Kinetic Studies of Iron Oxidation by Whole Cells of *Ferrobacillus ferrooxidans*

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A colorimetric assay was developed for studying the kinetics of iron oxidation with whole cells of the chemoautotroph, *Ferrobacillus ferrooxidans*. The assay was more advantageous than the conventional method of Warburg manometry because of its simplicity, rapidity, and the small amount of cells required. The assay measured Fe^{3+} as a chloride complex which absorbs at 410 nm. Kinetic analysis showed the apparent K_m for iron oxidation to be 5.4×10^{-3} M in an unbuffered system and 2.2×10^{-3} M in the presence of β-alanine-SO_4^{2-} buffer. Glycine and β-alanine buffers were used in the measurement of the pH optimum for iron oxidation; the optimum ranged from 2.5 to 3.8. The effect of pH was primarily on the V_{max} while the K_m remained constant. Added SO_4^{2-} was found to stimulate iron oxidation by increasing the V_{max} of iron oxidation by whole cells, but it did not affect the K_m. Results of assays of iron oxidation in systems containing various mole percentages of SO_4^{2-} and Cl^- indicated that Cl^- did not inhibit iron oxidation but that SO_4^{2-} was required. Sulfate could be partially replaced by HPO_4^{2-} and $HAsO_4^{2-}$ but not by BO_3^-, MoO_4^{2-}, NO_3^-, or Cl^-; formate and MoO_4^{2-} inhibited iron oxidation.

Iron-oxidizing bacteria (*Ferrobacillus-Thiobacillus* group) inhabit a narrow ecological zone where acid mine drainage, containing reduced iron and sulfur compounds, meets the aerobic atmosphere at the earth's surface. These organisms derive their sustenance by accelerating the oxidation of these reduced inorganic compounds and coupling the energy released to the reduction of carbon dioxide and the generation of reducing power.

The properties possessed by these autotrophic bacteria, permitting the oxidation of ferrous ion (or sulfur) in an acid environment (pH 2 to 4), may indicate a structural-functional relationship not found in heterotrophic organisms. Our interests lie in studying such relationships, and this paper is concerned with one phase of the problem, namely, a kinetic analysis of ferrous ion oxidation by intact cells. The latter is important in understanding any structural organization that might exist in the cell's iron oxidation enzyme system. Studies of growth patterns of bacteria as well as isolation and identification of cell substances can furnish only inferential information about the mechanics of iron oxidation. This paper reports on a kinetic analysis of ferrous ion oxidation and

shows that it follows Michaelis-Menten kinetics. Some of the parameters governing the rate of iron oxidation are discussed.

MATERIALS AND METHODS

Organism and culture procedure. *F. ferrooxidans*, obtained from the culture of Leathen et al. (6), was grown on the 9K medium and in the manner previously described (9) in 16-liter glass carboys under forced aeration. Cells were harvested in a Sharples centrifuge after 48 hr of growth, washed three times with distilled water (pH 3.0), and stored in the cold (6 C) in acidified 9K salts or β-alanine-SO_4^{2-} buffer (pH 3.6, 0.01 M). Stored cells were used within 4 days.

Development of an assay system. Initially, a direct colorimetric assay was used which measured the appearance of small amounts of Fe^{3+}, in the presence of variable anions, as a red complex formed with sodium thiocyanate (2). The reaction was stopped with 5 ml of 2 N HCl prior to analyzing for Fe^{3+}. The addition of HCl to tubes containing Fe^{3+} gave rise to a yellow color, presumably due to the formation of a ferric chloride complex, and it was possible to estimate the color spectrophotometrically at 410 nm. This Fe^{3+}-chloride complex formation served as a basis for the Fe^{3+} assay used in this report.

The assay procedure measuring the appearance of Fe^{3+} was as follows. Five acid-washed and thoroughly rinsed Bellco colorimeter tubes, each containing 3.8 ml of distilled water adjusted to pH 3.0 with H_2SO_4, were placed in a water bath at 35 C; 0.2 ml of a cell suspension (2 mg, dry weight of cells) was added to

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each tube, and O_2 was bubbled through the suspension via Pasteur pipettes. To each tube, 1 ml of $FeSO_4 \cdot 7H_2O$ solution containing 2.5 mg of Fe^{2+} was added in rapid succession by use of repeating syringe. The oxidation was stopped at 0, 1, 3, 6, and 10 min by rapidly adding 5 ml of 2 N HCl. The HCl was added before the addition of Fe^{2+} ion for the zero-time tube. Tubes were removed and analyzed for Fe^{3+} at 410 nm in a Bausch & Lomb Spectronic 20 colorimeter. The intensity of the color was directly related to the amount of Fe^{3+} formed and agreed well with Fe^{3+} determinations by the thiocyanate assay method.

Kinetic analysis of iron oxidation by whole cells. Five test tubes, each containing 3.8 ml of distilled water adjusted to *p*H 3.0 with H_2SO_4, were placed in a water bath at 35 C. This temperature had been established as optimal for the system. To each tube, 0.2 ml of cell suspension was added and O_2 was bubbled through the suspension via Pasteur pipettes. The rate of gas flowing through each tube was regulated by a needle valve to ensure a constant bubbling rate. At 15-sec intervals, 1 ml of an $FeSO_4$ solution containing 2.5 mg (500 µg/ml) of Fe^{2+} was added to each tube. The oxidation of iron was stopped in individual tubes by the addition of 5 ml of 2 N HCl at intervals of 2, 3.25, 6.50, and 10.75 min. The development of the yellow ferric chloride complex was estimated as above. For these assays, a reaction tube which received HCl to stop the reaction at 1 min served as the blank; the assay was not considered linear for the first minute. The optical density (OD) at 410 nm was plotted versus reaction time, and the rate of change as OD_{410} per min was obtained from a line fitted to the OD values of the reaction tubes.

The effect of whole cells and Fe^{2+} substrate concentrations on the rate of iron oxidation was followed by varying the concentration of cells or of iron in the appropriate reaction system.

The *p*H of the reaction mixture of the assay system was varied by using different amino acid buffers. Glycine and β-alanine were dissolved in excess dilute H_2SO_4 of known normality and back-titrated to the desired *p*H with KOH. The final concentrations of components in the assay system were: Fe^{2+}, 500 µg/ml; glycine or β-alanine, 0.05 M; SO_4^{2-}, 0.034 M. A variety of other buffers (Table 1) were also tested in order to determine whether they could replace the β-alanine-SO_4^{2-} buffer.

The effect of SO_4^{2-} and Cl^- on iron oxidation was investigated. The assay system contained Fe^{2+} (200 µg/ml, as $FeSO_4 \cdot 7H_2O$); the SO_4^{2-} content was increased from 0.0036 to 0.05 M by the addition of K_2SO_4. The Cl^- levels were varied by preparing a series of assay systems in which all cations were the same and the anions consisted of various mole percentages of Cl^- and SO_4^{2-}. The test systems contained 8.94×10^{-3} M Fe^{2+}, 10^{-2} M β-alanine, and a total anion concentration of 3.79×10^{-2} M; the *p*H was adjusted to 3.2 with KOH.

All assay results were reproducible and all experiments were done in triplicate and with different batches of cells.

Anionic replacement of SO_4^{2-} in the iron assay system. The assay system to test for replacement of other anions contained: 1.9×10^{-2} M Cl^-, 4.5×10^{-3} M Fe^{2+}, and 5×10^{-3} M β-alanine. The fact that Cl^- was not inhibitory at these levels and could not satisfy the anionic requirements for iron oxidation led to the use of a system containing Cl^- as the only anion to determine whether other anions could replace the SO_4^{2-} requirement for iron oxidation. The various test anions were added as the Na or K salt and were tested at 2.5×10^{-3} M, both in the presence and absence of 2.5×10^{-3} M SO_4^{2-}.

RESULTS

The bubble tube-type of iron assay measuring the ferric chloride complex at 410 nm shows a linear increase in Fe^{3+} after a brief initial lag (Fig. 1); the lag could be due to the mixing requirement. The Fe^{3+} levels can be compared to those measured by the SCN^- assay, which also shows a linear response. However, the SCN^- assay has limitations which restrict its use for kinetic analysis; these limitations are not pertinent to this paper. The rate of iron oxidation (Δ OD_{410}/min) was found to be directly proportional to the concentration of cells used in the assay over the ΔOD range of 0.02 to 0.085 (Fig. 2).

The effect of Fe^{2+} concentration (from 3.5 to 35.8 mM) on the rate of iron oxidation is shown in Fig. 3, and is expressed as a double reciprocal (Lineweaver-Burke) plot. The apparent K_m of this system was estimated to be 5.4×10^{-3} M.

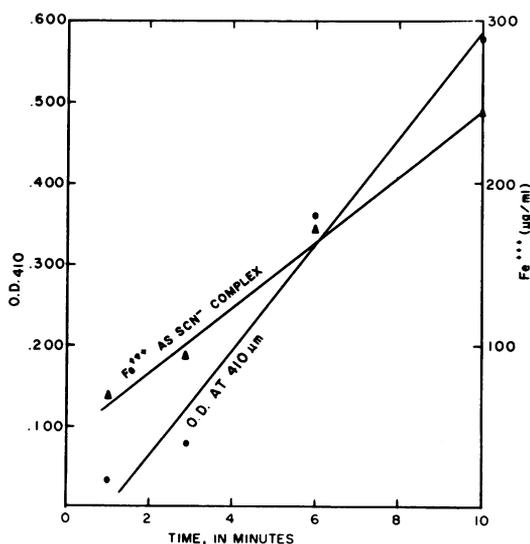The standard iron assay procedure and various

FIG. 1. *Bubble tube assay for iron oxidation by intact cells of F. ferrooxidans. Iron oxidation was followed by measuring Fe^{3+} with the SCN^- complex and by measuring the Fe^{3+}-chloride complex at 410 nm.*
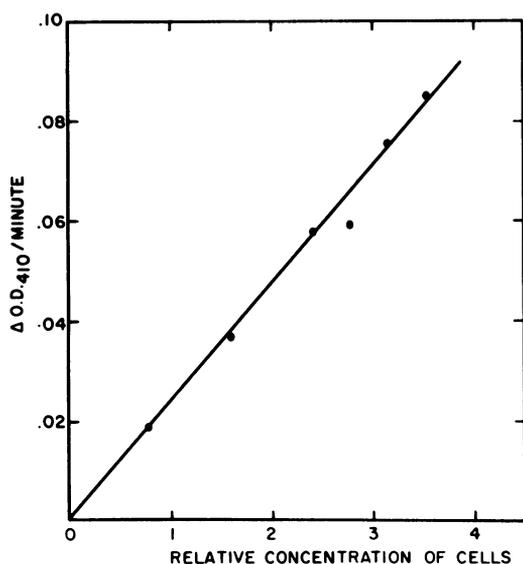
FIG. 2. *Rate of iron oxidation as a function of relative concentration of cells.*
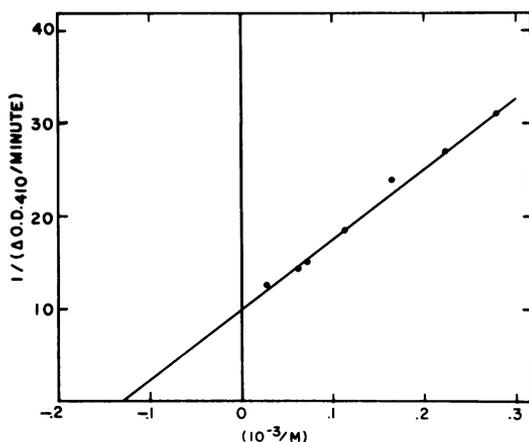

FIG. 3. *Lineweaver-Burke plot of substrate concentration versus the rate of iron oxidation.*

FIG. 4. *Effect of pH on the rate of iron oxidation. Curve A, glycine-SO_4^{2-} buffer; curve B, β-alanine-SO_4^{2-} buffer.*

FIG. 5. *Effect of pH on the V_{max} and K_m of iron oxidation.*

amino acid buffers were used to obtain more precise information concerning the effect of pH on iron oxidation by intact cells. Glycine-SO_4^{2-} buffer was used over the pH range of 1.8 to 3.6, and β-alanine-SO_4^{2-} buffer was used over the pH range of 3.2 to 4.8. Figure 4 illustrates the difference in the rate of iron oxidation in the two buffer systems. The pH optimum for Fe^{2+} oxidation was broad, extending from 2.4 to 3.6, and a rapid decrease in iron oxidation occurred above pH 3.6 and below 2.0.

The effect of pH on iron oxidation was investigated further by observing its effect on the two kinetic parameters, K_m and V_{max} (Fig. 5). When
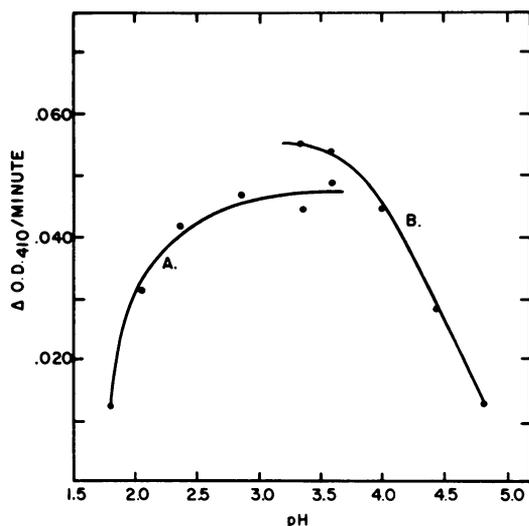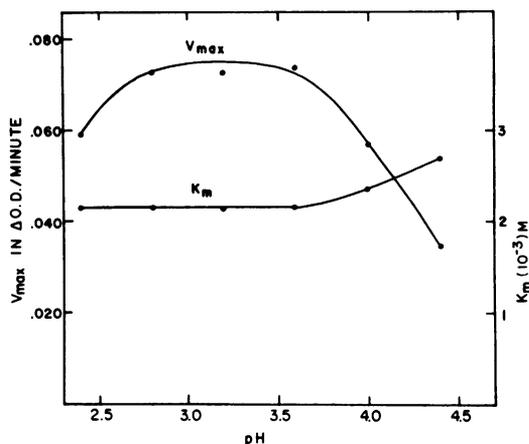
β-alanine-sulfate buffer was used in the assay system over a pH range of 2.4 to 4.4 with the Fe^{2+} concentration varied from 3.5 to 17.9 mM for each pH, the K_m remained constant throughout most of the pH range. The change in V_{max} was more significant in terms of the pH optimum noted in Fig. 4, since it decreased below pH 2.8 and above pH 3.6. The K_m determined in the presence of β-alanine-SO_4^{2-} buffer was 2.2×10^{-3} M.

Table 1 shows the effects of using various buffers in the assay system measuring Fe^{2+} oxidation by whole cells. Both formate and acetate buffer systems were inhibitory. Maleate, citrate, and malate buffers were also unsuitable, owing to inhibi-

TABLE 1. *Buffers tested for use in the iron oxidation assay*

Buffer system	pH tested	Results
Formate-formic acid[a]	3.2	Inhibition (70%) at 5×10^{-5} M
Acetate-acetic acid[a]	3.6	Inhibition (55%) at 5×10^{-2} M
Maleate-NaOH[a]	3.6	Slight auto-oxidation; no biological oxidation at 5×10^{-2} M
Citrate-NaOH	3.6	Slight biological oxidation; large auto-oxidation at 5×10^{-2} M
Malate-NaOH	3.6	Same as maleate
Glycine-SO_4^{2-}	2.1–3.7	No inhibition or stimulation; rate of oxidation the same as without buffer
β-Alanine-SO_4^{2-}	3.2	Slight stimulation of oxidation over the control with no buffer

[a] Described by Long (7).

tion of the biological oxidation or to some auto-oxidation of iron. The slight stimulation of iron oxidation observed with β-alanine may be due to additional SO_4^{2-} added to the system, but this did not occur in the case of glycine-SO_4^{2-}.

The iron assay system was also used to investigate the effects of SO_4^{2-} on iron oxidation. Addition of SO_4^{2-} to the reaction mixture, ranging from 0.0036 to 0.05 M, approximately doubled the rate of iron oxidation (Fig. 6). Table 2 shows the effect of the addition of SO_4^{2-}-containing salts. The four salts tested all caused a significant increase in iron oxidation. The low increase with $(NH_4)_2SO_4$ is probably due to a slight inhibition caused by NH_4^+ ions.

Figure 7 shows the effect of SO_4^{2-} on the K_m and V_{max} of the Fe^{2+} oxidation reaction. Curve A is a reciprocal plot of an oxidation rate in an unbuffered system as previously described (Fig. 3), whereas curve B is a reciprocal plot of oxidation rates of the same cell suspension but the reaction mixture contained 0.035 M K_2SO_4. The addition of SO_4^{2-} increased the velocity of the reaction but did not affect the K_m.

The effects of Cl^- on the rate of iron oxidation are shown in Fig. 8, in which the rate of iron oxidation is plotted against the mole per cent Cl^- in the assay system. A slight stimulation of iron oxidation occurred at low Cl^- levels, but the rate decreased rapidly at high Cl^- levels; no oxidation was observed when Cl^- was the only anion present. Since these levels of Cl^- do not inhibit iron oxidation, the data indicate a specific SO_4^{2-} requirement.

The fact that Cl^- was not inhibitory and that chloride could not satisfy the anionic requirements for iron oxidation led to the use of a system containing Cl^- as the only anion, to determine whether other anions could replace SO_4^{2-}. When various anions were added to the reaction system in the presence and absence of 2.5×10^{-3} M SO_4^{2-}
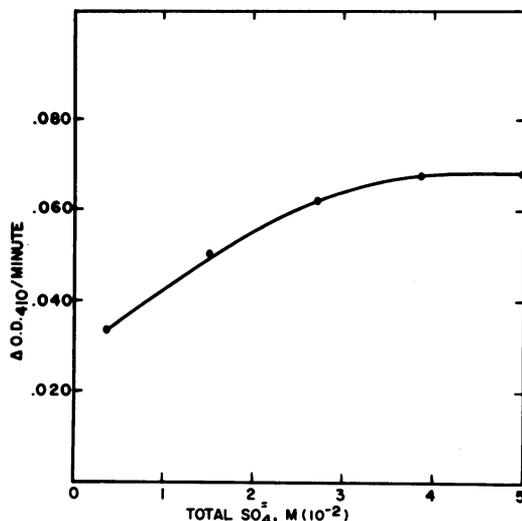

FIG. 6. *Effect of SO_4^{2-} concentration on the rate of iron oxidation. The assay system was unbuffered and contained 200 µg of Fe^{2+} per ml.*

TABLE 2. *Effects of various SO_4^{2-}-containing salts on the rate of iron oxidation*

SO_4^{2-} salt added	SO_4^{2-} in assay	ΔOD_{410}/min
	M	
None	0.0036	0.033
K_2SO_4	0.041	0.066
$MgSO_4$	0.041	0.060
Na_2SO_4	0.041	0.059
$(NH_4)_2SO_4$	0.041	0.046

to test for both the inhibitory effect of anions and their ability to replace SO_4^{2-}, it was noted that both HPO_4^{2-} and $HAsO_4^{2-}$ could partially replace SO_4^{2-} (Table 3). Both of these anions stimulated iron oxidation in the presence of SO_4^{2-}, but the
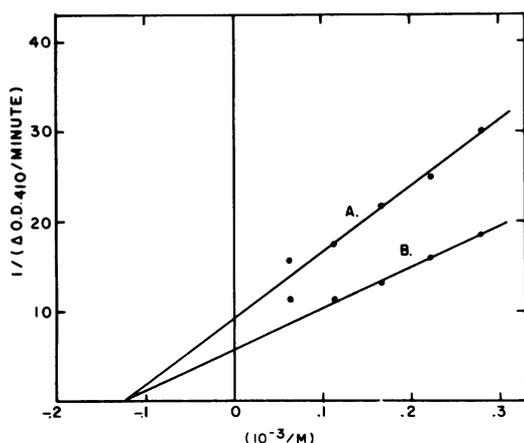
FIG. 7. *Double reciprocal plot showing the effect of added SO_4^{2-} on the kinetics of iron oxidation. Curve A represents the results obtained with no added SO_4^{2-}; curve B represents the results of identical assays in which 0.035 M K_2SO_4 was added to the assay system.*
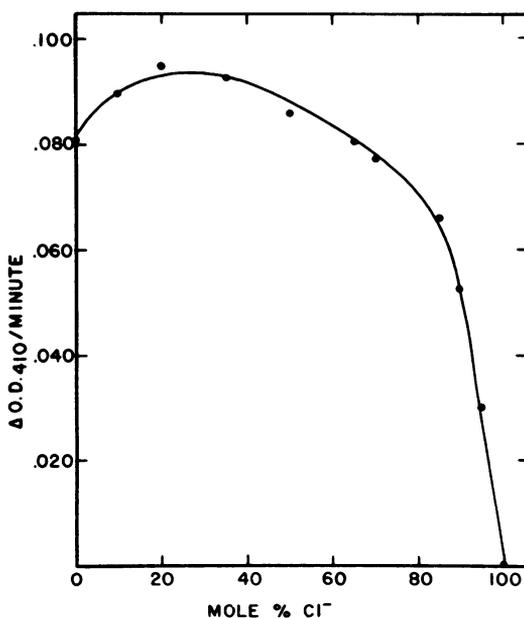

FIG. 8. *Effect of varying mole fractions of Cl^- and SO_4^{2-} on iron oxidation. The total molarity of anion in the system was maintained constant (3.79×10^{-2} M) but consisted of varying mole percentages of SO_4^{2-} and Cl^-.*

TABLE 3. *Effect of various anions on iron oxidation in a system containing only Cl^- and in the same system containing Cl^- plus SO_4^{2-}*

Anion added[a]	ΔOD_{410}/min	
	No SO_4^{2-}	2.5×10^{-3} M SO_4^{2-}
$HAsO_4^{2-}$	.005	.033
HPO_4^{2-}	.015	.035
BO_3^-	.000	.031
NO_3^-	.000	.016
MoO_4^{2-}	.000	.000
SO_4^{2-}	.030	.035[b]

[a] The assay system used to test anions other than SO_4^{2-} contained the following: 5×10^{-3} M β-alanine, 1.9×10^{-2} M Cl^-, and 4.5×10^{-3} M Fe^{2+}. The various test anions were added as the Na or K salt and were tested at 2.5×10^{-3} M both in the presence and absence of 2.5×10^{-3} M SO_4^{2-}.

[b] This assay contained a total of 5×10^{-3} M SO_4^{2-}.

strongly inhibitory. These preliminary results demonstrate that the requirement for SO_4^{2-} in iron oxidation is quite specific.

DISCUSSION

The assay procedure described satisfies the requirements for studying the reaction kinetics of the oxidation of ferrous to ferric iron by intact cells. Iron oxidation follows zero-order kinetics and the rate of the reaction is directly proportional to the concentration of cells added over a satisfactory range. The advantages of this assay are its simplicity and rapidity compared to Warburg respirometry. The assay system in its present form is not suited for studying iron oxidation by cell-free extracts, because of excessive forming; a good assay system for use with extracts remains to be developed.

An accurate estimation of the effects of pH on iron oxidation has been reported; such effects are important in establishing relationships between the organism and its natural acidic environment, as well as between substrate (ferrous ion) and "iron oxidase." The pH optimum for iron oxidation is broad; with intact cells, it is much lower than that reported for one of the enzymes involved in iron oxidation, namely, Fe^{2+}-cytochrome c reductase (1, 3). The reason for such a pH difference is unknown.

The major effect of pH is on the velocity (V_{max}) and not on the K_m of the iron oxidation reaction. The effects of pH could be due to the ionization of groups around the active site of those enzymes involved in iron oxidation or due to a loss of iron-binding groups known to be present in the cell envelope (Bacteriol. Proc., p. 123, 1968).

stimulation was no greater than that observed when the equivalent amount of additional SO_4^{2-} was added. Borate appeared to be without effect, and it did not replace SO_4^{2-}. Nitrate did not replace SO_4^{2-} and was inhibitory in the presence of SO_4^{2-}; molybdate did not replace SO_4^{2-} and was

Iron-oxidizing bacteria exhibit an interesting and somewhat unusual anion requirement in that SO_4^{2-} is required in substantial (not catalytic or nutrient) quantities for both growth and iron oxidation (5). This requirement may somehow be related to the fact that ferrobacilli can oxidize elemental sulfur to SO_4^{2-} (8, 10) and, in the cells' natural environment, high levels of reduced sulfur compounds are found. A role for SO_4^{2-} in the organism may be its involvement in the formation of high-energy sulfate compounds (5). Dugan and Lundgren (4) found that ferrous ions as well as sulfate ions are bound by the cell envelope of *F. ferrooxidans* and suggested that the binding of iron requires the participation of SO_4^{2-}. However, in light of the present kinetic studies, it doesn't appear likely that SO_4^{2-} affects the binding of Fe^{2+} at an enzymatic site, for the additions of various concentrations of SO_4^{2-} did not affect the K_m of the reaction. However, the studies do not rule out the possibility that sulfate might simply expose more enzymatic sites which could change the V_{max} without affecting the K_m.

The partial replacement of SO_4^{2-} by HPO_4^{2-} and to a lesser extent by $HAsO_4^{2-}$ indicates something about the nature of the anion requirement. At the pH of the assay, these compounds would probably exist as divalent anions, and the requirement may be for a suitable charge. This would explain the inability of Cl^- and NO_3^- to replace SO_4^{2-}. Also, since both HPO_4^{2-} and $HAsO_4^{2-}$ form insoluble precipitates with ferric iron, this may in part explain their inability to completely replace the SO_4^{2-} requirement. Divalent anions could be required for the depolarization of positively charged sites on the cell surface which prevent the approach of the positively charged Fe^{++} substrate. Alternatively, they may also be needed for the maintenance of some essential configuration of the iron-cytochrome *c* reductase enzyme.

The assay system developed for studying the kinetics of iron oxidation has practical significance, for it permits the study of iron oxidation inhibitors. In this study, formate and molybdate were found to be good inhibitors of iron oxidation. These inhibitors may be of importance in controlling iron oxidation which results in a water pollution problem, namely, acid mine wastes.

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