obligate chemoautotrophic iron-oxidizing bacterium *Ferrobacillus ferrooxidans* were studied manometrically using intact cells. Iron was oxidized at an unusually rapid rate ($Q_{O_2}(N)$ of from 2027 to 5131). Oxygen uptake was over 92 per cent of the theoretical amount required by the reaction:

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

The optimal pH and temperature for the iron-oxidizing system were 3.0 to 3.6 and 37 C, respectively, although no growth occurred at 37 C. There was no evidence of iron toxicity to the intact cells at concentrations as high as 500 $\mu$moles $Fe^{++}$ per Warburg vessel. During the oxidation of 50 $\mu$moles of iron, *F. ferrooxidans* assimilated $CO_2$ with an average efficiency of 20.5 $\pm$ 4.3 per cent. Phosphate moderately inhibited iron oxidation; citrate was strongly inhibitory. Resting cells were unable to oxidize ammonium, thiosulfate, di- and tetravalent manganese, cobalt, or nickel. However, a slow but significant oxidation of elemental sulfur was observed.

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