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Direct Method for Continuous Determination of Iron Oxidation by Autotrophic Bacteria

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A method for direct, continuous determination of ferric ions produced in autotrophic iron oxidation, which depends upon the measurement of ferric ion absorbance at 304 nm, is described. The use of initial rates is shown to compensate for such changes in extinction during oxidation, which are due to dependence of the extinction coefficient on the ratio of complexing anions to ferric ions. A graphical method and a computer method are given for determination of absolute ferric ion concentration, at any time interval, in reaction mixtures containing *Thiobacillus ferrooxidans* and ferrous ions at known levels of SO_4^{2+} and hydrogen ion concentrations. Some examples are discussed of the applicability of these methods to study of the rates of ferrous ion oxidation related to sulfate concentration.

Thiobacillus ferrooxidans is an acidophilic bacterium capable of chemolithotrophic growth using carbon dioxide as the sole source of carbon while obtaining its metabolic energy from the oxidation of ferrous iron or the sulfur atoms of thiosulfate and elemental sulfur. The oxidation of ferrous iron by *T. ferrooxidans* is not only of intrinsic interest due to its biological uniqueness, but is also of economic significance because of the ecological impact of such processes in natural environments.

In nature, the oxidation of iron by *T. ferrooxidans* in mine drainage waters results in the formation of sulfuric acid and ferric iron precipitates. These products account for the severe pollution problems that occur in surface waters which receive discharges from geological substrata containing reduced iron and sulfur minerals.

Various methods have been employed to study the oxidation of iron by *T. ferrooxidans*. Manometric techniques were used by Temple and Colmer (15), Silverman and Lundgren (13), Beck (2), Landesman et al. (7), and others. The data of these workers expressed ferrous iron oxidation as a function of oxygen uptake under various experimental conditions.

An additional approach has been to measure ferric iron concentration with colorimetric methods. For instance, Lazaroff (8), and Razzell and Trussell (10) colorimetrically measured the concentration of ferric-thiocyanate complex. Schnaitman et al. (11) reported a simplified colorimetric assay in which ferric iron was measured as the yellow chloride complex at 410 nm.

All of the colorimetric assays mentioned have the disadvantage of requiring the introduction of color reagents to the samples which are removed from the reaction mixtures for analysis. This necessitates stopping the reaction prior to the determination of absorbance.

It is essential for physiological studies that the absorbance of the colored iron complexes not be altered indeterminately by the experimental variables imposed on the bacteriological system under study. Such alterations which do occur must be compensated for by appropriate controls to obtain an accurate determination of oxidized iron. For example, if the effect of sulfate ions on bacterial iron oxidation is the subject of inquiry, it would be prerequisite to determine the effects of sulfate concentration on the molar extinction of the absorbing ferric iron species before the amount of oxidized iron could be accurately measured.

Hayon and Weiss (5) have used the ultraviolet absorbance of ferric ions as the basis for the measurement of ferrous iron oxidation in irradiated nonbiological systems. Previously, Whiteker and Davidson (17), Bastian et al. (1), and Heidt et al. (6) had described the ultraviolet photochemistry of ferrous and ferric ions in inorganic solution. Our investigations showed that the methods employed by these workers could be adapted to the study of the biological oxidation of ferrous iron by *T. ferrooxidans*. In contrast to previously used colorimetric tech-

niques, this adaptation has permitted the continuous, kinetic determination of ferric iron formation in a reaction mixture without stopping the reaction or adding colorimetric reagents during the course of reactions.

MATERIALS AND METHODS

Strain used. The strain of *T. ferrooxidans* used in this study was obtained from D. G. Lundgren (Syracuse University, Syracuse, N.Y.). Stock cultures were maintained aseptically in the 9K medium of Silverman and Lundgren (12). Working stocks for inoculating mass cultures were prepared from these and maintained aseptically in 250-ml shaking flasks at room temperature and were transferred weekly. The basal constituents of the 9K media were sterilized by autoclaving at 15 lb/in^2 for 15 min. Sterilization of the ferrous iron constituent was accomplished by forcing it through Metricel GA-8 filters (0.20-μm pore size) with a syringe.

Culture and purification of cells. Mass culture of the bacteria to obtain large quantities of cells for experimental purposes did not require aseptic conditions since the pH of 2.5 and the inorganic nature of the 9K medium inhibited growth of contaminating organisms. For mass cultures, 20-liter carboys each containing 10 liters of 9K medium were inoculated using the contents of an actively growing 250-ml shaken flask. Mass cultures were grown at room temperature and rigorously aerated by bubbling with compressed air through sintered-glass spargers.

One week after inoculation, the contents of the carboys were filtered through Whatman no. 1 filter paper to remove precipitated iron compounds. The cells were separated from the filtrate by continuous-flow centrifugation at 20,000 $\times$ *g*.

The sedimented cells were washed by suspension and sedimentation in dilute sulfuric acid (pH 2.5) at 5 C. Repetition of this procedure produced a cell suspension relatively free of solid contaminants. The final, washed cell suspensions contained approximately 3.0 $\times$ 10^{10} cells/ml, as determined microscopically with a Petroff-Hausser counting chamber.

Determination of iron absorption. A Cary model 15, double-beam, recording spectrophotometer was used for the determination of ferrous and ferric iron absorption spectra by scanning the ultraviolet-absorbing region of the spectrum between 340 and 220 nm. This instrument was also used to record the continuous production of ferric ions by measuring changes in absorbance at 304 nm. Using the sample cuvette as a reaction vessel containing the desired amounts of bacterial cells and substrate, kinetic data were obtained by comparing the sample at 304 nm to a reference cuvette which contained the appropriate concentrations of bacterial cells suspended in water acidified to pH 2.5 with H_2SO_4. Absorbance increases due to ferric iron production were plotted directly, versus elapsed time, on the strip-chart read-out of the instrument. Reaction rates were obtained from the slopes of these responses and plotted against the particular experimental variable employed.

When manometric determinations were made, the standard Warburg technique for oxygen uptake was used as described by Umbreit et al. (16).

RESULTS

The absorption maxima of ferric and ferrous ions are shown in Fig. 1. Since sulfate anions affect the rate of bacterial iron oxidation, it was important to note the differences in absorbance due to the replacement of sulfate by chloride anions. Ferric ions absorb strongly in the 295- to 304-nm region, in contrast to ferrous ions with major absorbance in the 220- to 250-nm region. In both cases, chloride anions cause shifts of the absorption peaks to lower wavelengths and overall increases in absorptivity. It is important to note that the extinction due to ferric complexes at 304 nm are approximately 300 times that of the corresponding ferrous species at the same wavelengths, a factor which permits the spectrophotometric determination of small amounts of ferric ions in solutions containing high concentrations of ferrous ions.

The small absorbance of ferrous iron solutions initially observed in the 300-nm region was virtually eliminated by treatment with stannous chloride, indicating that the absorbance was due to contaminating ferric ions (Fig. 2).

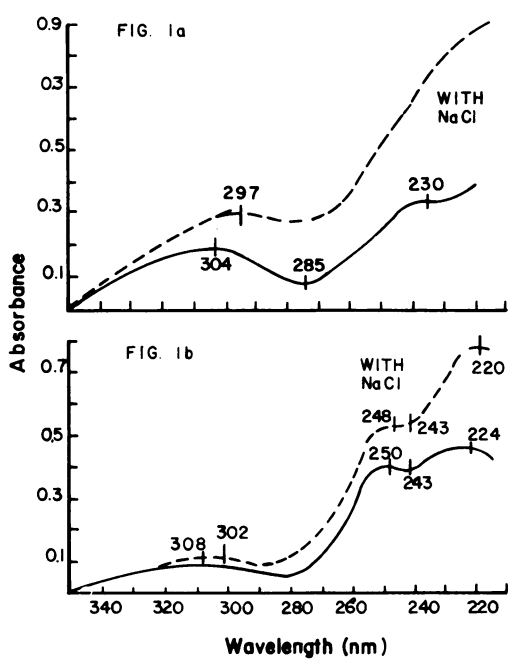

FIG. 1. *Absorption spectra of* $Fe_2^{3+}(SO_4)_3$ *and* $Fe^{2+}SO_4$. *(a)* 1.2 $\times$ 10^{-5} *M* $Fe_2^{3+}(SO_4)_3$ *alone and with equimolar NaCl. (b)* 3.23 $\times$ 10^{-3} *M* $Fe^{2+}SO_4$ *alone and with equimolar NaCl. All solutions adjusted to pH 2.5 with* H_2SO_4.

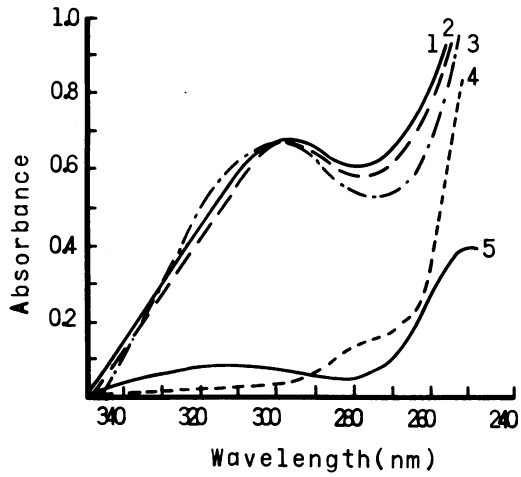

FIG. 2. *Absorbance spectra of 3.23 × 10^{-3} M $FeSO_4$ solutions treated as follows: (i) oxidized by a cell suspension of T. ferrooxidans, removed by filtration prior to determination; (ii) oxidized with 1 drop of 0.3% H_2O_2; (3) oxidized by irradiation with a 100-W mercury discharge lamp; (4) reduced with 9.7 μmol of $SnCl_2$ per cuvette containing 3 ml of sample solution; and (5) untreated. All solutions at pH 2.5.*

Conversely, the absorption of ferric solutions at wavelengths shorter than 250 nm was clearly not due to ferrous iron contamination. This is indicated by the disparity of the ferrous and ferric extinction coefficients, and by the stability of the absorption peaks in the 250- to 200-nm region after ferric solutions had been treated with hydrogen peroxide or by ultraviolet irradiation.

Because of the overlap of the ferrous and ferric iron absorption peaks in the region below 250 nm, as well as the large effects of complexing anions on short-wavelength absorbance, it was preferable to measure the formation of ferric ions in the 295- to 305-nm region. Any absorbance in this region is clearly attributable to ferric complexes with minimal interference from other species present in the reaction mixtures. This conclusion is in agreement with the observations of Bastian et al. (1), Whiteker and Davidson (17), and Heidt et al. (6).

A comparison was made of absorption spectra when identical ferrous sulfate solutions were oxidized biotically by a cell suspension of *T. ferrooxidans* and abiotically with hydrogen peroxide or by ultraviolet irradiation. These spectra indicate that the end products of ferrous ion oxidation are spectrophotometrically similar in all cases (Fig. 2). Therefore, it was feasible to measure directly the bacterial oxidation of ferrous ions by a spectrophotometric method dependent on ultraviolet absorbance specific for ferric ions. Any absorbance due to the cells themselves could be blanked out by using a cell suspension of *T. ferrooxidans* without substrate in the reference cuvette.

To obtain ferric iron formation in absolute units the molar extinction coefficients of ferric ions at 304 nm were determined. For the purpose of our investigations of bacterial iron oxidation, the effect of sulfate anion concentration on the molar extinction of ferric ions had to be considered.

Ferric ion absorbance was measured at 304 nm when ferrous solutions were oxidized with H_2O_2 in the presence of various concentrations of sulfate (Table 1). The oxidized solutions were allowed to stand overnight, and then were diluted 1:10 with H_2SO_4 (pH 2.5) prior to the determination of absorbance and calculation of the molar extinction coefficients.

The elevation of sulfate concentrations increased the absorbance at 304 nm. It was necessary to distinguish between two possible means by which sulfate anions could influence absorption: (i) higher sulfate concentrations could affect the equilibrium of the oxidation reaction, resulting in an increased yield of ferric ions; or (ii) the amounts of ferric ions formed were the same in each case, but increasing levels of sulfate resulted in sulfate-ferric ion complexes with greater specific extinctions.

To determine which of the above effects were operative, ferrous sulfate solutions were oxidized by the procedure described above, except that the sulfate levels were adjusted 1 h after the solutions had been oxidized. A comparison of these data shows that both procedures resulted in equivalent ferric ion absorbances at

TABLE 1. *Molar extinction coefficients (at 304 nm) of 3.23 × 10^{-3} M $FeSO_4$ solutions (pH 2.5) oxidized with 1 drop of H_2O_2*[a]

Total sulfate from $FeSO_4$ and Na_2SO_4 (× 10^{-3} M)	$[SO_4^{2-}]/[Fe^{2+}]$	Optical density	ε = A/Ml		Δε[b]
			Na_2SO_4 added before oxidation	Na_2SO_4 added after oxidation	
3.23	1	6.10	1,888	1,873	+15
16.56	5.1	6.40	1,981	1,935	+46
56.56	17.5	6.65	2,059	2,043	+16
216.56	67.0	7.60	2,353	2,353	0
669.89	207.4	8.30	2,570	2,554	+16

[a] Various concentrations of sulfate were added as Na_2SO_4 before and after iron oxidation. Total reaction mixture volumes were 3.0 ml.

[b] A paired *t* test value of $t = 2.64$ indicates that the differences between the two procedures are not statistically significant at the 5% level.

the same sulfate levels (Table 1). This indicates that increasing the sulfate concentration increases the molar extinction of the absorbing species, most probably by altering the nature of the sulfate-ferric ion complex in solution. Our molar extinction coefficients are in agreement with those of Heidt et al. (6), who showed a similar range of values for oxidized, acidic, ferrous sulfate solutions.

Figure 3 is a graphic presentation of the relationship between the molar extinction coefficients of ferric ions and the relative proportions of SO_4^{2-} to Fe^{3+}. This curve can be used to calculate the absolute quantities of ferric ions produced at different sulfate concentrations.

For example, a typical substrate for oxidation consisted of 3.23×10^{-3} M (final concentration) $FeSO_4$ adjusted to pH 2.5 with 0.42×10^{-3} M H_2SO_4, yielding a total sulfate concentration of 3.65×10^{-3} M. For any concentration of ferric ions up to the maximum of 3.23×10^{-3} M, the SO_4^{2-} to Fe^{3+} ratio can be calculated and used to obtain an appropriate molar extinction value from the curve shown in Fig. 3. By plotting Fe^{3+} concentration versus absorbance (where absorbance = $A = \epsilon M$, a curve is obtained which can be used to convert absorbance values to Fe^{3+} concentration. Such a curve is shown in Fig. 4. It may be noted that the relationship between the absorbance and molarity of Fe^{3+} is nearly linear at the lowest values. With increasing Fe^{3+}, the line curves slightly and then becomes linear again. This reflects the comparatively small change in extinction at high $[SO_4^{2-}]/[Fe^{3+}]$ ratios and the diminishing change in the $[SO_4^{2-}]/[Fe^{3+}]$ ratio as it approaches unity at higher Fe^{3+} molarity.

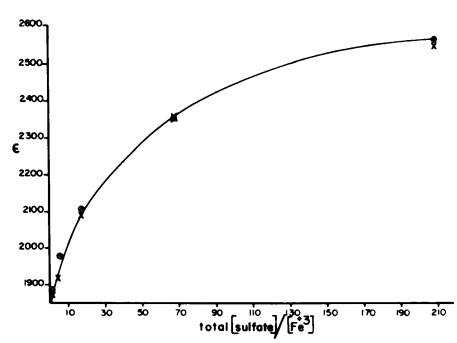

FIG. 3. *Effect of sulfate concentration on molar extinction coefficient of ferric ions (3.23 × 10⁻³ M Fe^{2+} [as $FeSO_4$] oxidized with 1 drop of 30% H_2O_2 and diluted 1 to 10 in H_2SO_4 [pH 2.5] for absorbance determinations). Symbols: (⊖), sulfate added before oxidation; (×), sulfate added after oxidation. All solutions were at pH 2.5.*

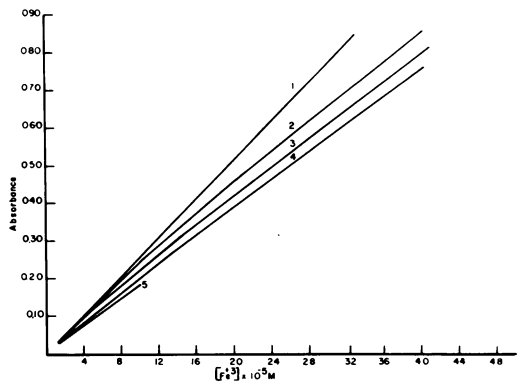

FIG. 4. *The calculated relationship of absorbance at 304 nm to increasing ferric ion concentration at five levels of sulfate ion concentration: (1) 1.0 × 10⁻¹ M SO_4^{2-}; (2) 1.0 × 10⁻² M SO_4^{2-}; (3) 3.65 × 10⁻³ M SO_4^{2-}; (4) 1.0 × 10⁻³ M SO_4^{2-}; (5) 1.0 × 10⁻⁴ M SO_4^{2-} (used experimentally).*

Figure 5 shows this even more strikingly by relating the calculated changes in the extinction coefficient, the $[SO_4^{2-}]/[Fe^{3+}]$ ratio, absorbance, and micromoles of Fe^{3+} formed. Since the extinction coefficient is maximal at the beginning of the reaction (due to the high ratio of sulfate to ferric ions), the use of initial rates for kinetics determinations appears amply justified. In addition, actual observations of absorbance changes measured by continuous spectrophotometric analysis are nearly linear from time zero during the first few minutes of oxidation, depending on the oxidation rate.

The practical limits for $[SO_4^{2-}]/[Fe^{3+}]$ ratios which may be treated in this method of analysis are fixed at an upper value by the solubility product for ferric sulfate in acid solution and at a lower value by a ratio which is slightly greater than the equivalence of sulfate to ferric ions. The latter situation would be represented by a ferrous sulfate solution containing a small amount of additional sulfate originating from pH adjustment to 2.5 with H_2SO_4. Lower ratios than 1 cannot be treated with our extinction coefficients, since anions other than sulfate would change the relationship shown in Fig. 3. (Although the extinction values in the presence of other anions can be determined, it should be recognized that the extinction coefficient would change very little unless competing anions were present in concentrations approaching the sulfate ion.)

Figure 4 shows the relationship between absorbance and Fe^{3+} concentrations in solutions containing four different levels of sulfate. The value for $[Fe^{3+}]$ may be approximated for absorbance measurements at any sulfate level by

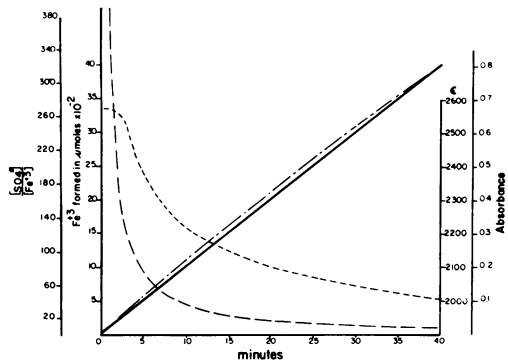

FIG. 5. *The calculated relationship of Fe^{3+} formation, $[SO_4^{2-}]$ to $[Fe^{2+}]$ ratio, ϵ_{304}, and absorbance to time, in a system containing 3.23 × 10^{-3} M $FeSO_4$ and 0.42 × 10^{-3} M H_2SO_4 (3.65 × 10^{-3} M $[SO_4^{2-}]$), with 1.0 × 10^{-2} μmol of Fe^{3+} formed/min. Key to lines:* (———), *μmol × 10^{-2} Fe^{3+} formed;* (—·—·—), *absorbance at 304 nm;* (– – –), *molar extinction coefficient (ϵ_{304});* (— — —), *$[SO_4^{2-}]/[Fe^{2+}]$ ratio.*

interpolation between the curves. However, a more precise value can be obtained by the graphical method described previously.

In addition, it is possible to obtain an equation which approximately expresses the curve in Fig. 3, relating the molar extinction coefficient (ϵ) of ferric ions to the $[SO_4^{2-}]/[Fe^{3+}]$ ratio. The correspondence of the experimental relationship to the least-squares curve (Fig. 6) is expressed by

$$Y = e\, C_0 + C_1 \ln X + C_2 (\ln X)^2 + C_3 (\ln X)^3$$

where $Y = \epsilon$, the molar extinction coefficient; $X = S/F$, $S = [SO_4^{2-}]$, $F = [Fe^{3+}]$; $C_0 = 7.797$, $C_1 = 0.243988$, $C_2 = 0.08920$, and $C_3 = 0.00782431$. Since $Y = A/F\,l$, where A = absorbance, F = molar concentration of ferric ions, and l = path length of cuvette in centimeters, then:

$$A_{304} = Fl \times e^{C_0 + C_1 (\ln S/F) + C_2 (\ln S/F)^2 + C_3 (\ln S/F)^3}$$

Thus, the absorbance can be calculated directly for any ferric ion concentration if the sulfate concentration is known. However, a more important application of this equation is to use a computer to evaluate F ($[Fe^{3+}]$) for any given absorbance and sulfate level. This eliminates the cumbersome operations involved in the construction of curves and the application of the graphic method for determining $[Fe^{3+}]$.

Under conditions employed for spectrophotometric determination, the rates of bacterial iron oxidation were proportional to the concentration of bacterial cells present in the reaction cuvette (Fig. 7). This indicates that neither the concentration of ferrous iron substrate nor oxygen availability were rate-limiting.

Figure 8 shows a Lineweaver-Burk treatment of data from an experiment in which the bacterial cell concentrations were constant but the ferrous sulfate concentrations were varied. The K_m and maximal velocity were calculated to be 5.68 × 10^{-4} mol/liter and 2.08 × 10^{-2} μmol/min, respectively.

The spectrophotometric method was applied in experiments to determine the effects of sulfate ion concentration on bacterial iron oxidation. By increasing the $[SO_4^{2-}]/[Fe^{2+}]$ ratio from 1 to 14, stimulation of ferric iron formation occurred (Fig. 9).

A comparison was made of these data with manometric determinations in which the concentrations of bacterial cells and ferrous iron substrate were constant and the concentration of sulfate was varied (Fig. 9). The manometric data indicated that a $[SO_4^{2-}]/[Fe^{2+}]$ ratio of 1.5 produced the greatest rate of oxygen uptake, but the further addition of sulfate sharply decreased this activity.

This pattern of response differed from the spectrophotometric determination of Fe^{3+} formation, prompting additional determinations of ferric iron formation at higher sulfate levels (Table 2). It can be seen that the rate of ferrous

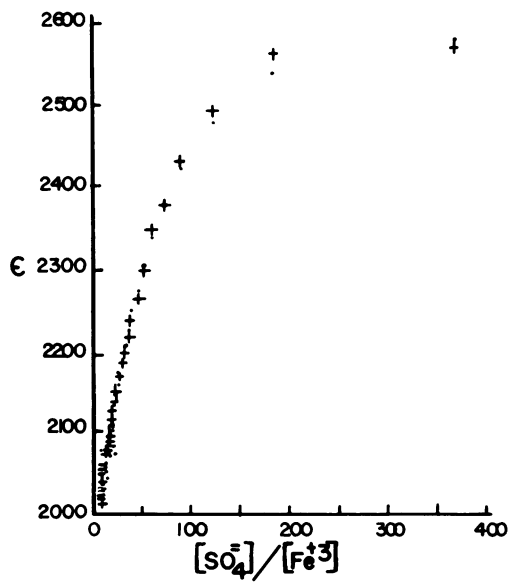

FIG. 6. *A comparison of the relationship between $[SO_4^{2-}]/[Fe^{3+}]$ to molar extinction (ϵ_{304}) and the least-squares function*

$$y = e\, C_0 + C_1 \ln X + C_2 (\ln X)^2 + C_3 (\ln X)^3$$

where: $y = \epsilon_{304}$; $x = [SO_4^{2-}]/[Fe^{3+}]$; $C_0 = 7.797$; $C_1 = 0.243988$; $C_2 = 0.08920$; and $C_3 = 0.00782431$. Symbols: (+) *experimental data and* (•) *least-squares function.*

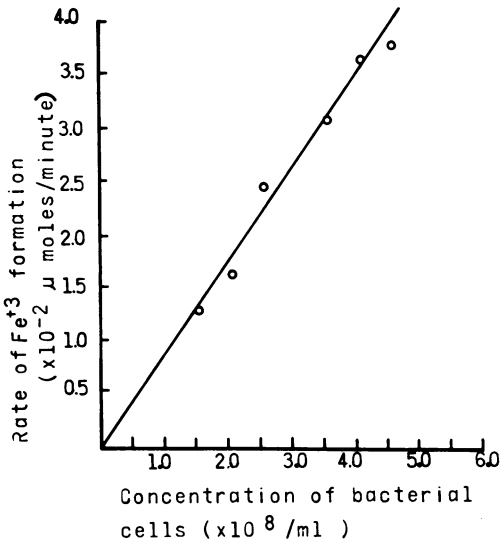

FIG. 7. *Dependence of rate of bacterial iron oxidation (measured as absorbance change at 304 nm) on concentration of bacterial cells. Substrate solutions contained 3.23 × 10^{-3} M $FeSO_4$ adjusted to pH 2.5 with H_2SO_4. The rate of ferric ion formation is expressed as micromoles × 10^{-2} per 3 ml of reaction mixture (in cuvette) per min. Bacterial concentration is given as cells × 10^8 per ml of reaction mixture.*

iron oxidation does decrease at elevated sulfate levels. However, this inhibition occurs at much higher sulfate concentrations than those which inhibit oxygen uptake in manometric determinations.

It was important to compare the spectrophotometric kinetics of bacterial iron oxidation with similar data describing the nonbiological oxidation of ferrous iron. Solutions containing identical concentrations of ferrous iron, but with varying sulfate levels, were placed in quartz cuvettes and irradiated with the full spectrum of a 100-W mercury discharge lamp. At timed intervals, the cuvettes were placed in the spectrophotometer and differentially scanned against distilled water. It was apparent that much higher $[SO_4^{2-}]/[Fe^{2+}]$ ratios are required for maximal rates of photochemical oxidation than for maximal rates of oxidation by *T. ferrooxidans* (Fig. 10).

DISCUSSION

The ultraviolet absorbance which results when *T. ferrooxidans* oxidizes ferrous ions coincides with that obtained from the nonbiological formation of ferric ions under comparable conditions of pH and anionic environment (1, 6, 17). Since our values obtained for the molar extinction coefficient of ferric ions at 304 nm are in agreement with the generally accepted values of others (6), we believe that these values may be used for converting spectrophotometric absorbance at 304 nm to absolute units of ferric ion formation during autotrophic iron oxidation.

The derived data indicate that the system exhibits consistent Michaelis-Menten kinetics even though the K_m of 5.68 × 10^{-4} M differs from that of Schnaitman et al. (11), who reported a value of 5.4 × 10^{-3} M. This 10-fold discrepancy may reflect several differences in the systems used for studying the rates of autotrophic, ferric-ion formation.

Probably most important is the temperature at which rate determinations were made. Our determinations were made at 22 C, in deference to the conditions under which *T. ferrooxidans* seems to grow best in nature and in the laboratory, whereas those of Schnaitman et al. (11) were conducted at 35 C. This 13° temperature difference would be expected to greatly alter the oxidation rate and to account in large measure for the observed differences in the maximal velocity and K_m.

Din and Suzuki (4) working with cell-free extracts, in which the reduction of cytochrome *c* by Fe^{2+} was measured spectrophotometrically, obtained a K_m of 5.9 × 10^{-4} M for Fe^{2+}, a value which closely approximates our result. Their determinations were carried out at approximately 25 C, although at a pH of 7.0. If we note that Schnaitman et al. found that the K_m of autotrophic oxidation was virtually independent of pH, then it may be concluded that our measurements agree precisely with the kinetics

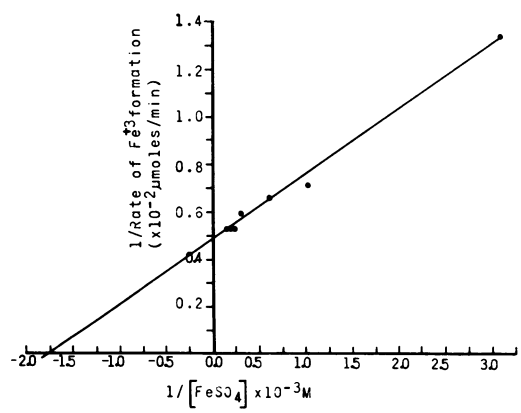

FIG. 8. *Lineweaver-Burk plot showing relationship of rate of bacterial ferric iron formation at 304 nm to initial ferrous ion concentration. Temperature of determinations was 22 C. All cuvettes contained 2.55 × 10^8 bacterial cells; all substrate solutions were adjusted to pH 2.5 with H_2SO_4.*

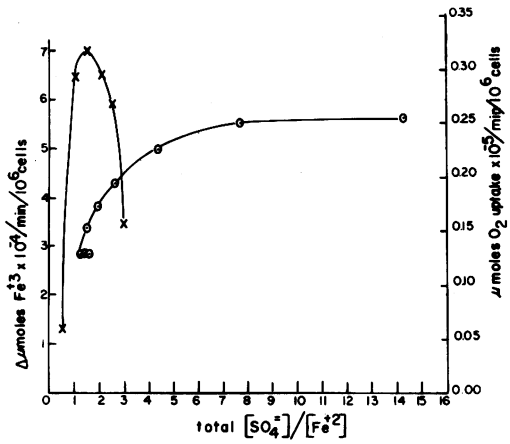

FIG. 9. *Effect of increasing sulfate concentration on the rate of oxygen uptake and ferric ion formation by Thiobacillus ferrooxidans. Ferric ion formation (—⊙—) determined spectrophotometrically at 304 nm. Initial concentration of Fe^{2+} (as $FeSO_4$) was 3.23 × 10^{-3} M. O_2 uptake (—×—) was determined manometrically; initial concentration of Fe^{2+} (as $FeSO_4$) was 4.44 × 10^{-2} M. For both determinations, the pH was adjusted to 2.5 with H_2SO_4, and additional sulfate was added as Na_2SO_4.*

of Fe^{2+}-cytochrome *c* reduction of Din and Suzuki.

It is pertinent to compare the spectrophotometric methods used previously for studying the autotrophic oxidation of ferrous ions. The thiocyanate method, whereby the absorbance of a ferric thiocyanate complex is measured, results in erroneous measurements due to instability of the complex in the presence of various anions. Stopping the reaction for sampling is a problem since the addition of hydrochloric acid or most other mineral reagents would decrease extinction due to the thiocyanate complex. The need for intermittent sampling or replication of reaction mixtures is cumbersome and prone to sampling error. This also applies to the chloride-complex method of Schnaitman et al.

Neither of these approaches allows measurement of initial rates. There is a large initial inflection of rate which could be caused by problems of mixing (11) or of nonconformance to the Beer-Lambert Law at low Fe^{3+} concentration. The latter possibility seems plausible when the data of Schnaitman et al. are examined, and it is noted that, in the course of 10 min, the amount of ferric ions increases almost 300-fold. Moreover, the ratio of complexing chloride ions to ferric ions or the presence of other anions would affect the specific molar extinction at the wavelength employed (410 nm). This can be concluded from the determinations of Rabinowitch and Stockmayer (9) of the extinction values of ferric ions between 400 and 460 nm at several HCl concentrations. The 410-nm extinction value of ferric ions at the HCl concentrations used by Schnaitman et al. would show considerable fluctuation with comparatively small changes in acidity and anionic concentration.

Our data at 304 nm allow linear extrapolation to zero time and can be reliably converted to absolute amounts of ferric ions formed at any point during the time course. The effects of variations in the anionic environment on ferric absorbance at 304 nm are comparatively small, but can be compensated for when absolute measurements are required by using an appropriate calibration of extinction coefficient versus anionic molarity.

It might seem that continuous measurement of ferric ion formation in a cuvette without provision of aeration would become quickly limited by the rate of oxygen diffusion. However, our determinations are proportional to cell concentration and are linear with time, showing that the rates are independent of oxygen concentration. This finding allowed us to dispense with the aeration used by Schnaitman et al. and to measure the oxidation as it occurred.

The significance of this lack of requirement for aeration is not completely understood, although our data have shown that oxygen utilization and ferric ion formation in autotrophic iron oxidation are not tightly coupled (Fig. 9).

The stimulatory effects of sulfate upon bacterial iron oxidation are in agreement with those of Lazaroff (8), Silverman (14), Schnaitman et al. (11), and others. In this respect, our results closely compare with those of Schnaitman et al. (11). These workers had shown that there is a point beyond which the addition of sulfate fails to produce continued stimulation of iron oxidation.

It is further indicated that sulfate concentrations greater than those previously employed

TABLE 2. *Relative effect of high sulfate concentration on the oxidation of 3.23 × 10^{-3} M $FeSO_4$ solutions by identical bacterial suspensions*[a]

Total sulfate from $FeSO_4$ and Na_2SO_4 (× 10^{-3} M)	$[SO_4^{2-}]/[Fe^{3+}]$	Fe^{3+} (μmoles) formed per min
56.56	17.5	0.267
216.56	67.0	0.210
669.89	207.4	0.088

[a] All reaction mixtures were at pH 2.5.

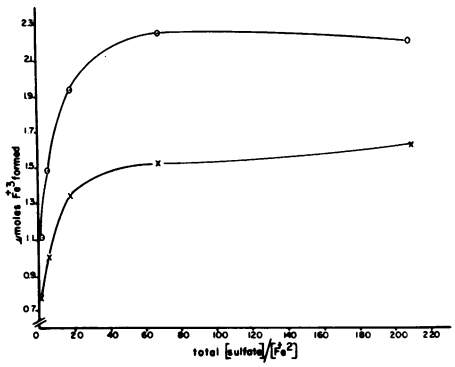

FIG. 10. *The relationship of* $[SO_4^{2-}]/[Fe^{2+}]$ *to amounts of ferric ions, formed from* $FeSO_4$ *solution after 5 min (—×—) and 10 min of ultraviolet irradiation (—⊙—). The* Fe^{2+} *substrate was* 3.23×10^{-3} *M (as* $FeSO_4$*). Reaction mixtures were adjusted to pH 2.5.*

begin to inhibit iron oxidation in a pattern similar to that observed using manometric determinations of oxygen uptake. However, our measurements and those of Schnaitman et al. (11) show that the levels of sulfate (relative to ferrous iron substrate) required for maximal stimulation of bacterial iron oxidation are much greater when Fe^{3+} formation is determined spectrophotometrically, than when oxygen consumption is determined manometrically. This again suggests that biological oxidation of ferrous iron by the autotrophic system is not always paralleled by oxygen reduction.

The effects of sulfate on ferrous iron oxidation in biological and nonbiological systems are similar but not identical. The stimulatory effect of sulfate occurs in both the biological and nonbiological systems. However, bacterial iron oxidation was inhibited at sulfate concentrations above 0.216 M. Similar solutions oxidized by ultraviolet irradiation showed a deceleration of the rate of oxidation rather than a net inhibitory effect.

The information obtained so far is consistent with the interpretation that the important role of sulfate in bacterial iron oxidation is a consequence of its inorganic complexing of iron. Increasing concentrations of sulfate result in different complexes with either the ferrous iron substrate or the ferric iron product. This could favor the oxidation reaction thermodynamically and therefore accelerate the observed rates in both biological and nonbiological systems. However, this does not preclude the participation of sulfate in some additional role in bacterial iron oxidation as suggested by Lazaroff (8) or Dugan and Lundgren (5).

In conclusion, we think that the method developed for the direct spectrophotometric determination of autotrophic iron oxidation possesses a number of advantages over previous colorimetric techniques. (i) The analysis of ferric ion production can be carried out continuously as a monitoring technique which does not require that the reaction be stopped for analysis or that color reagents be added. This greatly facilitates laboratory studies, allowing rapid analysis of oxidation kinetics with maximum precision due to elimination of manipulative and sampling error. (ii) The extinction coefficient for ferric ions is consistent for initial rate determinations in all experimental protocols employed. In such systems, therefore, initial increases in absorbance will be strictly due to ferric ion formation. On the other hand, if comparisons of ferric ion concentrations in absolute terms are desired for systems which differ greatly in anionic composition, temperature, or pH, it is possible to calibrate the extinction coefficient accordingly. (iii) The high molar extinction coefficient of ferric ions at 304 nm makes this technique extremely sensitive, as compared with previous colorimetric methods. (iv) A mathematical computer solution has been developed that can be used to convert absorbance values to absolute $[Fe^{3+}]$ in systems of known sulfate concentrations.

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