The Specificity of the Anionic Requirement for Iron Oxidation by *Thiobacillus ferrooxidans*

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The anionic requirement for the oxidation of ferrous ions by suspensions of *Thiobacillus ferrooxidans* was satisfied by selenate as well as sulphate. Selenate was toxic to the organism and suppressed growth in ferrous iron media, even in the presence of high concentrations of sulphate. After treatment with dilute hydrochloric acid at 0 °C, *T. ferrooxidans*, which specifically required SO_4^{2-} or SeO_4^{2-} for iron oxidation, showed no activity in response to 12 other anions tested. However, after the introduction of SO_4^{2-} or SeO_4^{2-} , addition of anions such as TeO_4^{2-} , WO_4^{2-} , AsO_4^{3-} or PO_4^{3-} further enhanced the rate of iron oxidation. Under these conditions, Cl^- , $\text{B}_4\text{O}_7^{2-}$ and ClO_3^- had no significant effect at low concentration, whereas Br^- , NO_3^- and MoO_4^{2-} were inhibitory. These observations distinguish between a specific and a non-specific anionic requirement for the oxidation of ferrous ions by *T. ferrooxidans*. The specific requirement is satisfied only through the uptake of SO_4^{2-} or SeO_4^{2-} by the bacteria. The non-specific requirement is satisfied by any one of several anions, including SO_4^{2-} and SeO_4^{2-} , which are presumed to act as ligands for iron in solution.

INTRODUCTION

Inorganic anions such as Cl^- and NO_3^- inhibit iron oxidation as well as growth of the autotrophic bacterium *Thiobacillus ferrooxidans* (Lazaroff, 1963; Razzell & Trussel, 1963; Schnaitman, Korczynski & Lundgren, 1969; Tuovinen, Niemella & Gyllenberg, 1971). To account for these effects, it has been proposed that sulphate ions are required for autotrophic iron oxidation and that the inhibitory anions compete with sulphate for some metabolic site essential for iron oxidation. The principal support for this hypothesis comes from the observation that sulphate ions stimulate the oxidation of ferrous chloride solutions by suspensions of *T. ferrooxidans* (Lazaroff, 1963; Tuovinen, Kelley & Nicholas, 1975). The stimulation of iron oxidation by sulphate ions is manifested by increases in both the rate of oxygen uptake and the rate of ferric ion formation (Steiner & Lazaroff, 1974).

The concept of a sulphate requirement for iron oxidation fits the observed facts and seems the simplest explanation for sulphate reversal of the anionic inhibition of ferrous ion oxidation. Indeed, Dugan & Lundgren (1965) demonstrated a rapid uptake of sulphate by cell envelopes of *T. ferrooxidans* during iron oxidation. In their model of the oxidation of ferrous ions, occurring at the periphery of the cell envelope, the combination of sulphate, phosphate and ferrous ions is an initial step. However, the mechanism by which sulphate effects ferrous ion oxidation still requires elucidation, and relevant to this question is whether SO_4^{2-} is a specific requirement for Fe^{2+} oxidation or whether other anions can substitute for it.

In studying the specificity of the sulphate requirement, selenate was of special interest as it is chemically similar to sulphate, can substitute for sulphate in its assimilation and become incorporated into selenium analogues of the naturally occurring sulphur amino

acids (Stadtman, 1974). Sulphate-selenate interchangeability also occurs in the inhibition of chloroplast photophosphorylation (Pick & Avron, 1973): in this case, selenate and sulphate are almost equally effective in competing with the phosphate required.

METHODS

Organism and growth. *Thiobacillus ferrooxidans*, Leathen strain, was cultivated routinely in the 9K medium of Silverman & Lundgren (1959). Modifications of the 9K medium with FeCl_2 (and HCl) instead of FeSO_4 (and H_2SO_4), or with all SO_4^{2-} replaced by Cl^- , were used to test for the effect of sulphate and selenate additions on the growth of *T. ferrooxidans*. The 9K medium had the following composition: 23 mM- $(\text{NH}_4)_2\text{SO}_4$, 2.0 mM- MgSO_4 , 160 mM- FeSO_4 , 6.0 mM- H_2SO_4 , 1.3 mM- KCl , 0.061 mM- $\text{Ca}(\text{NO}_3)_2$, and 2.9 mM- K_2HPO_4 . Where appropriate, sulphate salts were replaced by chlorides to give the same concentrations of the required cations (NH_4^+ , Mg^{2+} , Fe^{2+} , H^+).

All components of culture media, with the exception of the ferrous iron source, were sterilized by autoclaving at 120 °C for 15 min. Solutions of iron salts for the culture media were made up just before use at 0.37 M and acidified with the appropriate acid to pH 2.5. Where required, the iron solutions were sterilized by filtration through membrane or sintered-glass filters before combining with the acidified sterile basal medium. Growth experiments were usually carried out with continuous shaking in 250 ml Erlenmeyer flasks containing 100 ml medium, but to obtain large quantities of bacteria cultures were grown in 20 l bottles containing 10 l unsterile medium using forced aeration. All cultures were grown at room temperature.

Suspensions for iron oxidation experiments were prepared by filtering a culture from an aerated bottle through Whatman no. 1 paper. The residues on the filter paper were washed with cold 1.6 mM- H_2SO_4 and the washings were combined with the first filtrate. The bacteria were collected by centrifuging the filtrate either through a Sharples continuous flow centrifuge or at 30000g for 20 min, and then resuspended in 10 mM- H_2SO_4 . Sedimentation and resuspension were repeated to remove oxidized iron particles.

Measurement of ferric ion formation. Ferric ions were initially determined by a modification of the thiocyanate method (Kolthoff *et al.*, 1969). Samples were removed from reaction mixtures, and each was mixed with an equal volume of 6 M- HCl to stop oxidation, diluted to a suitable volume with water and a portion containing from 2 to 25 μg Fe^{3+} was mixed with 0.5 ml 6 M- HCl and 0.5 ml 3 M- KSCN . The volume was brought to 5.0 ml and E_{490} was determined. Standards were prepared from solutions of $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$. Additional measurements of the rates of Fe^{3+} formation were carried out continuously in a Cary model 15 recording spectrophotometer at 304 nm, according to the method of Steiner & Lazaroff (1974).

Measurement of oxygen uptake. Oxygen uptake of bacteria oxidizing iron was measured manometrically in Warburg flasks. The usual protocol involved placing 0.2 ml of an acidified suspension of *T. ferrooxidans* in the sidearm, and the substrate and any other additions, to 1.6 ml, in the main chamber of the flask. The centre well received 0.2 ml of 20% (w/v) KOH and a filter paper wick. After equilibration and mixing, measurements were made conventionally at 28 °C. Specific activities were estimated on the basis of total cell counts determined with a Petroff-Hauser counting chamber and a phase contrast microscope.

RESULTS

Effect of anions on oxygen uptake during iron oxidation

Bacteria grown in FeSO_4 medium then washed and suspended in 1.6 mM- H_2SO_4 exhibited a slow uptake of oxygen in FeCl_2 solutions. The rate of oxygen uptake was markedly increased by adding either SO_4^{2-} or SeO_4^{2-} , indicating that either substance can satisfy the anionic requirement for the oxidation of Fe^{2+} . As the anion concentration was increased the rate of oxygen consumption rose to a maximum, and then diminished as the SO_4^{2-} or SeO_4^{2-} concentration exceeded an optimal level (Fig. 1*a*). In 44 mM- Fe^{2+} solutions, the rate of oxygen consumption was maximal when the individual or combined concentrations of SO_4^{2-} and SeO_4^{2-} were about twice the Fe^{2+} concentration. A similar relationship between SO_4^{2-} concentration and oxygen consumption was observed in the presence of phosphate which further stimulated the rate of oxygen uptake (Fig. 1*a*). Anions such as NO_3^- and Cl^- inhibited oxygen uptake due to iron oxidation, but this inhibition was reversed by adding SO_4^{2-} or SeO_4^{2-} (Table 1). However the inhibition of oxygen uptake produced by adding 44 mM- $\text{B}_4\text{O}_7^{2-}$, succinate or acetate (anions which form undissociated acids at pH 2.5) was not counteracted by increasing the SO_4^{2-} concentration.

Table 1. *Effect of various anions on oxygen uptake by T. ferrooxidans in FeSO₄ solutions*

Reaction mixtures contained 44 mM-FeSO₄, 15 mM-KH₂PO₄ and the anions indicated (as sodium salts). All solutions were adjusted to pH 2.5 with H₂SO₄. Each result is expressed as a percentage of the control rate determined for a basal solution to which a portion of the same bacterial suspension was added at the same time.

Additions	Oxygen uptake ($\mu\text{l min}^{-1}$)	Percentage of control
None	1.31	—
130 mM-Cl ⁻	0.61	46.6
130 mM-Cl ⁻ , 44 mM-SeO ₄ ²⁻	0.79	60.3
44 mM-NO ₃ ⁻	0.24	18.3
44 mM-NO ₃ ⁻ , 44 mM-SeO ₄ ²⁻	0.46	35.1
44 mM-NO ₃ ⁻ , 44 mM-SO ₄ ²⁻	0.46	35.1

Fig. 1. Effect of sulphate and selenate on the rates of oxygen uptake and Fe³⁺ formation by *T. ferrooxidans* oxidizing Fe²⁺.

(a) Oxygen uptake. This was determined manometrically, see Methods, with 1.6 ml Fe²⁺ substrate in the main chamber and 0.2 ml suspension (3.8×10^9 bacteria ml⁻¹) in the sidearm. Substrates were: (○) 44 mM-FeSO₄, 6.7 mM-KH₂PO₄ plus increments of Na₂SO₄; (●) 44 mM-FeSO₄ plus increments of Na₂SeO₄; (□) 44 mM-FeCl₂ plus increments of Na₂SeO₄. The rates of oxygen uptake were linear for at least the first 20 min.

(b) Fe³⁺ formation. 1×10^9 bacteria were added to 50 ml Erlenmeyer flasks containing 0.1 M-Fe²⁺ substrate, 17 mM-KH₂PO₄ plus increments of Na₂SO₄ or Na₂SeO₄ in a total volume of 10 ml. The pH was adjusted to 2.5 with HCl or H₂SO₄ as appropriate. Temperature was maintained at 30 °C. All flasks contained approximately 0.016 mM additional H₂SO₄ introduced with the bacterial suspensions. (○) FeSO₄ plus Na₂SO₄; (●) FeSO₄ plus Na₂SeO₄; (□) FeCl₂ plus Na₂SO₄; (■) FeCl₂ plus Na₂SeO₄.

Effects of sulphate and selenate on growth

Growth of *T. ferrooxidans* was not detected after several weeks incubation in a modified 9K medium in which all SO₄²⁻ had been replaced by Cl⁻, despite the introduction of small amounts of SO₄²⁻ with the inoculum. In 9K medium in which only FeSO₄ had been replaced by FeCl₂ (25 mM SO₄²⁻ instead of 191 mM) slow growth and visible oxidation of iron were observed 11 days after inoculation, while control flasks with FeSO₄ showed growth and oxidation by 3 days. Bacteria from either FeCl₂ medium or FeSO₄ medium failed to grow after subculture in FeCl₂ or 50 % FeCl₂/50 % FeSO₄ media unless large inocula ($> 10^6$ bacteria) were used, indicating that slow growth in media containing low concentrations of SO₄²⁻ does not select for organisms which do not require SO₄²⁻ for oxidizing Fe²⁺.

Table 2. *Effect of selenate on growth of T. ferrooxidans in media containing FeSO₄ or FeCl₂*

Additions to basal medium* (Na salts)	After 4 days in 0.16 M-FeSO ₄		After 4 days in 0.16 M-FeCl ₂		After 8 days in 0.16 M-FeCl ₂	
	10 ⁻⁶ × no. of bacteria ml ⁻¹	Fe ³⁺ produced (mg ml ⁻¹)	10 ⁻⁶ × no. of bacteria ml ⁻¹	Fe ³⁺ produced (mg ml ⁻¹)	10 ⁻⁶ × no. of bacteria ml ⁻¹	Fe ³⁺ produced (mg ml ⁻¹)
None	5.2	7.13	0.0	0.23	0.0†	0.88
0.1 mM-SeO ₄ ²⁻	4.3	6.13	0.0	0.05	1.8	1.77
1 mM-SeO ₄ ²⁻	0.95	5.70	0.0	0.06	0.0	0.35
5 mM-SeO ₄ ²⁻	0.0	0.28	0.0	0.07	0.0	0.48
10 mM-SeO ₄ ²⁻	0.0	ND	0.0	ND	0.0	ND
0.1 mM-SO ₄ ²⁻	4.4	6.33	0.0	0.18	2.8	2.7
1 mM-SO ₄ ²⁻	5.3	5.8	0.0	0.09	3.0	4.3
5 mM-SO ₄ ²⁻	5.3	5.8	0.0	0.11	1.3	1.0
10 mM-SO ₄ ²⁻	11.7	5.7	1.5	2.85	ND	ND
100 mM-SO ₄ ²⁻	11.2	6.2	0.7	2.02	ND	ND

ND, Not determined.

* Basal 9K medium without FeSO₄ contained 25 mM-SO₄²⁻ as (NH₄)₂SO₄ and MgSO₄.

† Bacteria could be counted after 11 days.

Selenate inhibited the growth of *T. ferrooxidans* in FeCl₂ and FeSO₄ media (Table 2). After 8 days incubation, only the lowest concentration of SeO₄²⁻ (0.1 mM) permitted growth in FeCl₂ medium. At 1 mM, selenate inhibited growth in FeSO₄ medium containing 190 mM-SO₄²⁻. Since SO₄²⁻ and SeO₄²⁻ affect iron oxidation similarly (Fig. 1*b*), the inhibition of growth by selenate in Fe²⁺ media is probably not related to the process of iron oxidation but results from some biochemical process in which the exclusion or replacement of SO₄²⁻ by SeO₄²⁻ is injurious.

Effects of anions on Fe³⁺ formation

The suspensions of *T. ferrooxidans* grown in FeSO₄ medium which were routinely used contained sufficient SO₄²⁻ to allow a slow uptake of oxygen and oxidation of Fe²⁺ in FeCl₂ solutions. Increased rates of oxidation were obtained on addition of SO₄²⁻ or SeO₄²⁻ (Fig. 1*b*). Therefore, the oxidation of Fe²⁺ in chloride solutions would presumably be diminished or eliminated by washing the bacteria to remove SO₄²⁻. When the bacteria were washed by sedimentation and resuspension in HCl instead of H₂SO₄ at room temperature, much of the iron-oxidizing activity for both FeSO₄ and FeCl₂ was lost (Fig. 2*a*). Activity was not restored by resuspending bacteria in 1.6 mM-H₂SO₄ or adding further SO₄²⁻. When packed suspensions of bacteria grown in FeSO₄ medium (sulphate bacteria) were diluted in 3.2 mM-HCl at 0 °C instead of room temperature, the bacteria (chloride bacteria) displayed little or no oxidation of iron as FeCl₂ but responded strongly on addition of SO₄²⁻ or SeO₄²⁻ (Fig. 2*b*). These chloride bacteria maintained their activity in the absence of substrate at 0 °C but, unlike the sulphate bacteria, steadily lost activity at room temperature.

The two types of bacteria differed in the specificity of their response to added anions. The chloride bacteria required SO₄²⁻ or SeO₄²⁻ to oxidize Fe²⁺; all other anions tested were inactive (Table 3). However, the iron-oxidizing activity of sulphate bacteria was stimulated by several anions, including PO₄³⁻, AsO₄³⁻, WO₄²⁻ and TeO₄²⁻. The iron-oxidizing activity of chloride bacteria was enhanced by those anions only if SO₄²⁻ or SeO₄²⁻ was also added.

The conversion of sulphate bacteria to chloride bacteria is not simply a result of dilution of free sulphate in suspensions, since when sulphate bacteria were diluted or washed in distilled water, the specificity of their response to the addition of anions was unchanged (Fig. 2*c*). This suggests that at low temperatures Cl⁻ ions reversibly displace bound SO₄²⁻ from loci requisite for iron-oxidizing activity, but at higher temperatures the displacement of SO₄²⁻ leads to further irreversible changes.

Fig. 2. Effect of the anionic environment on the formation of Fe^{3+} by *T. ferrooxidans*. Fe^{3+} formation was measured continuously by the increase in E_{304} at room temperature. All solutions were adjusted to pH 2.5 with HCl or H_2SO_4 as appropriate.

(a) Inactivation of bacteria by Cl^- at room temperature. Bacteria were diluted 1:10 in 3.2 mM-HCl or 1.6 mM- H_2SO_4 at room temperature. Cuvettes contained: 1.0 ml suspension (6.8×10^9 bacteria ml^{-1}), 1.0 ml 1.0 mM- Fe^{2+} substrate and 1.0 ml 3.2 mM-HCl or 1.6 mM- H_2SO_4 . (1) FeSO_4 substrate, bacteria added 130 min after dilution in H_2SO_4 ; (2) FeSO_4 , immediately after dilution in H_2SO_4 ; (3) FeSO_4 , immediately after dilution in HCl, or FeCl_2 , immediately after dilution in H_2SO_4 ; (4) FeCl_2 , immediately after dilution in HCl; (5) FeSO_4 , 145 min after dilution in HCl; (6) FeCl_2 , 60 min after dilution in HCl.

(b) Effect of various anions on the activity of bacteria diluted in HCl at 0 °C. Cuvettes contained: 0.5 ml bacterial suspension diluted 1:10 in 3.2 mM-HCl, 1.0 ml 1.0 mM- FeCl_2 and one of the following solutions, adjusted to pH 2.5 with HCl: (1) 1.0 ml 10 mM- Na_2SO_4 plus 0.5 ml 3.2 mM-HCl; (2) 1.0 ml 10 mM- Na_2SO_4 plus 0.5 ml 10 mM- Na_2TeO_4 ; (3) 1.0 ml 10 mM- Na_2SO_4 plus 0.5 ml 10 mM- NaH_2PO_4 ; (4) 0.5 ml 1.0 mM- Na_2SO_4 plus 0.5 ml 1.0 mM- NaH_2PO_4 plus 0.5 ml 3.2 mM-HCl; (5) 0.5 ml 1.0 mM- Na_2SO_4 plus 1.0 ml 3.2 mM-HCl, bacteria added immediately after dilution; (6) 1.0 ml 1.0 mM- Na_2SO_4 plus 0.5 ml 3.2 mM-HCl, 120 min after dilution; (7) 1.5 ml 3.2 mM-HCl, or any of the following (where necessary made up to 1.5 ml with 3.2 mM-HCl): 0.5 ml 10 mM- Na_2TeO_4 ; 0.5 ml 1.0 mM- NaH_2PO_4 ; 0.5 ml 1.0 mM- NaH_2PO_4 and 1.0 ml 10 mM- Na_2TeO_4 ; 1.0 ml 1.0 mM- Na_2WO_4 ; 0.5 ml 1.0 mM- Na_3AsO_4 ; 1.5 ml 1.0 mM- $\text{Na}_2\text{B}_4\text{O}_7$; or 0.5 ml 10 mM- NaClO_3 .

(c) Activity of bacteria diluted in H_2O at 0 °C. Cuvettes contained: 1.0 ml bacterial suspension diluted 1:20 in H_2O at 0 °C, 1.0 ml 1.0 mM- FeCl_2 and 1.0 ml of one of the following solutions, adjusted to pH 2.5 with HCl: (1) 1.0 mM- Na_2SO_4 , bacteria added 15 min after dilution; (2) 1.0 mM- Na_2SO_4 , 120 min after dilution; (3) 1.0 mM- Na_2SeO_4 , 30 min after dilution; (4) 1.0 mM- Na_2TeO_4 , 45 min after dilution; (5) 3.2 mM-HCl, immediately after dilution; (6) 3.2 mM-HCl, 105 min after dilution; (7) 1.0 mM- Na_2MoO_4 , 70 min after dilution.

DISCUSSION

In several biochemical systems, SO_4^{2-} and SeO_4^{2-} have similar activity indicating that the enzymes involved do not distinguish between these anions (Shrift, 1958). Their identical behaviour in relation to iron oxidation could stem from close correspondence in the properties of their iron complexes. Thus, the reported stability constant K_1 at 25 °C for the sulphate complex of Fe^{2+} is 10 and for the selenate complex is 12 under the same conditions (Wells & Salam, 1968). By contrast, K_1 for the chloride complex is less than 0.5 and for the HPO_4^{2-} complex is 1.5×10^7 (Sillen & Martell, 1971). If the rate of iron oxidation were dependent on the concentration of a sulphate complex of Fe^{2+} , inhibition due to complex formation by other anions should depend on the stability constants of the competing complexes. However, this is not the case; some anions which form weak complexes (Cl^-) are inhibitory but those which form strong complexes (HPO_4^{2-}) stimulate oxidation if SO_4^{2-} or SeO_4^{2-} is present.

Nearly all of the inorganic ligands stabilize the oxidized cationic species more strongly than

Table 3. *Effects of various anions on the oxidation of Fe²⁺ by suspensions of T. ferrooxidans*

Reaction mixtures contained bacterial suspension diluted in water or 3.2 mM-HCl, 1 mM-FeCl₂ and the anion indicated (as the sodium salt) at a final concentration of 1 mM. Results are expressed as the rate of increase in E_{304} relative to that with SO₄²⁻ as the added anion.

Anion added	Relative rate of E_{304} increase	
	Bacteria diluted in H ₂ O	Bacteria diluted in HCl
None	31.7	0.0
Cl ⁻	33.5	0.0
Br ⁻	0.0	0.0
NO ₃ ⁻	0.0	0.0
SO ₄ ²⁻	100.0	100.0
SeO ₄ ²⁻	70.0	83.4
TeO ₄ ²⁻	60.0	0.0
B ₄ O ₇ ²⁻	41.4	0.0
ClO ₃ ⁻	19.9	0.0
MoO ₄ ²⁻	0.0	0.0
WO ₄ ²⁻	50.0	0.0
CrO ₄ ²⁻	*	*
VO ₄ ³⁻	*	*
AsO ₄ ³⁻	62.2	0.0
PO ₄ ³⁻	83.2	0.0

* Slow chemical oxidation of Fe²⁺ occurred in the absence of bacteria, and there was no stimulation of abiotic oxidation in the presence of *T. ferrooxidans*.

the reduced species (Quagliano & Rebertus, 1956). This would facilitate oxidation of Fe²⁺ by increasing the negative free energy change of the reaction. Although such properties of chloride and phosphate as ligands can account for their enhancement of the rate of chemical oxidation of Fe²⁺ (Wells & Salam, 1968), the anionic requirement for biological oxidation by *T. ferrooxidans* is satisfied only by SO₄²⁻ or SeO₄²⁻. The anionic requirement for autotrophic growth, with Fe²⁺ as the sole source of utilizable energy, is satisfied only by SO₄²⁻ since the SeO₄²⁻ concentrations which sustain appreciable chemolithotrophic iron oxidation are toxic to growth of *T. ferrooxidans*.

The inability of other anions which form complexes with Fe²⁺ in acid solutions to replace SO₄²⁻ does not rule out the possibility that complexing of the entering ferrous substrate or the leaving ferric product is necessary for the oxidation of iron by the bacteria. The oxidized iron product deposited by the activities of *T. ferrooxidans* is an aquated sulphate complex (jarosite) which differs from the form of iron precipitated after abiotic oxidation (Ivarson, 1973). Nevertheless, a specific biochemical function of SO₄²⁻ in iron oxidation is likely to be separate from its role as ligand for the metabolized iron species.

High concentrations of SO₄²⁻, which inhibit oxygen uptake, do not concomitantly inhibit the formation of Fe³⁺: this may indicate that Fe²⁺ oxidation can be partially uncoupled from the reduction of oxygen or that the stoichiometry of iron oxidation and oxygen utilization can be altered toward increased efficiency of oxygen reduction (Steiner & Lazaroff, 1974). Although Tuovinen *et al.* (1975) have reported that SO₄²⁻ taken up during Fe²⁺ oxidation is, at least in part, converted to adenosine phosphosulphate, no evidence is yet available that this is part of an energy-generating mechanism, or that sulphate, in any form, is reduced during iron oxidation.

The requirement for sulphate in iron oxidation by *T. ferrooxidans* has been confirmed by investigations with whole cells (Schnaitman *et al.*, 1969; Tuovinen *et al.*, 1971) and with cell envelopes (Barvinchak, 1975). Cell-free studies of iron oxidase activity have not clearly shown a sulphate requirement, although chloride has been reported to inhibit Fe²⁺:cytochrome *c* reductase activity (Din, Suzuki & Lees, 1967). The specificity of the anionic require-

ment for iron oxidation by *T. ferrooxidans* agrees with the model proposed by Dugan & Lundgren (1965) in which the ferrous substrate is bound as a sulphate and phosphate adduct to the bacterial cell envelope. Several earlier reports had indicated that the addition of phosphate stimulated oxygen uptake during Fe^{2+} oxidation (Lazaroff, 1963; Beck & Shafia, 1964; Tuovinen *et al.*, 1971). This is consistent with the present finding that TeO_4^{2-} , WO_4^{2-} , AsO_4^{3-} or PO_4^{3-} stimulate oxidation if SO_4^{2-} or SeO_4^{2-} is also present even though there is no absolute requirement for anions other than SO_4^{2-} or SeO_4^{2-} .

The overall effect of the anionic environment can best be interpreted as the result of two different requirements for anions in the iron oxidation process. The first involves non-specific iron complex formation in acid solution for which several anions including SO_4^{2-} and SeO_4^{2-} may serve as ligands. The second function specifically requires SO_4^{2-} or SeO_4^{2-} interaction with the bacteria for oxidation of the ferrous ion complex to occur.

REFERENCES

- BARVINCHAK, G. (1975) *Investigation of the mechanism of iron oxidation by cells and cell envelopes of Thiobacillus ferrooxidans*. Thesis, Syracuse University, U.S.A.
- BECK, J. V. & SHAFIA, F. M. (1964). Effect of phosphate ion and 2,4-dinitrophenol on the activity of intact cells of *Thiobacillus ferrooxidans*. *Journal of Bacteriology* **88**, 850–857.
- DIN, G. A., SUZUKI, I. & LEES, H. (1967). Ferrous iron oxidation by *Ferrobacillus ferrooxidans*. Purification and properties of Fe^{2+} :cytochrome *c* reductase. *Canadian Journal of Biochemistry* **45**, 1523–1546.
- DUGAN, P. R. & LUNDGREN, D. G. (1965). Energy supply for the chemoautotroph *Ferrobacillus ferrooxidans*. *Journal of Bacteriology* **89**, 825–834.
- IVARSON, K. C. (1973). Microbiological formation of basic ferric sulfates. *Canadian Journal of Soil Science* **53**, 315–323.
- KOLTHOFF, I. M., SANDELL, E. B., MEEHAN, E. J. & BRUCKENSTEIN, S. (1969). *Quantitative Chemical Analysis*, pp. 1049–1062. Toronto: The Macmillan Co.
- LAZAROFF, N. (1963). Sulfate requirement for iron oxidation by *Thiobacillus ferrooxidans*. *Journal of Bacteriology* **85**, 78–83.
- PICK, U. & AVRON, M. (1973). Inorganic sulfate and selenate as energy transfer inhibitors of photophosphorylation. *Biochimica et biophysica acta* **325**, 297–303.
- QUAGLIANO, J. V. & REBERTUS, R. L. (1956). Stabilization of valence states through coordination. In *Chemistry of the Coordination Compounds*, pp. 398–415. Edited by J. C. Bailar Jr. New York: Reinhold Publishing Corp.
- RAZZELL, W. E. & TRUSSEL, P. C. (1963). Isolation and properties of an iron oxidizing *Thiobacillus*. *Journal of Bacteriology* **85**, 595–603.
- SCHNAITMAN, C. A., KORCZYNSKI, M. D. & LUNDGREN, D. G. (1969). Kinetic studies of iron oxidation by whole cells of *Ferrobacillus ferrooxidans*. *Journal of Bacteriology* **99**, 552–557.
- SHRIFT, A. (1958). Biological activities of selenium compounds. *Botanical Review* **24**, 550–583.
- SILLEN, L. G. & MARTELL, A. E. (1971). *Stability Constants of Metal Ion Complexes*, special publication no. 25. London: The Chemical Society.
- SILVERMAN, M. P. & LUNDGREN, D. G. (1959). Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. *Journal of Bacteriology* **77**, 642–647.
- STADTMAN, T. C. (1974). Selenium biochemistry. *Science*, **183**, 915–921.
- STEINER, M. R. & LAZAROFF, N. (1974). Direct method for continuous determination of iron oxidation by autotrophic bacteria. *Applied Microbiology* **38**, 872–880.
- TUOVINEN, O. H., NIEMELLA, S. I. & GYLLENBERG, H. G. (1971). Effect of mineral nutrients and organic substances on the development of *Thiobacillus ferrooxidans*. *Biotechnology and Bioengineering* **13**, 517–527.
- TUOVINEN, O. H., KELLEY, B. C. & NICHOLAS, D. J. D. (1975). The uptake and assimilation of sulphate by *Thiobacillus ferrooxidans*. *Archives of Microbiology* **105**, 123–127.
- WELLS, C. F. & SALAM, M. A. (1968). Complex formation between iron(II) and inorganic anions. Part II. The effect of oxyanions on the reaction of iron(II) with hydrogen peroxide. *Journal of the Chemical Society (A)*, 308–315.