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CHARACTERIZATION OF URANIUM MINE ISOLATES AND
LABORATORY CULTURES OF THIOBACILLUS FERROOXIDANS WITH
EMPHASIS ON THE OXIDATION AND CELLULAR ACCUMULATION OF
URANIUM AND BIOENERGETIC COMPARISON WITH IRON

The Ohio State University

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DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

Alan Angelo DiSpirito, B.S., M.S.

* * * * *

The Ohio State University

1983

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ACKNOWLEDGEMENTS

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PUBLICATIONS


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TECHNICAL REPORTS


PUBLISHED ABSTRACTS


UNPUBLISHED CONFERENCE AND SEMINAR PRESENTATIONS

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Sorption of *Thiobacillus ferrooxidans* on 
solid surfaces and its effect on iron oxidation. 
Joint Meeting of the Indiana and Ohio Branches 
of the American Society for Microbiology, 
October 1979.

DiSpirito, A.A. and Tuovinen, O.H. Oxidation of 
U(IV) compounds by acidophilic thiobacilli. 
Joint Meeting of the Indiana and Ohio Branches 
of the American Society for Microbiology, 
October 1980.

Determination of Ubiquinone-8 in *Thiobacillus 
ferrooxidans*. Microbial Chemoautotrophy 
Colloquium, The Ohio State University, Columbus, 
Ohio. Presented also at the Annual Meeting of 
the Indiana and Ohio Branches of the American 
Society for Microbiology, October 1982.

DiSpirito, A.A. Uranium oxidation and uptake by 
*Thiobacillus ferrooxidans*. Department of 
Biology, Illinois Institute of Technology, 
This thesis consists of the following papers arranged in separate chapters:


The first chapter in this thesis is an examination of the surface morphology of 13 strains of T. ferrooxidans and 2 strains of T. acidophilus. Microscopic evidence is presented in support of...
the original description of *T. ferrooxidans* as a motile bacterium by means of a single polar flagellum. This chapter also demonstrates morphological variation in different isolates with the observation of peritrichous flagella in three strains of iron-oxidizing thiobacilli. In addition to the variation in flagella number and dimensions, the presence or absence of pili also differed in several isolates of *T. ferrooxidans*.

Chapters II-IV of this thesis deal with the oxidation of tetravalent uranium to its hexavalent form. In addition to the oxidation of U⁴⁺ by *T. ferrooxidans*, these chapters will also examine the electron flow during this oxidation, its coupling to the phosphorylation system, and the mechanism through which this oxidation may occur.

Chapter V deals with the isolation and characterization of ubiquinone-8 in this bacterium. A comparatively simple and fast method of isolating, purifying, and characterizing isoprenoid quinones from bacterial systems is also presented.

For chapter VI the uptake and cellular distribution of uranium in *T. ferrooxidans* was investigated.
This chapter also examines the involvement of metabolic activity and some environmental factors in the cellular accumulation of uranium.
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CHAPTER I

FLAGELLA AND PILI OF IRON-OXIDIZING THIOBACILLI ISOLATED FROM A URANIUM MINE IN NORTHERN ONTARIO, CANADA

Five strains of Thiobacillus ferrooxidans, which included three recent isolates from a uranium mine, possessed flagella. Three of the strains had several pili per cell. The dimensions, fine structure, and orientation of the flagella were different. Both polar and peritrichous flagella were observed, indicating strain-dependent ultrastructural variation in acidophilic thiobacilli. Neither flagella nor pili were detected in eight other strains of T. ferrooxidans and two strains of Thiobacillus acidophilus by electron microscopy, although all of the cultures contained motile cells.

Introduction

Thiobacillus ferrooxidans, a motile acidophilic autotroph, is reported to possess a single polar flagellum characteristic of the Pseudomonadaceae (Vishniac, 1974), the presence of which has not been confirmed by electron microscopy. Flagella have been
studied in sulfur-grown *Thiobacillus thiooxidans* (Doetsch et al., 1967), a closely related acidophile which does not oxidize ferrous iron. Gromova et al. (1978) reported the presence of pili in sulfur-grown *T. ferrooxidans*, but did not detect these structures when ferrous iron was used as growth substrate. Pili have also been found in *Thiobacillus kabobis* (Reynolds et al., 1981) and in thermophilic sulfur-oxidizing bacteria (Weiss, 1973; Berry and Murr, 1980). Fimbriae have been detected in *Thiobacillus* sp. strain A2 (Korhonen et al., 1978).

Recently, the hypothesis has been proposed that the iron-oxidizing thiobacilli may not be one distinct species but, rather, a group of metabolically similar bacteria (Silver, 1978). This premise is based on the differences in the physiological characteristics (Silver and Torma, 1974) and guanine and cytosine contents of DNA (Guay et al., 1976) of different isolates of *T. ferrooxidans*. This report extends the examination of different strains of these bacteria by investigating the presence and external morphology of flagella and pili in a number of different cultures.
of acidophilic thiobacilli, including iron oxidizers which are currently being used in the commercial extraction of uranium at Agnew Lake, Ontario. The physical and chemical characteristics and the concentrations of iron-oxidizing and heterotrophic bacteria in the leach liquors from various points in the extraction circuit have been reported (Tuovinen et al., 1981). Several isolates of the iron-oxidizing thiobacilli from this mine were found to possess plasmids; they were also characterized (Tuovinen et al., 1981).

Materials and Methods

All of the cultures were grown at room temperature (22°C) in unagitated 20-ml test tubes containing 10 ml samples of media. The ferrous iron (pH 1.5), tetra-thionate (pH 4.5), and glucose (pH 2.0) media have been described elsewhere (Shafia and Wilkinson, 1969; Tuovinen and Kelly, 1973; Tuovinen and Kelly, 1974c). The strains were subcultured at least four times before examination.

Strains TFI-1, TFI-4, TFI-6, TFI-7, TFI-9, TFI-10, and TFI-13 of _T. ferrooxidans_ were isolated from various
locations of the Agnew Lake uranium mine of northern Ontario (Tuovinen et al., 1981). \textit{T. ferrooxidans} strain TFI-17 was obtained as a derivative of \textit{T. ferrooxidans} ATCC 19859 from A.P. Harrison (University of Missouri, Columbia), TFI-23 was obtained from G.J. Olson (National Bureau of Standards, Washington, D.C.), TFI-27 and TFI-29 were obtained from A.E. Torma (New Mexico Institute of Mining and Technology, Socorro), and strain TFI-30 was obtained from E.J. Brown (University of Alaska, Fairbanks); strain TFI-35 is a derivative of the original isolation of \textit{Ferrobacillus ferrooxidans} (Leathen et al., 1956), which has been continually maintained on ferrous iron (Tuovinen and Kelly, 1973). All of the \textit{T. ferrooxidans} strains were grown on ferrous iron, and strains TFI-7 and TFI-35 were also grown on tetrathionate. \textit{Thiobacillus acidophilus} strains AFG-2 and AFG-3 were derived from the original culture of \textit{T. acidophilus} (Guay and Silver, 1975) and were grown on glucose and on tetrathionate.

Samples for electron microscopy were taken from the surfaces of the static liquid cultures. Under these conditions, glucose-grown thiobacilli form a
surface pellicle. A light surface film formed in tetrathionate-grown thiobacilli developed a white, thin surface film, with pellicle formation at the solution-glass interface. At pH values greater than 1.8, this pellicle formation becomes indistinguishable owing to precipitates formed by the hydrolysis of ferric iron. Samples were placed on Parlodion and carbon-coated grids and were negative stained with 2% (wt/vol) uranyl acetate for 2 to 5 min. A negative stain with 1% phosphotungstic acid was also tested, but it was excluded because of its inferior resolution in the present procedure. The grids were examined and photographed under a Zeiss model 9A electron microscope. The length measurements of flagella and pili were determined from electron micrographs, using a Numonics digitizer.

Results

The Agnew Lake uranium mine, the environment from which iron-oxidizing strains of thiobacilli TFI-1 to TFI-13 were isolated, currently employs surface
heap leaching and flood leaching of the underground stopes for the recovery of uranium (Tuovinen et al., 1981). Table 1.1 shows the chemical analysis and numbers of iron-oxidizing and heterotrophic bacteria of the leach solutions in January, 1981. These solutions contained up to 0.11 g of uranium per liter and up to 1.23 g of thorium per liter. Iron is present at concentrations between 4 and 5 g/liter, mostly in the trivalent state, and acidophilic thiobacilli are present in all parts of the leach circuit with the exception of the tailings pond. The concentrations of iron-oxidizing thiobacilli and heterotrophic bacteria are similar to those found in other mining operations (Beck, 1967; Bhappu et al., 1969; Brierley and Lockwood, 1977; Wichlacz and Unz, 1981) or in the Agnew Lake mine at different times of the year (Tuovinen et al., 1981).

All of the cultures contained cells that were motile, but the percentage of motile bacteria in each culture varied. Electron microscopic examination showed the presence of flagella in only five of the strains when grown on ferrous iron, TFI-1, TFI-9, TFI-10, TFI-27, and TFI-35. The inability to detect flagella
Table 1.1. Chemical analysis and bacterial enumeration of leach liquor samples from the Agnew Lake uranium mine.

<table>
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<tr>
<th>Mine location</th>
<th>pH (mV)</th>
<th>Eh (mV)</th>
<th>U (g/L)</th>
<th>Th (g/L) (pc1/L)</th>
<th>SO₂⁻-S (g/L)</th>
<th>Fe²⁺ (g/L)</th>
<th>Total Fe (g/W)</th>
<th>Iron Oxidizers (cells/ml)</th>
<th>Glucose Oxidizers (cells/ml)</th>
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<td>700-5-E</td>
<td>1.75</td>
<td>570</td>
<td>0.030</td>
<td>1.18</td>
<td>230</td>
<td>10.7</td>
<td>0.73</td>
<td>4.77</td>
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<tr>
<td>1,000-5-E</td>
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<td>550</td>
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<td>590</td>
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<td>10.4</td>
<td>0.82</td>
<td>4.48</td>
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</tr>
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<td>474</td>
<td>0.0034</td>
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<td>0.91</td>
<td>4.11</td>
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<td>450</td>
<td>&lt;0.001</td>
<td>&lt;0.001e25</td>
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<td>&lt;0.001</td>
<td>0.00008</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
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<tr>
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<td>0.0020</td>
<td>1.13</td>
<td>110</td>
<td>10.4</td>
<td>0.99</td>
<td>4.18</td>
<td>4 x 10³</td>
</tr>
</tbody>
</table>

The chemical methods and viable count technique (MPN) are explained in Tuovinen et al. (1981).

The stopes of the mine are numbered in the following manner: depth (feet)-ore zone-East/West. Uranthorite, the main uranium mineral, occurs in two separate areas of the ore body designated zones 3 and 4. The solution flow in the mine was described previously (Tuovinen et al., 1981).
Fig. 1.1. A, Flagellum and pili of negatively stained \textit{T. ferrooxidans} TFI-35. Bar, 0.5 \( \mu \text{m} \). B, Pili of \textit{T. ferrooxidans} TFI-35, Bar, 0.2 \( \mu \text{m} \).
Fig. 1.2. Flagella of negatively stained *T. ferrooxidans* TFI-10. Bar, 0.5 μm.

Fig. 1.3. Flagella of negatively stained *T. ferrooxidans* TFI-10; sites of attachment can be seen on the cell surface. Bar, 0.2 μm.
Fig. 1.4. Pili (arrow) of negatively stained *T. ferrooxidans* TFI-10. Bar, 0.2 μm.

Fig. 1.5. A, Flagellum of negatively stained *T. ferrooxidans* TFI-35. Bar, 0.1 μm. B, Flagella of *T. ferrooxidans* TFI-10 showing light and dark staining regions. Bar, 0.1 μm.
in the other cultures is probably due to the low percentage of flagellated cells and to their tendency to detach from the cell upon sample preparation.

Strain TFI-35 (Fig 1.1) has a single polar flagellum, with a maximum length of 5.4 μm and an average diameter of 18.9 nm. These cells have up to five pili, with a maximum length of 3.6 μm and an average diameter of 5.4 nm. Only a small proportion of these bacteria retained their flagella after a number of subcultures. Strain TFI-1 was similar to strain TFI-35, with a maximum flagellum length of 9 μm and an average diameter of 19.9 nm. Cells of strain TFI-1 also contained up to five pili, with a maximum length of 1.5 μm and an average diameter of 8.9 nm. Figures 1.2 and 1.3 show that cells of T. ferrooxidans TFI-10 have at least four peritrichous flagella (average diameter, 18.2 nm). These flagella are extremely fragile, detaching easily from the cell and breaking into segments despite all precautions taken during the preparation for electron microscopy. This strain also has pili (Fig. 1.4), with an average diameter of 4.9 nm. Strains TFI-9 and TFI-27 also possessed peritrichous flagella. The maximum flagellum lengths of TFI-9 and TFI-27
were 3.9 and 4.6 μm, respectively, with average diameters of 19.3 and 16.9 nm. Pili were not, however, observed in either strain of TFI-9 or TFI-27.

The fine structures of flagella from strains TFI-35 and TFI-10 (fig. 1.5) appear different. The flagella of strain TFI-35 stained evenly, whereas those of TFI-10 showed alternating light and dark regions of approximately 3 nm each. Flagella and pili were not detected by electron microscopy in any isolate of *T. ferrooxidans* grown on tetrathionate or in *T. acidophilus* grown on either glucose or tetrathionate despite their motility under phase-contrast microscopy.

**Discussion**

The presence of polar flagella of strains TFI-1 and TFI-35 confirms the present classification character of *T. ferrooxidans* (Vishniac, 1974). In contrast, the presence of multiple flagella in a peritrichous arrangement in *T. ferrooxidans* strains TFI-9, TFI-10, and TFI-27 shows that there is structural variation in different strains of the iron-oxidizing thiobacilli. Pili and flagella are involved in the attachment of
bacteria to surfaces. These organells of the Agnew Lake uranium mine isolates TFI-1, TFI-9, and TFI-10 are of significance, as are those of strains TFI-27 and TFI-35, as the interaction of the iron-oxidizing thiobacilli with metal sulfide minerals requires a close contact and even attachment of the bacteria with the crystal surface (Kingma et al., 1980). Although the occurrence of pili and flagella on iron-oxidizing bacteria attached to ore particles has not been demonstrated, these organelles are now shown to be present on \textit{T. ferrooxidans} isolates from an environment in which these bacteria are known to oxidize sulfide minerals (Tuovinen et al., 1981). The concentrations of thiobacilli associated with mineral particles are at least equal to or greater than those in the bulk solution phase (Bhappu et al., 1969; Brierley and Lockwood, 1977; Murr and Brierley, 1978).

The pili of \textit{T. ferrooxidans} may be involved in the transfer of DNA, but unlike those of many other bacteria, this function has not been demonstrated in the thiobacilli. Many different plasmids have been detected in a variety of strains from the Agnew Lake uranium mine (Tuovinen
et al., 1981), including one of about 13 megadaltons which may encode resistance to uranium. The structure-function relationships of both flagella and pili of these organisms warrant further study in view of their ability to function in conditions of extreme acidity.

Variations in physiological and genetic characteristics of the iron-oxidizing thiobacilli have been previously demonstrated (Guay et al., 1976; Martin et al., 1981; Silver, 1978; Silver and Torma, 1974; Tuovinen et al., 1981). In this report, we have demonstrated that morphological variations of taxonomic importance also exist in different isolates of these bacteria. This supports the hypothesis that the iron-oxidizing thiobacilli may not belong to one distinct species, but may comprise a group of metabolically similar, but taxonomically distinct, bacteria.
The oxidation of uranous compounds by washed cell suspensions of *Thiobacillus ferrooxidans* and *T. acidophilus* was monitored with a Clark oxygen electrode. The bacteria were able directly to oxidize U-IV, which was supplied as uranous sulfate or as uranous oxide. The rates of oxygen uptake were dependent on: the cell concentration; the previous growth history of the organisms; the amounts of U-IV and of inhibitors; pH and the presence of iron sulfates.

Introduction

*Thiobacillus ferrooxidans* is an obligate acidophile often associated with acid mine effluents and with leach liquors (Tuovinen and Kelly, 1974d; Bosecker and Kürsten, 1978; Unz et al., 1979). Because of their ability to oxidize iron and sulfur compounds for energy and growth, these bacteria can produce copious amounts of ferric salts and sulfuric acid from ore and rock materials containing metal sulfides. Iron-oxidizing thiobacilli are employed in leaching
processes that have been developed for the recovery of base metals and uranium both by dump leaching and by heap and stope leaching (Torma, 1977; Brierley, 1978; Lundgren and Silver, 1980). These iron-oxidizers also oxidize cuprous and stannous compounds (Nielsen and Beck, 1972; Golding et al., 1974; Lewis and Miller, 1977). Microcalorimetric and manomeric studies have indicated that \emph{T. ferrooxidans} may also respire on uranous compounds (Ivarson, 1980; Soljanto and Tuovinen, 1980). The bacterial oxidation of U-IV compounds is of particular interest since \emph{T. ferrooxidans} enhances uranium recovery in hydrometallurgical processes (MacGregor, 1969; McCreedy et al., 1969; Duncan and Bruynesteyn, 1971). In the acid leaching, U-IV is oxidized by ferric iron to the hexavalent form, which is readily soluble in dilute sulfuric acid. In the reaction, iron is reduced to Fe$^{2+}$, which is then regenerated to Fe$^{3+}$ by iron-oxidizing thiobacilli (Derry et al., 1976; Guay et al., 1976, 1977; Manchee, 1977):

\[
\begin{align*}
\text{UO}_2 + \text{H}_2\text{SO}_4 + 0.5\text{O}_2 & \rightarrow \text{UO}_2\text{SO}_4 + \text{H}_2\text{O} \\
\text{UO}_2 + \text{Fe}_2(\text{SO}_4)_3 & \rightarrow \text{UO}_2\text{SO}_4 + 2\text{FeSO}_4
\end{align*}
\]
The present work presents data indicating that *T. ferrooxidans* can directly oxidize both uranous sulfate and uranous oxide without the involvement of ferrous or ferric salts. Similar evidence is presented for a closely related species, *T. acidophilus*. The results suggest that a direct mechanism exists for the bacterial oxidation and solubilization of uranium from ores, and that the practical significance of this mechanism is determined by the level of Fe\(^{2+}\) present in the system.

**Materials and Methods**

**Bacteria:**

An iron-oxidizing culture of *T. ferrooxidans* (Tuovinen and Kelly, 1973), designated as strain TFI-35 was grown on 120 mM FeSO\(_4\) at pH 1.5 in 20 l-carboys with forced aeration. The bacteria were harvested by centrifugation at 13,200 x g for 20 min at 4°C. The cells were washed three times and were then resuspended in 0.1 N H\(_2\)SO\(_4\). Residual inorganic compounds
were removed by three successive centrifugations at 1000 × g each for 10 min at 4°C. Strain TFI-7, a uranium-resistant strain of *T. ferrooxidans*, had been originally isolated from the Agnew Lake Uranium Mine leach liquor and had been maintained on FeSO₄ in the presence of 10 mM uranyl sulfate. The cells were grown, harvested, and washed exactly as described for TFI-35. Tetrathionate-oxidizing *T. ferrooxidans* (strain TFK-35) was derived from TFI-35 and was grown on K₂S₄O₆ as described by Tuovinen and Kelly (1974). The harvesting and washing of these bacteria were also carried out exactly as described above for TFI-35 except that distilled water was used instead of H₂SO₄.

*T. acidophilus* cultures were grown on glucose (strain AFG-1) (Mao et al., 1980) and on tetrathionate (strain AFK-1, derived from AFG-1). The plasmid patterns in AFG-1 (Mao et al., 1980) and in AFK-1 were identical, thereby suggesting culture homogeneity. The plasmid DNA patterns of *T. ferrooxidans* TFI-35 and of *T. ferrooxidans* TFK-35 were also identical. The original description of *T. acidophilus* indicated that these organisms utilize glucose and elemental sulfur as growth substrates (Guay and Silver, 1975). Subsequent work demonstrated
the genetic and physiological heterogeneity of *T. acidophilus* (Tuovinen et al., 1978), and the organism was re-isolated and maintained as a pure culture (Mao et al., 1980). The growth of *T. acidophilus* on tetra-thionate in the present work demonstrates that these bacteria are not limited to elemental sulfur as the energy substrate for the chemolithotrophic growth. *T. acidophilus* strain AFG-1 and AFK-1 were harvested and washed exactly as previously described for TFK-35. The growth media both for *T. ferrooxidans* TFK-35 and for *T. acidophilus* strains AGF-1 and AFK-1 did not receive any added iron or other metals. The cell suspensions were calibrated by optical density and by protein determinations (Lowry et al., 1951).

U-IV compounds:

Uranous oxide (UO$_2; O/\text{U}>2$) was obtained from Alfa Products (Danvers, MA). Uranous sulfate was prepared by electrolytic reduction, under nitrogen atmosphere, of 48 mM uranyl sulfate in 0.1 N $\text{H}_2\text{SO}_4$. The electrolysis was carried out at the Canada Centre for Mineral and Energy Technology (Ottawa, Ontario).
and the uranous sulfate solution was obtained through the courtesy of K.C. Ivarson (Agriculture Canada, Ottawa, Ontario).

Oxygen uptake:

The oxygen uptake studies were carried out using a Clark oxygen electrode which was accommodated in a 1.9-ml cell. The system was maintained at a constant temperature of 30°C by circulating water through the outer jacket. Chemical and bacterial controls were run frequently to ensure the reduced state of the uranium stock solution. The controls included the chemical oxidation of graded amounts of $U^{4+}$ and the use, as a chemical oxidant, of ferric sulfate, which was reduced by $U^{4+}$ to $Fe^{2+}$ and was then re-oxidized by adding bacteria to the reaction mixture. The electrode response was monitored by using a recorder. The oxygen scale on the chart was calculated by completely oxidizing graded amounts of $Fe^{2+}$ with *T. ferrooxidans*. This reaction has a stoichiometry of $4Fe^{2+}/O_2$. 
The total iron in the cell suspensions and in the uranous sulfate stock solution was analyzed by atomic absorption spectrometry. The amount of total iron as impurity never exceeded 50 nmoles·ml⁻¹ reaction mixture.

Results

Oxidation of U-IV compounds by iron-grown *T. ferrooxidans*:

Uranous sulfate was gradually oxidized in dilute sulfuric acid as shown in Figure 2.1 and Table 2.1. The rate of chemical oxidation of U⁴⁺ was concentration-dependent (Figure 2.1) and was influenced by the pH (Table 2.1). The maximum rate of chemical oxidation at pH 2.0 was 0.07 μmoles O₂·min⁻¹, which equals 0.14 μmoles U⁴⁺ oxidized·min⁻¹. The rate decreased by almost 40% when the pH was lowered to pH 1.6 (Table 2.1).

In the presence of washed cells, the oxidation rates were enhanced (Table 2.1). At pH 1.7, in the absence of bacteria and in their presence, the maximum oxidation rates were respectively 0.052 and 0.096 μmoles O₂·min⁻¹. Both Table 2.1 and Figures 2.1 and
2.2 demonstrate that the oxidation rates were dependent on the cell density, as well as on the substrate concentration. At pH 1.5 to pH 1.6 with low cell density (0.16 mg protein·ml⁻¹), the bacterial enhancement of U⁴⁺ oxidation declined, whereas at pH 1.7 with 0.16 mg to 2.76 mg protein·ml⁻¹, the oxygen uptake was increased by 80% to 450%, as compared with the chemical oxidation (Table 2.1). Figure 2.2 illustrates for comparison the oxidation rates of Fe²⁺ and of U⁴⁺ and shows that much faster rates were obtained with Fe²⁺ as the substrate. Pentachlorophenol (1.3 mM) enhanced the chemical oxidation of U⁴⁺, but it completely inhibited the bacterial activity. NaN₃ and KCN (each at 1.3 mM) were also tested as inhibitors, but the oxidation rates became erratic since these inhibitors affected the chemical oxidation of uranous sulfate. The addition of autoclaved cells did not enhance the chemical oxidation of U⁴⁺ (Table 2.1).

Figure 2.3 illustrates the time course for the oxygen uptake coupled with uranous sulfate oxidation by T. ferrooxidans. The rate of oxidation was linear until it became limited by oxygen depletion. Uranous
Fig. 2.1. The effect of \( U^{4+} \) concentration on the rate of chemical and bacterial oxidation of uranous sulfate. Key: \( \Delta \), chemical oxidation; \( \circ \), bacterial oxidation (TFI-35, 0.16 mg protein ml\(^{-1}\)); \( \Theta \), differential rate (= the difference between the bacterial and chemical oxidation).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein mg·ml(^{-1})</th>
<th>Treatment</th>
<th>pH</th>
<th>(Pb^{2+}) umole·ml(^{-1})</th>
<th>(D^{2+}) umole·ml(^{-1})</th>
<th>(O_2) uptake</th>
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<td>11.50</td>
</tr>
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<td>0.64</td>
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\(^a\)All reaction mixtures contained 50 moles Fe·ml\(^{-1}\) as an impurity.

\(^b\)PCP = pentachlorophenol.
Fig. 2.2. The effect of cell concentration on Fe$^{2+}$ oxidation and on U$^{4+}$ oxidation by T. ferrooxidans TFI-35. Key: □, iron oxidation; ○, uranium oxidation.
Fig. 2.3. $^{4+}\text{U}$ oxidation by washed cell suspensions of iron-grown *T. ferrooxidans* strains TFI-35 and TFI-7. The latter strain is resistant to 10 mM uranyl sulfate. 1: Strain TFI-35 (0.3 mg protein) was incubated for 5 minutes before addition (arrow) of 25 μmoles of uranyl sulfate. 2: Chemical oxidation of 12 μmoles of uranous sulfate added at arrow. 3: Autoclaved cells (0.3 mg protein) of TFI-35 were incubated for 5 minutes before the addition (arrow) of 12 μmoles of uranous sulfate. 4: TFI-35 (0.3 mg protein) was incubated for 5 minutes prior to the addition (arrow) of 12 μmoles of uranous sulfate. 5: The uranium-resistant strain TFI-7 (0.19 mg protein) was incubated for 5 minutes before the addition (arrow) of 12 μmoles of uranous sulfate.
oxide in a powdered form was also tested as a substrate (fig. 2.4). Although chemically more stable than uranous sulfate and oxidizable by the bacteria (fig. 2.4), UO\(_2\) was not used in further experiments because it sorbed to the electrode membrane, thereby rapidly poisoning the electrode.

The uranium-resistant strain TFI-7 consumed oxygen at a faster rate than did the cells that had not previously been exposed to uranium (Table 2.1). Figure 2.3 illustrates that uranyl sulfate did not enhance endogenous respiration.

The addition of excess Fe\(^{3+}\) instantaneously depleted U\(^{4+}\) by oxidizing it to UO\(_2^{2+}\) (fig. 2.5). Oxygen consumption was not associated with this oxidation-reduction reaction. The oxygen uptake was resumed upon the addition of bacteria, suggesting that the cells had oxidized the ferrous iron that had been formed during the reaction of Fe\(^{3+}\) with U\(^{4+}\) (fig. 2.5).

Oxidation of U\(^{4+}\) by tetrathionate-grown T. ferrooxidans:

Table 2.2 indicates that S\(_4\)O\(_6^2-\)-grown T. ferrooxidans oxidized Fe\(^{2+}\) as well as U\(^{4+}\), at rates comparable to
Fig. 2.4. Oxidation of $U^{IV}O_2$ by washed cells of
$T.\text{ferrooxidans}$ strains TFI-35 and TFI-7.
1: Chemical oxidation of 25 μmoles of
$UO_2$ (arrow). 2: TFI-35 (0.304 mg protein)
was incubated for 5 minutes before the
addition of 25 μmoles of $UO_2$ (arrow).
TFI-7 (0.10 mg protein) was incubated
for 5 minutes before the addition of
25 μmoles of $UO_2$ (arrow).
Fig. 2.5. \( U^{4+} \) oxidation mediated by ferric iron and washed cells of *T. ferrooxidans* TFI-35. Additions were as follows: 1: arrow A, 13 \( \mu \)moles of uranous sulfate; arrow B, 24 \( \mu \)moles of Fe\(^{3+} \). 2: arrow A, 13 \( \mu \)moles of uranous sulfate; arrow B, 2.4 \( \mu \)moles of Fe\(^{3+} \). 3. arrow A, 13 \( \mu \)moles of uranous sulfate; arrow B, 24 \( \mu \)moles Fe\(^{3+} \); arrow C, TFI-35 (0.304 mg protein).
Table 2.2. $\text{U}^{4+}$ oxidation by *T. ferrooxidans* TFK-35 and by *T. acidophilus* strains AFG-1 and APK-1<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein mg·ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;2+&lt;/sup&gt; pmole·ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>$\text{U}^{4+}$ pmole·ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Oxygen Uptake µl·min&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>µl·min&lt;sup&gt;-1&lt;/sup&gt;·mg&lt;sup&gt;-1&lt;/sup&gt; protein</th>
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<td>-</td>
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<td>APK-1</td>
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<td>18.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>pH was adjusted to pH 1.7 for all experiments.

<sup>b</sup>See footnote to Table 2.1.
those which the iron-grown cells oxidized them.

Oxidation of $U^{4+}$ by *T. acidophilus*:

*T. acidophilus* was grown with glucose and with tetrathionate, and the culture homogeneity was confirmed by analysis of the respective plasmid DNA as described in the above section on Materials and Methods. Table 2.2 and fig. 2.6 indicate that glucose-grown *T. acidophilus* was unable to oxidize $U^{4+}$, although it did oxidize both Fe$^{2+}$ and glucose. The tetrathionate-grown *T. acidophilus* cells consumed oxygen in the presence of either $U^{4+}$ or Fe$^{2+}$ as a substrate (Table 2.2). Since the iron-oxidation activity by washed cell suspensions of *T. acidophilus* contrasted with the original description of *T. acidophilus* (Guay and Silver, 1975), growth studies were initiated for testing FeSO$_4$ as a growth substrate. Neither AFG-1 nor AFK-1 were able to grow with FeSO$_4$; this indicated that in these strains, whose plasmid patterns were identical, the lack of growth on FeSO$_4$ was due to enzyme repression or to the loss of a coupling factor, thereby dissociating the iron oxidation from the energy conservation essential for carbon dioxide fixation and growth.
Fig. 2.6. $U^{4+}$ oxidation by washed cell suspensions of *T. acidophilus* grown on either tetra-thionate (APK-1) or on glucose (AFG-1). The bacteria were incubated for 5 minutes before the following additions: 1: Chemical oxidation of 12 μmoles $U^{4+}$ added at arrow. 2: AFG-1 (0.23 mg protein) received 12 μmoles of $U^{4+}$ at arrow. 3: APK-1 (0.286 mg protein) received 12 μmoles of $U^{4+}$ at arrow.
Discussion

In the present work, both the bacteria that grew on $\text{S}_4\text{O}_6^{2-}$ and those that grew on glucose were cultured without an iron supplement in order to minimize the amount of inorganic Fe in the washed cell suspension. After harvest, all cultures were also vigorously washed, and iron analysis indicated that less than 50 nmole Fe·ml$^{-1}$ was present in the final reaction mixture. It is questionable whether trace amounts of iron, some of it also incorporated into cell constituents, could be recycled by bacteria at rates that would be compatible with the observed oxygen uptake during U$^{4+}$ oxidation. For example, the measured rates indicated that the bacteria also oxidized U$^{4+}$ at rates of up to 0.4 µmoles·min$^{-1}$. If 50 nmole Fe·ml$^{-1}$ were the oxidizing agent, then this amount of iron would have to be regenerated 12 times in order to account for the stoichiometry of U/2Fe (i.e., 0.4 µmoles U/0.8 µmoles Fe$^{3+}$). In fact, this is an underestimation of the regeneration rate for iron, since not all iron would be freely dissociated as Fe$^{2+}$ or as Fe$^{3+}$, but, rather, some Fe would be either peptized or bound in the cell suspensions. On the basis of oxygen demand, it is
not possible to distinguish between the direct bacterial oxidation of uranous ion and that mediated by ferric-ferrous iron recycling, since both activities would require equal amounts of oxygen. The present work, however, indicates that the bacteria were able directly to oxidize uranous compounds. The rates of oxygen uptake were dependent on the cell density and on the substrate concentration, as well as on inhibitors and on the prior growth history of the organisms. These relationships are indicative of biological activity, but they do not exclude the possibility of iron recycling being the major mechanism for uranous ion oxidation. The glucose-grown cell suspensions of \textit{T. acidophilus} oxidized Fe$^{2+}$, as did all the other strains tested in the present study. The glucose-grown bacteria, however, did not oxidize U$^{4+}$. This observation suggests that the recycling of residual iron did not account for U$^{4+}$ oxidation; were this otherwise, there should have been oxygen uptake by glucose-grown \textit{T. acidophilus} with uranous sulfate as a substrate. Moreover, the oxidation rates also indicate that U$^{4+}$ was directly oxidized by bacteria. For example, the strain TFI-35, that had never previously been exposed to uranium com-
pounds, and the uranium-resistant strain TFI-7 oxidized Fe\(^{2+}\) at equal rates, but TFI-7 showed a much faster rate of oxygen uptake with uranous sulfate as a substrate. Similarly differential rates were evident when the rates of oxygen uptake by TFI-7 were compared with those found for TFK-35, AFK-1, or AFG-1. Thus, it can be concluded that the major path of U\(^{4+}\) oxidation by bacteria did not involve recycling of inorganic iron.

Although there is energy released during the oxidation of U-IV compounds, it remains to be established whether the cells are able to conserve the energy to support growth-related functions. From previous studies it is known that UO\(_2^{2+}\) is toxic to the iron oxidizers in a fashion similar to that of the uncoupling agent 2,4-dinitrophenol (Tuovinen and Kelly, 1974b). The tolerance to uranium develops in these bacteria (Tuovinen and Kelly, 1974a), but the resistance may not enable the bacteria to conserve energy from U\(^{4+}\) oxidation; rather, the resistance may alleviate the inhibitory effects of UO\(_2^{2+}\) on the iron oxidation. The uranium-resistant strain, however, consistently showed a faster rate of oxygen uptake with U\(^{4+}\) than that of any of the other tested strains. It was also established that the bacteria
oxidized uranous compounds rather than endogenous metabolites released from cells in the presence of uranium, since appropriate controls showed that endogenous respiration was not enhanced in the presence of UO$_2$SO$_4$. When autoclaved cells, glucose-grown cells, or pentachlorophenol-inhibited bacteria were used the oxygen uptake in the presence of uranous sulfate was negligible, suggesting that bacterial activity was essential for U$^{4+}$ oxidation.

Since both strains of *T. acidophilus* oxidized iron while strain AFK-1 also oxidized uranium, the taxonomic description of *T. acidophilus* may be deficient and warrants further clarification. In the present work, special attention was given to culture purity since previous studies have indicated heterogeneity in cultures of acidophilic thiobacilli (Tuovinen and Nicholas, 1977; Tuovinen et al., 1978; Harrison et al., 1980). Culture homogeneity in the present study was accomplished by the re-isolation and routine monitoring (Mao et al., 1980) of the plasmid patterns in the AFG-1 and the AFK-1 strains. Enzyme repression and a lack of a coupling factor may account for the
lack of growth of *T. acidophilus* on FeSO₄, though these bacteria actively oxidized this substrate in cell suspensions; more work, however, is required to elucidate these considerations.

The chemical characteristics of naturally occurring uranium compounds suggest that uranium in natural waters may persist as a mobile species by the formation of colloids and strong complexes (Martin et al., 1978; Li et al., 1980). The present data indicate that acidophilic organisms contribute to uranium mobilization both by direct oxidation and by ferric iron-mediated oxidation of uranous compounds. The present understanding of uranium metabolism is primarily limited to the characterization of uranium uptake systems in various organisms. Very few microbes are known that can either reduce or oxidize uranium compounds in a respiratory fashion (Taylor, 1979). The present study particularly highlights that, in the bacterial leaching of uranium ores, several concurrently-functioning mechanisms contribute to uranium solubilization: namely, the production of acidic ferric salts and their regeneration, as well as the direct bacterial oxidation of U from rock material. In
leach liquors, iron is abundantly present; but the accessibility of bacteria, iron compounds, and oxygen to uranium minerals in the ore material determines both the kinetics and the rate-limiting reactions. Thus, although ferric iron is the primary oxidizing agent, the bacteria, when sorbed to the material such as uraninite, have a virtually unlimited contact time and may directly solubilize the mineral in the absence of suitable iron compounds.
CHAPTER III
URANOUS ION OXIDATION AND CARBON DIOXIDE FIXATION BY

**THIOBACILLUS FERROOXIDANS**

The stoichiometric oxidation of uranous- to uranyl-uranium by *Thiobacillus ferrooxidans* is demonstrated. Fixation of $^{14}\text{CO}_2$ and the effect of inhibitors demonstrate that energy is conserved during the oxidation and used for energy dependent "reverse electron flow" and carbon dioxide fixation.

Introduction

*Thiobacillus ferrooxidans* is an acidophilic chemolithothrophic capable of using inorganic iron compounds for energy:

\[
4\text{Fe}^{2+} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Fe}^{3+} + 2\text{H}_2\text{O}
\]

\[\Delta G_{30^\circ\text{C}} = -38.0 \text{kJ} \cdot \text{mol}^{-1}\]

The energy thus derived is utilized to drive the reverse electron flow (Aleem et al., 1963) and the fixation of carbon dioxide (Gale and Beck, 1967). The oxidation of cuprous copper and stannous tin...
has also been demonstrated with washed cell suspensions of \textit{T. ferrooxidans} (Nielsen and Beck, 1972; Lewis and Miller, 1977) but it is not known whether these oxidations are coupled with the phosphorylation system in this bacterium. Studies with Cu\textsuperscript+ and Sn\textsuperscript{2+} are complicated because both ions are susceptible to chemical oxidation by oxygen in acid solutions.

Similarly, there is now evidence that uranous compounds oxidized by \textit{T. ferrooxidans} (Ivarson, 1980; Soljanto and Tuovinen, 1980; DiSpirito and Tuovinen, 1981). The role of Fe\textsuperscript{3+}/Fe\textsuperscript{2+} in cyclically mediating the oxidation of U(IV) has been recognized as one mechanism (Tuovinen and Kelly, 1974d):

\[
\text{UO}_2^+ + \text{Fe}^{3+} \rightarrow \text{UO}_2^{2+} + 2\text{Fe}^{2+}
\]

Results have also been presented for the direct biological oxidation of tetravalent uranium (Soljanto and Tuovinen, 1980; DiSpirito and Tuovinen, 1981):

\[
\text{UO}_2 + \frac{1}{2}\text{O}_2 + 2\text{H}^+ \rightarrow \text{UO}_2^{2+} + \text{H}_2\text{O}
\]

Uranous oxide (UO\textsubscript{2}), a general formula of natural uraninite, is not readily soluble in acid solution.
without accompanying oxidation to uranyl ion by $O_2$ and/or $Fe^{3+}$. The tetravalent uranium in dilute sulfuric acid as uranous sulfate is soluble essentially as $U^{4+}$ and the oxidation of soluble uranous sulfate has also been reported (DiSpirito and Tuovinen, 1981).

uranous ion is oxidized by *T. ferrooxidans* with a stoichiometry of $2U^{4+} : 1 O_2$ as will be shown in the present paper:

$$2U^{4+} + O_2 + 4H^+ \rightarrow 2U^{6+} + 2H_2O$$

The hexavalent uranium occurs at pH 2.5 as uranyl ion:

$$2U^{6+} + 4H_2O \rightarrow 2UO_2^{2+} + 8H^+$$

Therefore, the net reaction of oxidation should be written as follows:

$$2U^{4+} + O_2 + 2H_2O \rightarrow 2UO_2^{2+} + 4H^+$$

($\Delta G_{30^\circ C} = -130.4 \text{ kJ mol}^{-1}$)

It should be noted that the net reaction does not allow for the requirement for hydrogen ions in the reduction of $O_2$ to $H_2O$. For the biological system
the first reaction of $U^{4+}$-oxidation applies but the intermediate $U^{6+}$ is only hypothetical because of the formation of uranyl ion $UO_2^{2+}$.

In previous studies both the oxygen uptake and heat evolution were monitored during biological oxidation of uranous compounds (Ivarson, 1980; Soljanto Tuovinen, 1980; DiSpirito and Tuovinen, 1981). In the present work, oxygen uptake was also determined but additionally, carbon dioxide fixation was used as an indication of energy transduction involved in the oxidation. Ferrous iron was used in parallel studies as a reference substrate for comparison and for modeling the electron transport scheme for the two substrates in separate systems.

Materials and Methods

Bacteria. *Thiobacillus ferrooxidans* strain TFI-35 (Martin et al., 1981) was grown in a mineral salts solution supplemented with 120 mM ferrous sulfate at pH 1.5 (Tuovinen and Kelly, 1973) in 20 l carboys under forced aeration. The bacteria at the late logarithmic growth phase were harvested by centrifugation
at 13,200 x g for 20 min at 4°C. Insoluble compounds were removed by three successive centrifugations at 1,000 x g for 10 min at 4°C. The cells were washed three times with 0.11 N H$_2$SO$_4$ by centrifuging at 13,200 x g for 20 min at 4°C. The washed cell suspensions in 0.11 N H$_2$SO$_4$ were standardized by protein determinations (Lowry et al., 1951).

**Chemicals and reagents.** Uranous sulfate stock was prepared by the electrolytic reduction under nitrogen of 53.75 mM uranyl sulfate in 100 mM H$_2$SO$_4$. The electrolysis was carried out at the Canada Centre for Mineral and Energy Technology (Ottawa, Ontario) and the uranous sulfate stock solution was a gift from Dr. Marvin Silver.

Sodium azide and potassium cyanide were used in aqueous stock solutions. Other inhibitors were dissolved in ethanol. TTFA (thenoyl trifluoroacetone), rotenone, quinacrine, and HOQNO (2-heptyl-4-hydroxy-quinoline-N-oxide) were purchased from Sigma Chemical Co. 8-HQ (8-hydroxy-quinoline) was obtained from Matheson, Coleman & Bell.
Oxygen uptake. The oxygen uptake activity of washed cell suspensions of *T. ferrooxidans* at pH 1.5 was determined with a Clark oxygen electrode and a Warburg respirometer maintained at a constant temperature of 30°C. Both chemical and bacterial controls were routinely included in the experiments to ensure the tetravalent state of the uranous sulfate stock solution. The Clark oxygen electrode was calibrated as previously described (DiSpirito and Tuovinen, 1981). The values obtained from the calibrations were also checked with the Warburg respirometer. In the inhibitor studies the cells were preincubated for 5 min at 30°C with the inhibitor before the reaction was started by the addition of the substrate. The Clark oxygen electrode was used in the inhibitor studies and the relative rates were compared on a percentage basis. The Warburg respirometer was used in the determination of the stoichiometry of uranium and iron oxidation.

Carbon dioxide fixation. Assimilation of $^{14}$CO$_2$ was tested in sealed 6 or 125 ml vials at 23°C. The reaction mixture contained (per ml mineral salts
solution): washed cells, 1 mg protein; 0-10 μmol Fe^{2+} or 0-5 μmol Cu^{2+}, and 0.27 μmol \((^{14}\text{C})\text{NaHCO}_3\) (5 μCi).

For inhibitor studies, the bacteria were preincubated with the inhibitor for 5 min before the reaction was started by the addition of the substrate. Control experiments with ethanol (up to 80 μl per ml) indicated that the solvent itself did not influence the oxygen uptake and carbon dioxide fixation activity of *T. ferrooxidans*.

Aliquots (250 μl) of the reaction mixture were removed and filtered through a 0.45 μm cellulose nitrate membrane filter (Micro Filtration Systems). Filters were dried in a vacuum oven and immersed in 9 ml of Preblend 2a70 scintillation cocktail (Research Products International) in 20 ml vials. The radioactivity was counted in a Beckman LS-355 liquid scintillation spectrometer.

**Rusticyanin.** Washed cells in the mineral salts solution (3 g wet wt./15 ml) were passed through a French Press six times at 140 MPa. The homogenate was centrifuged at a high speed (144,000 x g) for 2 h at 4°C.
Rusticyanin in the supernatant fraction ($S_{144}$) was incubated for 5 min in the presence of 10 µmol of either Fe$^{2+}$, Fe$^{3+}$, or UO$_2^{2+}$ before scanning of the absorption spectrum (450-700 nm) of the sample (1 ml) with a Bausch & Lomb Spectronic 2000 spectrophotometer. Rusticyanin in the oxidized form has a broad absorption peak around the 590 nm range owing to the blue color of copper in the molecule (Cobley and Haddock, 1975). The blue color is abolished upon the reduction of rusticyanin by reducing agents such as Fe$^{2+}$ (Cobley and Haddock, 1975).

Results

Stoichiometry and oxygen uptake. The results of the manometric studies of ferrous iron oxidation by *Thiobacillus ferrooxidans* are presented in Fig. 3.1. The oxygen uptake coupled with ferrous iron showed a stoichiometry of 3.7 Fe(II)/$\text{O}_2$ which was 92.5% of the theoretical value of 4Fe(II)/$\text{O}_2$. The uranous ion oxidation was measured at concentrations of up to 15 mM (Fig. 3.2) and indicated a stoichiometry of 2.15 U(IV)/$\text{O}_2$ which was 107% of the theoretical value.
Fig. 3.1. Time course of iron oxidation by *Thiobacillus ferrooxidans* at pH 1.5. The reaction mixtures contained washed cells (4 mg protein) and either 0 µmol (□), 40 µmol (▲), 100 µmol (△), 200 µmol (●), or 300 µmol (○) ferrous iron in a total volume of 4 ml.

Fig. 3.2. Time course of uranium oxidation by *T. ferrooxidans* at pH 1.5. The reaction mixtures contained washed cells (4 mg protein) and either 0 µmol (□), 10 µmol (▲), 20 µmol (△), 40 µmol (●), or 60 µmol (○) of uranous ion in a total volume of 4 ml.
of $2\text{U(IV)}/10_2$. The rate of iron oxidation was linear whereas the uranium ion oxidation proceeded at phases of slightly different rates at each test concentration (Fig. 3.2). The rate of chemical oxidation of iron was negligible and that of uranium was less than 0.6% of the biological oxidation.

**Carbon dioxide fixation.** Carbon dioxide fixation associated with iron and uranium oxidation is presented in Fig. 3.3 and 3.4, respectively. On an equimolar substrate basis twice as much $^{14}\text{C}$ was fixed during uranium oxidation as with iron. This conforms with the number of electrons transferred during the oxidation of these substrates ($2\text{e}^- \text{from } \text{U}^{4+}; 1\text{e}^- \text{from } \text{Fe}^{2+}$)

Table 3.1 indicates the efficiency of carbon dioxide fixation coupled with the substrate oxidation. The relative efficiencies per electron pair were within the same range for both uranium (10.85%) and iron (11.44%). These efficiencies were calculated with the assumption that $2\text{Fe}^{2+}$ were required for 1 ATP ($2\text{Fe(II)} \rightarrow 2\text{Fe(III)} + 2\text{e}^-$) while $1\text{U}^{4+}$ was required for 1 ATP in the electron transport phosphorylation ($\text{U(IV)} \rightarrow \text{U(VI)} + 2\text{e}^-$). The reverse
Fig. 3.3. Time course of carbon dioxide fixation coupled to iron oxidation by *T. ferrooxidans* at pH 1.25. O, 5 mM Fe$^{2+}$; □ 10 mM Fe$^{2+}$; △ 5 mM Fe$^{2+}$ and 2.5 mM KCN; ▲, 10 mM Fe$^{2+}$ and 2.5 mM KCN.

Fig. 3.4. Time course of carbon dioxide fixation coupled to uranium oxidation by *T. ferrooxidans* at pH 1.5. O, 2.5 mM U$^{4+}$; □, 5.0 mM U$^{4+}$; △ 2.5 mM U$^{4+}$ and 2.5 mM KCN; ▲ 5.0 mM U$^{4+}$ and 2.5 mM KCN.
electron flow was assumed to expend 2ATP for each NAD\(^+\) reduced and 80% of the available ATP balance was assumed to be used for carbon dioxide fixation. If the efficiency of CO\(_2\) assimilation was based on the free energy changes of the Fe(II)/Fe(III) and U(IV)/U(VI) couples, energy from the ferrous iron oxidation is utilized more efficiently. However, since both substrates are probably coupled at the cytochrome \(_c\) level, only one ATP can be formed during the down-hill electron flow and the greater free energy change of U(IV)/U(VI) couple cannot be utilized.

**Electron transport inhibitors.** The effect of electron transport inhibitors on oxygen uptake and carbon dioxide fixation by \(T.\)ferrooxidans is present and in Table 3.2 for ferrous iron oxidation. The oxygen uptake was relatively insensitive to HOQNO, rotenone, quinacrine and 8-HQ suggesting entry of electrons at the cytochrome \(_c\) level. The relatively strong inhibition by TTFA of both oxygen uptake and carbon dioxide fixation may indicate an additional component of electron transfer between rusticyanin and cytochrome \(_c\), labeled \(X\) in Figure 3.6. The general pattern of inhibition of carbon dioxide fixation indicates the
Table 3.1. Assimilation of carbon dioxide by *Thiobacillus ferrooxidans*, oxidation of uranous uranium and ferrous iron at pH 1.5.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (nmol)</th>
<th>CO₂ Assimilated (nmol)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous Iron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.96</td>
<td>10.85</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>4.46</td>
<td>10.03</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>11.07</td>
<td>12.45</td>
<td></td>
</tr>
<tr>
<td>3000</td>
<td>16.59</td>
<td>12.44</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>11.44 ± 1.11</td>
</tr>
<tr>
<td>Uranous Uranium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.91</td>
<td>10.29</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>5.32</td>
<td>11.96</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>9.16</td>
<td>10.31</td>
<td></td>
</tr>
<tr>
<td>1500</td>
<td>13.03</td>
<td>9.77</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>10.58 ± 0.95</td>
</tr>
</tbody>
</table>

*The relative efficiency of CO₂ fixation decreases at higher substrate concentrations as also evident by comparison with data given in Fig. 3.3 and 3.4.*
Table 3.2 Effect of electron transport inhibitors on the oxygen uptake and carbon dioxide fixation during iron oxidation by T. ferrooxidans.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>% Inhibition/Stimulation&lt;sup&gt;*&lt;/sup&gt;</th>
<th>O₂ Uptake</th>
<th>¹¹C₂O₂ fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>50 µM</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 µM</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>13</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Quinacrine</td>
<td>10 µM</td>
<td>1</td>
<td>31&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>37&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>4</td>
<td>26&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>TTF A</td>
<td>250 µM</td>
<td>19</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 µM</td>
<td>42</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>92</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>S-HQ</td>
<td>1.0 mM</td>
<td>40</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 mM</td>
<td>22</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>HQQNO</td>
<td>25 µM</td>
<td>4</td>
<td>5&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>15</td>
<td>7&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>73</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>KCN</td>
<td>100 µM</td>
<td>98</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 µM</td>
<td>99</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>99</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>NaN₃</td>
<td>500 µM</td>
<td>99</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 mM</td>
<td>100</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>
involvement of cytochrome b, quinones, flavoprotein, and possible an iron-sulphur protein in the reverse electron flow.

Table 3.3 demonstrates the effect of electron transport inhibitors on the oxygen uptake and carbon dioxide fixation coupled with uranous oxidation. The oxygen uptake was enhanced by the inhibitors with the exception of the terminal inhibitors. The general pattern of inhibition of carbon dioxide fixation was similar for both U^{IV} and Fe^{II} but the inhibitory concentrations were higher for uranous ion oxidation. The uncoupling effects were promoted in the presence of uranous ion. Previously uranyl ion has been shown to act as an uncoupling agent on ferrous iron oxidation (Tuovinen and Kelly, 1974b) and a similar additive uncoupling effect may explain the pattern of inhibition in the present work.

Rusticyanin. The oxidation of rusticyanin in the S_{144} fraction by ferric iron (Fig. 3.5) resulted in an increase in the absorption around the 590 nm range in accordance with its known absorption spectrum.
Table 3.3. Effect of electron transport inhibitors on the oxygen uptake and carbon dioxide fixation during uranium oxidation by *T. ferrooxidans*.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>% Inhibition/Stimulation</th>
<th>( \text{O}_2 ) Uptake</th>
<th>( \text{CO}_2 ) Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>50 ( \mu \text{M} )</td>
<td>14*</td>
<td>25*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 ( \mu \text{M} )</td>
<td>26*</td>
<td>30*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 ( \mu \text{M} )</td>
<td>85*</td>
<td>73*</td>
<td></td>
</tr>
<tr>
<td>Quinacrine</td>
<td>10 ( \mu \text{M} )</td>
<td>69*</td>
<td>51*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 ( \mu \text{M} )</td>
<td>57*</td>
<td>39*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 ( \mu \text{M} )</td>
<td>83*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>TFFA</td>
<td>250 ( \mu \text{M} )</td>
<td>101*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 ( \mu \text{M} )</td>
<td>101*</td>
<td>99*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ( \text{mM} )</td>
<td>88*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>8-HQ</td>
<td>1.0 ( \text{mM} )</td>
<td>97*</td>
<td>33*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 ( \text{mM} )</td>
<td>10*</td>
<td>99*</td>
<td></td>
</tr>
<tr>
<td>NOQNO</td>
<td>25 ( \mu \text{M} )</td>
<td>82*</td>
<td>59*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 ( \mu \text{M} )</td>
<td>68*</td>
<td>73*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 ( \mu \text{M} )</td>
<td>97*</td>
<td>99*</td>
<td></td>
</tr>
<tr>
<td>KCN</td>
<td>100 ( \mu \text{M} )</td>
<td>49*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 ( \mu \text{M} )</td>
<td>70*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 ( \text{mM} )</td>
<td>100</td>
<td>99*</td>
<td></td>
</tr>
<tr>
<td>NaN3</td>
<td>500 ( \mu \text{M} )</td>
<td>50*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 ( \text{mM} )</td>
<td>69*</td>
<td>99*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0 ( \text{mM} )</td>
<td>77*</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0 ( \text{mM} )</td>
<td>100</td>
<td>100*</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.5. Oxidation of rusticyanin by Fe$^{3+}$ and UO$_2^{2+}$ in the S$_{44}$ fraction of cell extract. A, cell extract plus Fe$^{3+}$; B, cell extract plus UO$_2^{2+}$; C, cell extract; D, cell extract plus Fe$^{2+}$. 
(Cobley and Haddock, 1975). The 590 nm absorption was abolished due to the reduction of rusticyanin upon the addition of \( \text{Fe}^{2+} \). Fig. 3.5 also demonstrates the oxidation of rusticyanin following addition of \( \text{UO}_2^{2+} \) in the reaction mixture, thereby suggesting that rusticyanin can be involved as an electron carries with both uranium and iron. The reduction of rusticyanin by uranous ion could not be demonstrated since uranous ion even at 2 \( \mu \text{mol} \cdot \text{ml}^{-1} \) strongly absorbs in the 400-680 nm range.

**Discussion**

The present results demonstrate the ability of *Thiobacillus ferrooxidans* to oxidize reduced uranium and that metabolic energy is derived from this oxidation. Distinct phases in the rate of uranous ion oxidation were observed during the time course but the significance of this phase effect can not be characterized at the present time.

The coupling of uranous ion oxidation to carbon dioxide fixation was also demonstrated in the present work. Per electron pair, the efficiency of \( ^{4+} \text{U} \)-oxidation was 10.58 ± 0.95\% compared with 11.44 ± 1.11\%
for Fe$^{2+}$-oxidation. The similarity in the efficiency of CO$_2$-fixation between these two substrates, based on 2 e$^-$ transfers, indicates a similar electron transport system and coupling. The molar ratio of substrate oxidized to CO$_2$ fixed was 198.1 to 1 (Fe$^{2+}$/CO$_2$) and 107.1 to 1 (U$^{4+}$/CO$_2$) in the present work. The estimated ratio for iron is close to the value of 182 given by Beck (1969) (2.2 CO$_2$/100 O$_2$); for uranyl ion no previous values are available.

Rusticyanin has been shown to be the initial electron acceptor for ferrous iron oxidation by T. ferrooxidans (Ingledew et al., 1977; Cox and Boxer, 1978). The standard assay for rusticyanin is based on the reversal of the reaction; i.e., the oxidation of rusticyanin by ferric iron because in the reduced form it lacks an absorption spectrum in the 590 nm range. It was demonstrated qualitatively that both ferric iron and uranyl ion were able to oxidize rusticyanin. Thus, rusticyanin is likely to also be involved in the uranous ion oxidation. The absorbancy of uranous ion in the 400-680 nm range does not allow the demonstration of rusticyanin reduction nor the
characterization of the cytochrome spectra associated with $U^{4+}$-oxidation. The pattern of inhibition by electron transport inhibitors indicated that the reverse electron flow operated during iron and uranium oxidation. The inhibitors indicated the involvement of cytochrome b, quinones, flavoproteins and possibility an iron sulfur protein in the reverse electron flow, but the iron-sulfur component needs to be confirmed. The relatively strong inhibition by TTFA of the oxygen uptake during iron oxidation may be indicative of an additional electron acceptor between rusticyanin and cytochrome c. Alternatively, TTFA is an inhibitor of either rusticyanin or cytochrome c but this is unlikely in view of previous findings (King and Drew, 1975). The inhibitors (except $NaN_3$ and KCN) showed pronounced uncoupling effects with uranous ion as the substrate and this may be a consequence of the slightly promoting effect of uranium itself. However, uranium alone at the concentrations tested did not appear to uncouple the $U^{4+}$ oxidation from carbon dioxide fixation since the relative efficiency was in the same range as that for iron. Based on the effects of inhibitors on the oxygen uptake and carbon dioxide
fixation, an electron transport scheme is presented for both ferrous iron and uranous ion in Fig. 3.6.

Based on the similarity between iron and uranium in the efficiencies of CO₂ fixation another possible mechanism for uranous ion oxidation involves the direct reduction of membrane-bound ferric iron by uranous ion. Thermodynamically the electron transfer from uranous ion to ferric iron is feasible (U(IV)/U(VI) = +334 mV; Fe(II)/Fe(III) = +770 mV). However, the rusticyanin data illustrate that uranyl ion can directly oxidize rusticyanin although not as efficiently as ferric iron. In addition, the differences in the oxidation kinetics, as demonstrated in the accompanying paper (DiSpirito and Tüovinen, 1982) do not support this theory.
Fig. 3.6. Electron transport scheme of uranous ion and ferrous iron oxidation.
CHAPTER IV

KINETICS OF URANOUS ION AND FERROUS IRON OXIDATION BY
THIOBACILLUS FERROOXIDANS

Kinetic constants for the oxidation of uranous and ferrous ions Thiohacillus ferrooxidans were estimated. The kinetics indicate a direct biological mechanism for uranium oxidation. Uranous ion was a competitive inhibitor of ferrous iron oxidation. The complex interrelations of ferric, uranyl and uranous ion inhibition are considered.

Introduction

The catalytic involvement of Thiobacillus ferrooxidans in the solubilization of uranium from ore materials has been known for a couple of decades (Miller et al., 1963; Fisher, 1966; Harrison et al., 1966; Tuovinen and Kelly, 1974d). Unlike some transition metals commercially extracted in acid leaching operations, uranium does not usually occur as sulfides but as insoluble oxides.

Uranium minerals are often associated with metal sulfides such as iron, pyrite, pyrrhotite,
and chalcopyrite. In the case of metal sulfides, the production of sulfuric acid and soluble ferric iron is one mechanism by which *T. ferrooxidans* indirectly accelerates the leaching of uranium ores:

\[
2\text{FeS}_2 + 7.5\text{O}_2 + \text{H}_2\text{O} \rightarrow 4\text{SO}_4^{2-} + 2\text{H}^+ + 2\text{Fe}^{3+}
\]

\[
\text{UO}_2 + 2\text{Fe}^{3+} \rightarrow \text{UO}_2^{2+} + 2\text{Fe}^{2+}
\]

\[
2\text{Fe}^{2+} + 0.5\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{Fe}^{3+} + \text{H}_2\text{O}
\]

Sum: \[
2\text{FeS}_2 + 8\text{O}_2 + \text{UO}_2 \rightarrow 2\text{Fe}^{3+} + \text{UO}_2^{2+} + 4\text{SO}_4^{2-}
\]

This indirect biological mechanism of uranium leaching has been demonstrated by the requirement of iron for enhanced leaching rates (Harrison et al., 1966). Moreover, a dialysis cultivation technique (Tomizuka et al., 1976; Tomizuka and Yagisawa, 1978) demonstrated that a direct contact between *T. ferrooxidans* and the uranium ore was not required for the microbiological catalysis of the leaching in the presence of iron salts at acidic pH. In addition, the resistance to the metals solubilized, especially to U and Th, during the acid leaching of ores is a required characteristic if the bacteria are to be involved in the process.
As in the case of metal sulfides, the direct oxidation of reduced uranium compounds has been demonstrated (Ivarson, 1980; Soljanto and Tuovinen, 1980; DiSpirito and Tuovinen, 1981). In the accompanying paper it was proposed that U^{4+} is biologically oxidized by a mechanism somewhat similar to that of Fe^{2+} (DiSpirito and Tuovinen, 1982). The coupling of CO_{2}-assimilation to U(IV)-oxidation was also demonstrated. For the present paper a kinetic evaluation was undertaken to characterize both the uranous ion and ferrous iron oxidation in single and mixed substrate experiments. Since several strains of *Thiobacillus ferrooxidans* were included in the study, data are also presented on the resistance to actinides (UO_{2}^{2+}, Th^{4+}) of these strains.

**Materials and Methods**

*Thiobacillus ferrooxidans* strains TFI-1, TFI-4, TFI-7, TFI-9, TFI-10 and TFI-13 were originally isolated from the Agnew Lake uranium mine, Canada (Tuovinen et al., 1981). These strains as well as TFI-23, TFI-29, TFI-30 and TFI-35 (DiSpirito et al., 1981) were
grown at pH 1.5 and 2.5 in 120 mM FeSO₄ (Tuovinen and Kelly, 1973) in 10 ml culture tubes without shaking. The tubes containing 5 ml of the medium and 10% inocula were incubated at room temperatures for 14 days. The cultures were grown in the presence of 0 to 5 mM uranyl sulfate or 0 to 5 mM thorium chloride in increments of 0.1, 0.2 and 0.5 mM followed by 0.5 mM increases up to 5.0 mM. Thorium chloride (IGN Pharmaceuticals) was used because of its greater solubility in water than that of thorium sulfate. For preparing stock solutions, the formula was taken to be ThCl₄·7H₂O. Uranyl sulfate (UO₂SO₄·3H₂O) was obtained from Alfa Products. Strains TFI-1, TFI-29, and TFI-35 were also cultured with ferrous iron in 20 l carboys under forced aeration at pH 1.5. The biomass was harvested and washed as described in the accompanying paper (DiSpirito and Tuovinen, 1982). The washed cell suspensions were standardized by the protein determination (Lowry et al., 1951).

The rates of uranium and iron oxidation were determined at 30°C with a Clark oxygen electrode and a Warburg respirometer as previously described (DiSpirito and Tuovinen, 1982).
Table 4.1 illustrates the resistance of the 13 strains of *Thiothrix ferrooxidans* to uranyl and thorium salts. In general, the inhibitory concentration range was similar for both uranium and thorium. An increase of pH 1 somewhat relieved the inhibition caused by both metals, but the increase in resistance with pH varied from one strain to another. Uranium resistance of TFI-9 was not altered by the change from pH 1.5 to pH 2.5; nor was the thorium resistance of strains TFI-30, TFI-33, and TFI-35 influenced by the pH change.

Based on the uranium and thorium resistance at pH 1.5, strains TFI-1, TFI-29, and TFI-35 were chosen for kinetic evaluation U⁴⁺-oxidation by strain TFI-1, TFI-29, and TFI-35 were similar. However, the apparent $K_M$ of the strain TFI-1 showing the greatest resistance was lower by a factor of 4. The reason for the greater affinity of the uranium resistant strain TFI-1 for uranium cannot be characterized at this time.

The $V_{max}$ and apparent $K_M$ of ferrous iron oxidation were similar for strains TFI-1, TFI-29, and TFI-35
Table 4.1. Uranium and thorium resistance of *Thiobacillus ferrooxidans* test strains. The values represent the highest concentration of uranium and thorium at which growth occurred within 14 days.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH 1.5</th>
<th>pH 2.5</th>
<th>pH 1.5</th>
<th>pH 2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPI-1</td>
<td>3.5</td>
<td>3.0</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>TPI-4</td>
<td>0.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>TPI-7</td>
<td>0.5</td>
<td>1.5</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>TPI-9</td>
<td>1.0</td>
<td>1.0</td>
<td>1.5</td>
<td>3.5</td>
</tr>
<tr>
<td>TPI-10</td>
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<td>5.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>TPI-11</td>
<td>0.5</td>
<td>2.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>TPI-13</td>
<td>1.0</td>
<td>1.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>TPI-23</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>TPI-27</td>
<td>1.0</td>
<td>5.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>TPI-29</td>
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<td>5.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>TPI-30</td>
<td>0.5</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TPI-33</td>
<td>0.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TPI-35</td>
<td>0.2</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
(Table 4.2). The $V_{\text{max}}$ varied from 2.13 and 2.47 $\mu$mol Fe$^{2+}$ oxidized/min·mg protein and the apparent $K_M$ from 1.41 and 1.58 mM. These values are similar to the values reported elsewhere (Kelly and Jones, 1978) for a derivative of TFI-35.

Lineweaver-Burk plots of uranous ion and ferrous ion by TFI-35 are shown in Fig. 4.1 and 4.2. The rate of ferrous iron oxidation increased up to about 5.0 mM FeSO$_4$ and showed a $V_{\text{max}}$ and $K_M$ of 2.23 $\mu$mol Fe$^{2+}$ oxidized/min·mg protein and 1.51 mM Fe$^{2+}$, respectively (Fig. 4.1). The rate of uranous ion oxidation increased up to about 2.5 mM U$^{4+}$ (Fig. 4.2). Above 10 mM the rate of uranous ion oxidation declined. The $V_{\text{max}}$ of U$^{4+}$ oxidation was 0.077 $\mu$mol U$^{4+}$ oxidized/min·mg protein with an apparent $K_M$ of 0.132 mM U$^{4+}$.

Mixed substrate studies with varying ferrous iron concentrations and fixed uranous ion concentration indicated a predominantly competitive type of inhibition by uranium on iron oxidation (Fig. 4.3). Similar concentrations of UO$_2^{2+}$ had little or no effect on the rate of ferrous iron oxidation. At concentrations of uranous ion above 2.5 mM a mixed type of inhibition was observed (Table 4.3).
Table 4.2. Kinetic parameters of uranous ion and ferrous iron oxidation by *T. ferrooxidans* strains TFI-1, TFI-29 and TFI-35. $V_{\text{max}}$ is given in umol substrate oxidized/min·mg protein and $K_{\text{app}}^{\text{m}}$ as mM.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Uranium Oxidation</th>
<th>Iron Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_{\text{app}}^m$</td>
</tr>
<tr>
<td>TFI-1</td>
<td>0.056</td>
<td>0.031</td>
</tr>
<tr>
<td>TFI-29</td>
<td>0.069</td>
<td>0.126</td>
</tr>
<tr>
<td>TFI-35</td>
<td>0.077</td>
<td>0.132</td>
</tr>
</tbody>
</table>
Fig. 4.1 A, The effect of ferrous iron concentration on the rate of oxygen uptake by *Thiobacillus ferrooxidans* strain TFI-35.

Fig. 4.1 B, Lineweaver-Burk plot of 1A (\(v_o\) in \(\mu l O_2/min \cdot mg\) protein; \(Fe^{2+}\) in mM).
Fig. 4.2. A, The effect of uranous ion concentration on the rate of oxygen uptake by \textit{T. ferrooxidans} strain TFI-35.

Fig. 4.2. B, Lineweaver-Burk plot of 2A (\(v_0\) in \(\mu l \text{O}_2/\text{min·mg protein}; U^{4+}\) in mM).
Fig. 4.3. Lineweaver-Burk plot of iron oxidation by *T. ferrooxidans* strain TPI-35 with increasing concentration of uranous ion. The rate ($v_0$) is given in $\mu$l $O_2$/min·mg protein and [S] refers to the combined concentration (mM) of the two substrates. Symbols: 0 mM $U^{4+}$ ($\square$); 0.1 mM $U^{4+}$ ($\bigcirc$); 0.25 mM $U^{4+}$ ($\bullet$); 1.0 mM $U^{4+}$ ($\triangle$); 2.5 mM $U^{4+}$ ($\Delta$).
### Table 4.3. Kinetic parameters of ferrous iron oxidation by T. ferrooxidans strain TF-35 in the presence of varying concentrations of uranous ion. $V_{\text{max}}$ is given as mmol substrate oxidized/min·mg protein and $K_{M}^{\text{app}}$ as mM (Fe$^{2+}$ + U$^{4+}$).

<table>
<thead>
<tr>
<th>$U^{4+}$ (mM)</th>
<th>$1/V_{\text{max}}$</th>
<th>$V_{\text{max}}$</th>
<th>$K_{M}^{\text{app}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.45</td>
<td>2.23</td>
<td>1.51</td>
</tr>
<tr>
<td>0.10</td>
<td>0.53</td>
<td>1.90</td>
<td>1.65</td>
</tr>
<tr>
<td>0.25</td>
<td>0.53</td>
<td>1.88</td>
<td>2.39</td>
</tr>
<tr>
<td>1.00</td>
<td>0.52</td>
<td>1.94</td>
<td>3.86</td>
</tr>
<tr>
<td>2.50</td>
<td>0.61</td>
<td>1.64</td>
<td>6.53</td>
</tr>
<tr>
<td>5.00</td>
<td>1.72</td>
<td>0.58</td>
<td>7.84</td>
</tr>
<tr>
<td>10.0</td>
<td>3.22</td>
<td>0.31</td>
<td>9.78</td>
</tr>
</tbody>
</table>
By varying the uranous ion concentration with fixed concentrations of ferrous iron mixed effects were observed (Fig. 4.4). At low concentrations, the uranous ion either acted as an inhibitor or had little effect on the rate of oxygen uptake depending on the ferrous iron concentration. Accelerated rates were observed between 0.5 and 1.0 mM U⁴⁺ in all cases (Fig. 4.4). Above 1 mM U⁴⁺-concentration a saturation level was reached and a slight decline was observed. Thus, a valid kinetic analysis of this series was impossible due to the non-linearity of the oxygen uptake.

Figure 4.5 illustrates the rate of oxygen uptake coupled with the oxidation of 0.25 mM U⁴⁺ and low concentrations of Fe²⁺. No increase in the rate of oxygen uptake was observed at or below 0.5 μM Fe²⁺. A gradual increase in the rate was evident in the 0.5 μM-100 μM Fe²⁺ range followed with a rapid acceleration up to 720 μM Fe²⁺. The oxidation of ferrous iron alone was barely detectable below 100 μM concentrations. The cell suspension and uranous sulfate mixtures used in the oxygen uptake studies were found
Fig. 4.4. The rate of oxygen uptake at varying concentrations of uranous ion and ferrous iron; 0 mM Fe$^{2+}$ (□); 0.25 mM Fe$^{2+}$ (□); 0.5 mM Fe$^{2+}$ (○); 0.75 mM Fe$^{2+}$ (O); 1.0 mM Fe$^{2+}$ (▲); 5.0 mM Fe$^{2+}$ (△).
Fig. 4.5. The effect of low levels of Fe$^{2+}$ on the rate of oxygen uptake. The rates of oxygen uptake are shown for the oxidation of 0.25 mM U$^{4+}$ in the presence of Fe$^{2+}$ (•) and for the oxidation of Fe$^{2+}$ alone (○) on a logarithmic scale.
to contain approximately 50 nM contaminating iron. Thus, the results indicated that about 20-100 times the contaminating iron in the reaction mixture was required to increase the oxygen uptake rates. It follows, then, that the recycling of small quantities of iron (\(<0.5 \mu\text{M}\)) does not appear to be a major factor in the oxidation of $U^{4+}$ in this system.

**Discussion**

The results presented in this paper demonstrate that the rate ($V_{\text{max}}$) of uranous ion oxidation by *Thiobacillus ferrooxidans* is independent of the resistance to uranium and thorium. This observation is in contradiction to earlier observations that uranium resistant strains of *T. ferrooxidans* oxidize uranous ion at faster rates than do uranium sensitive strains (DiSpirito and Tuovinen, 1981). The difference between the two studies can be explained by the growth conditions used. Unlike in previous studies, these strains were cultured in the absence of uranyl sulfate for 6 months prior to the onset of the present study. It is known that the resistance of *T. ferrooxidans* to uranium decreases when the cells are cultured
in the absence of uranium (Tuovinen et al., 1981).

The apparent $K_M$ during uranous ion oxidation was similar in strains TFI-29 and TFI-35. In the strain previously showing the highest levels of resistance (TFI-1), however, the apparent $K_M$ was by a factor of 4 lower. The significance of the greater affinity of TFI-1 for uranous ion is not clear at this time.

The apparent $K_M$ and $V_{\text{max}}$ values of 0.132 mM $U^{4+}$ and 0.077 μmol $U^{4+}$ oxidized/min·mg protein, and of 1.51 mM $Fe^{2+}$ and 2.23 μmol Fe/min·mg protein indicate that there is a biological oxidation system for uranous ion in *T. ferrooxidans*. The relative difference in the apparent $K_M$ between TFI-1 and the other two strains, and the difference as contrasted with the apparent $K_M$ for ferrous iron oxidation as well as the lack of enhancement of the oxidation at low levels of $Fe^{2+}$, all suggest that the direct uranous ion oxidation is separate from the ferric-iron-catalyzed electron transfer between the two substrates. Upon maintaining uranyl sulfate in the medium to sustain
the resistance to uranium, different oxidation rates can be observed with different strains relative to their resistance to UO$_2^{2+}$ (DiSpirito and Tuovinen, 1981). In the present work, the resistance was allowed to elapse by prolonged subculture in the absence of uranyl sulfate and little difference was observed in the kinetic parameters with the exception of a low apparent $K_M$ for TFI-1. There is now evidence that uranium resistance is likely to involve a plasmid of approximately 13 megadaltons which can be detected in the population only in the presence of the selection pressure (Tuovinen et al., 1981). In general, the resistance to uranium of the test strains was in the same range as reported for TFI-35 (Tuovinen and Kelly, 1974a) and other T. ferrooxidans cultures elsewhere (Ebner and Schwartz, 1974; Barbini, 1977; Roy and Mishra, 1981). For thorium resistance, no previous data are available for comparison.

Uranous ion as a substrate is a competitive inhibitor of iron oxidation at concentrations at or below 2.5 mM U$^{4+}$. The competitive nature of inhibition by U$^{4+}$ of Fe$^{2+}$-oxidation may stem from the
difference between the oxidation rates of these two substrates. The lack of inhibition by uranyl ion at comparable concentrations supports this view. Above 2.5 mM U\textsuperscript{4+} a mixed type of inhibition is observed. Since uranous ion is oxidized concurrently with ferrous iron, due to its direct biological oxidation and to that mediated by Fe\textsuperscript{3+}, the concentration of uranyl ion (UO\textsubscript{2}\textsuperscript{+}) increases and makes the interpretation of the inhibition more complex. Oxygen uptake studies using varying concentrations of uranous ion with fixed concentrations of Fe\textsuperscript{2+} showed mixed effects. At high ferrous iron concentrations, uranous ion 0.5 mM decreases the rate of oxygen uptake while the uranous ion is concurrently oxidized both directly and via the Fe\textsuperscript{3+}-Fe\textsuperscript{2+} recycling. The rate of ferrous iron oxidation was determined to be approximately 30 times faster than the rate of uranous ion oxidation. At higher uranous ion concentrations the rates increased but these rates actually represented combined effects of Fe\textsuperscript{2+} and U\textsuperscript{4+}-oxidation, recycling of Fe\textsuperscript{3+}, and inhibition of iron oxidation due to U\textsuperscript{4+}, UO\textsubscript{2}\textsuperscript{+}, and Fe\textsuperscript{3+} in the mixture. It has been shown that ferric
iron and uranyl ion act as inhibitors of ferrous iron oxidation (Kelly and Jones, 1978) although the levels were higher than those in the present study. The interactions between these substrates and products make the separation of single events impossible in the present context.
CHAPTER V

DETERMINATION OF UBIQUINONE-8 IN THIOBACILLUS FERROOXIDANS

Isoprenoid quinones were extracted from T. ferrooxidans by three different methods and purified by thin layer chromatography. The quinone extracts were analyzed by thin layer chromatography, spectrometry, high performance liquid chromatography, and mass spectrometry. The only isoprenoid observed in T. ferrooxidans was ubiquinone-8.

Introduction

Thiobacillus ferrooxidans oxidizes reduced sulfur compounds, metal sulfides, Fe^{2+}, U^{4+}, and other reduced metal cations. In chemolithotrophic thiobacilli the electrons from reduced inorganic compounds are transported to oxygen and also utilized for the energy-dependent reduction of nicotinamide adenine dinucleotides (Aleem et al., 1963; Gale and Beck, 1967; Kelly, 1978). In T. ferrooxidans the coupling of inorganic substrate oxidations is indicated at
the cytochrome c level of the electron transport chain and cytochrome a\textsubscript{1} complex is involved as an electron carrier before the terminal reduction of O\textsubscript{2} (Blaylock and Nason, 1963; Ingledew and Cobley, 1980). In the reverse electron flow cytochrome b, ubiquinone, and flavoproteins are electron carriers before the reduction of NAD\textsuperscript{+} (Aleem et al., 1983; DiSpirito and Tuovinen, 1982).

In the present work an isoprenoid quinone was isolated and identified from T. ferrooxidans. As in T. thiooxidans (Cook and Umbreit, 1963); T. neapolitanus and T. thioparus (Sadler and Johnson, 1972), the component was identified as ubiquinone-8. Previously Dugan and Lundgren (1964) suggested the involvement of ubiquinone-6 in the electron transport system of T. ferrooxidans, but they indicated insufficient verification and several peaks in the chromatograph remained unidentified. Based on the data in the present work ubiquinone-8 was verified as the only isoprenoid quinone in T. ferrooxidans.
Materials and Methods

Preparation of cell homogenate. *T. ferrooxidans* TFI-35 was cultured with 120 mM ferrous sulfate and mineral salts at pH 1.5 in 20 l carboys under forced aeration (DiSpirito and Tuovinen, 1982). Bacteria were harvested from 100 l of the medium and washed with mineral salts (pH 2) as previously described (DiSpirito and Tuovinen, 1981). Cells (approx. 6 g wet weight) were resuspended in 15 ml of 0.2 M acetate buffer (pH 5.6) and either cryo-impacted (Smucker and Pfister, 1975) twice or passed through a French press six times at 140 MPa before the cell homogenate was sonicated for a total of 30 min in 3 min sets at 4°C.

Extraction of ubiquinones. After sonication, isoprenoid quinones were extracted by three different methods. **Method 1:** the cell slurry was centrifuged at 20,000 x g for 20 min at 4°C and the supernatant was filtered through a 0.45 µm membrane filter. The filtrate was centrifuged at 144,000 x g for 2 h at 4°C and the quinones were extracted from the membrane pellet (P_{144}) by the Redfearn method (Redfearn, 1967). **Method 2:** the P_{144} fraction was resus-
pended in 60 ml of 100% methanol and stirred for 30 min at 60°C. The quinone-containing methanol was extracted three times with 60 ml of n-hexane in a separatory funnel. The n-hexane phase was collected and extracted twice with water (90 ml) as a clean-up procedure. Approx. 5 g sodium sulfate was added to the n-hexane fraction to remove the remaining water. The n-hexane was flash evaporated to a yellow oil which was redissolved in 2 ml of spectral grade ethanol (Crane and Barr, 1971). Method 3: following the sonication step the cell homogenate (15 ml) was mixed with 100% acetone (180 ml) and shaken at 60 rev/min for 12 h at 4°C. The yellow supernatant was dried in a vacuum evaporator at 40°C and redissolved in n-hexane. As in Method 2, the quinone-containing n-hexane fraction was extracted with water, dried with sodium sulfate, and flash evaporated. The resulting oil was redissolved in 2 ml of ethanol. The full procedure of Method 3 is given as a flow scheme in Fig. 5.1

Thin layer chromatography. The ubiquinone extract in ethanol was further purified by preparative TLC (Silica Gel G) using 100% benzene as the solvent.
Approx. 6 g wet wt. bacteria in 15 ml of 0.2 M acetate buffer (pH 5.0)

Cryo-impact or French Press

Sonication (30 min)

Add 150 ml acetone (12 h, 4°C)

Centrifuge at 9000 x g (20 min, 4°C)

Filter the supernatant (Whatman No. 1)

Evaporate the filtrate under vacuum

Add 180 ml n-hexane

Add 90 ml water (repeat five times)

Remove residual water with ~ 5 g Na₂SO₄

Flash evaporate

Redissolve in a minimum volume (1-5 ml) of ethanol

Fig. 5.1. Flow scheme of the extraction of quinones by Method 2.
Quinones were detected by spraying with 0.5% Rhodamine B in ethanol and visualized as quenched regions under ultraviolet light. The silica gel in the quenched regions was scraped off the plate and extracted with a minimum volume of ethanol. To identify the isoprenoid chain length, ubiquinones were separated via reverse-phase TLC, using paraffin impregnated Silica Gel G with acetone-water (95 + 5) as the solvent system (Bolliger, 1965).

Spectroscopy. The characteristics of ubiquinone samples were confirmed by absorption spectra in the 240-440 nm range using a Bausch & Lomb Spectronic 2000 spectrophotometer. The oxidized and reduced spectra (240-340 nm) of purified ubiquinones were determined with a Cary 118 spectrometer adapted with a scatter apparatus. Spectra for reduced ubiquinones were determined after addition of a few crystals of sodium borohydride to the sample.

High performance liquid chromatography. HPLC was performed on an Altex 100A pumping system, equipped with a manual 20 µl loop injector. The column was an Altex Spherisorb ODS (4.6 x 250 mm; particle size
5 μm). The solvent system was 80/20 (vol/vol) methanol/isopropanol. The column effluent (flow rate 0.5 ml/min) was monitored at ambient temperature at 275 nm with a Hitachi 100-40 spectrometer adapted with an Altex flow cell.

Mass spectrometry. Quinone samples from T. ferrooxidans were purified by HPLC, collected manually, and dried under a stream of N₂. The crystals were analyzed on an Associated Electronic Industries MS9 solid probe mass spectrometer operated at 70 eV.

Ubiquinone standards. The purity of UQ-7 (Calbiochem-Behring Corporation), UQ-6 and UQ-10 (Sigma Chemical Company) was confirmed by HPLC. UQ-9 (Sigma Chemical Company) was found to be contaminated with UQ-7 and their separation was resolved by HPLC. UQ-8 was extract from 15 g of lyophilized Escherichia coli (Sigma Chemical Company; Crooke's strain) by a modification of the Crane and Barr (1971) method. The lyophilized bacteria were resuspended in 100% acetone, sonicated for 30 min at 4°C and shaken for 12 h at 60 rev/min at 4°C. Quinones from E. coli were extracted as described for T. ferrooxidans (Method 3).
Results

Isolation of ubiquinones. The problems listed by Redfearn (1967) in the isolation of ubiquinones from bacterial systems by Method 1 were also encountered in the present study. Although there was no indication of menaquinones in the isoprenoid extracts of *T. ferroxidans* (Methods 2 and 3), the presence of large amounts of material which absorbed in the same spectral region as ubiquinones made analysis impossible. Methods 2 and 3 both gave good yields of ubiquinones from *T. ferroxidans*. However, the elimination of the steps required for the isolation of cell membranes and the methanol extraction step makes Method 3 a simpler choice.

Spectral characterization. The absorption spectra of the quinone extract from *T. ferroxidans* are shown in Fig. 5.2. The sample (oxidized form) showed a typical broad absorption peak with a maximum at 274 nm. The addition of a few crystals of sodium borohydride to the extract shifted the absorption maximum to 287 nm and reduced the absorbance, both of which are characteristic of ubiquinones.
Fig. 5.2. The absorption spectra of the oxidized (-----) and sodium borohydride reduced (----) UQ-8 from *T. ferrooxidans*.
Reverse-phase thin layer chromatography. The quinones from *T. ferrooxidans* migrated as a single spot and the same distance as the UQ-8 reference sample (Table 5.1; Fig. 5.3). The UQ-8 spot became darker when an aliquot of the quinone extract of *T. ferrooxidans* was added to a mixture of ubiquinone standards (Fig. 5.3). The second spot observed in the UQ-9 standard was shown to be UQ-7 by HPLC analysis.

Characterization of quinones by high performance liquid chromatography. Figure 5.4 illustrates the HPLC analysis of the partially purified quinone extract of *T. ferrooxidans*. The retention time of the quinone extract in the HPLC column was the same as that of the UQ-8 standard (Table 5.1). When a mixture of UQ-6, UQ-7, UQ-8, UQ-9, and UQ-10 was spiked with the quinone sample from *T. ferrooxidans*, the UQ-8 peak increased while those of UQ-6, UQ-7, UQ-9, and UQ-10 decreased by dilution.

Analysis by mass spectrometry. Ubiquinone preparations from *T. ferrooxidans* purified by HPLC showed the characteristic m/e of 728 (M + 2)^+ (relative intensity 12) peak. The m/e of 727 (M + 1)^+ (relative
Table 5.1. $R_f$ values and HPLC retention times of the ubiquinones standards and the *Thiobacillus ferrooxidans* sample. The $R_f$ values were measured from paraffin impregnated Silica Gel G TLC plates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_f$</th>
<th>HPLC retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UQ-6</td>
<td>0.76</td>
<td>13.3</td>
</tr>
<tr>
<td>UQ-7</td>
<td>0.74</td>
<td>14.5</td>
</tr>
<tr>
<td>UQ-8</td>
<td>0.69</td>
<td>16.2</td>
</tr>
<tr>
<td>UQ-9</td>
<td>0.63</td>
<td>18.0</td>
</tr>
<tr>
<td>UQ-10</td>
<td>0.56</td>
<td>20.9</td>
</tr>
<tr>
<td><em>T. ferrooxidans</em></td>
<td>0.68</td>
<td>16.1</td>
</tr>
</tbody>
</table>
Fig. 5.3. Separation of ubiquinones on TLC plates. A: mixture of UQ-6, UQ-7, UQ-8, UQ-9, and UQ-10; B: the mixture of ubiquinones standards was spiked with the quinone extract of *T. ferrooxidans*. Tf: quinone extract of *T. ferrooxidans*. The individual quinone standards are also indicated.
Fig. 5.4. HPLC analysis of quinones from *T. ferrooxidans*. A: quinone extract from *T. ferrooxidans* (TPI-35); B: mixture of standards (---) and a mixture of standards and the quinone extract from *T. ferrooxidans* (-----).
intensity 7) and 726 (M)⁺ (relative intensity 4) were also detected. Several low molecular weight (<400) compounds with a relative intensity of 100 were observed as impurities in the sample.

Discussion

UQ-8 was the only isoprenoid quinone detected in T. ferrooxidans. This electron transport component has also been identified in other species of thiobacilli, viz. T. thiooxidans, T. neapolitanus, and T. thioparus (Cook and Unbreit, 1963; Sadler and Johnson, 1972; Adair, 1968). The previous tentative identification of UQ-6 in T. ferrooxidans (Dugan and Lundgren, 1964) probably resulted from analytical problems, whereas the UQ-8 was identified and verified by several methods in the present study and confirms the common occurrence of this quinone type in chemolithotrophic thiobacilli.
CHAPTER VI

ACCUMULATION AND CELLULAR DISTRIBUTION OF URANIUM IN
THIOBACILLUS FERROOXIDANS

The uptake and cellular distribution of UO$_2^{2+}$ was investigated in washed cell suspensions of Thiobacillus ferrooxidans. The uptake was dependent on the external concentration of uranium (0.01-10.0 mM) and was influenced by the pH of the reaction mixture, but not by 1 mM transition metals ions or by the previous growth history of the cells. Cells inactivated by either ultraviolet radiation or potassium cyanide accumulated about 40% more uranium than did viable cells especially at a high, toxic UO$_2^{2+}$ concentration. Most of the uranium was associated with the cell wall and membrane fractions and relatively little uranium was detected in the cytoplasmic, lipopolysaccharide, and periplasmic space fractions. Transmission electron micrographs of thin sections also indicated the localization of uranium in the cell wall and membrane of the bacteria exposed to uranium for 3 h. Ferrous iron, when present as an energy substrate, did not alter the distribution profile.
of uranium in the cell fractions. Cells poisoned with potassium cyanide were found to have an 8 to 11 fold increase in the cytoplasmic concentration.

Introduction

The projected increase in the use of nuclear energy has prompted studies in the use of microorganisms in the recovery of uranium from aqueous environments. The majority of the studies on the biosorption of uranium have been restricted to the uptake by freshwater or marine algae (Sakaguchi et al., 1978; Nakajima et al., 1981; Horikoshi et al., 1981; Sprey and Bochem, 1981), although the potential use of other microorganisms has also been considered (Shumate et al., 1980; Strandberg et al., 1981). The emphasis of these studies has been directed towards the investigation of the possibility of concentrating uranium from the low levels present in sea water. The results of these studies varied with the microorganisms and environmental conditions used. For example, uranium uptake by *Dunaliella salina* was proportional to the external uranium concentration, but the organism did not concentrate uranium beyond that present in the environment (Sprey
and Bochem, 1981). On the other hand, Horikoshi et al.,
(1981) screened 52 species of actinomycetes, bacteria,
yeasts, and fungi for the ability to accumulate
uranium. All microorganisms tested accumulated
uranium above the level of the reaction mixtures al­
though the concentration coefficients varied in each
species.

Unlike natural surfaces waters, effluents originat­
ing from mines contain high concentrations of metals
because of the leaching of exposed ore surfaces and
because the low pH permits high metal solubility.
Microorganisms found in acid effluents and leach liquors
are typically acidophilic and able to tolerate elevated
concentrations of many toxic metals. Tolerance of
metal ions by thiobacilli such as Thiobacillus fer­
rooxidans, which is commonly encountered in such
extreme environments, has prompted surveys of metal
ion concentrations toxic to these bacteria. Little
is known, however, about the accumulation of metals
into thiobacilli in acid solutions although this
would seem crucial to characterize in order to better
understand the high level of tolerance. The incorpora­
tion of silver, chromium, magnesium, and potassium
into *T. ferrooxidans* biomass was investigated by Norris and Kelly (1978) and Wong et al. (1982). Up to 4 μg Ag·mg⁻¹ dry weight bacteria accumulated in *T. ferrooxidans* during growth whereas substantially less chromium, magnesium, and potassium was associated with the cells (Norris and Kelly, 1978; Wong et al., 1982). Distribution of metal ions in cell fractions was not analyzed in these studies. Silver, as compared with transition metals, is a potent inhibitor of *T. ferrooxidans* and its uptake into *T. ferrooxidans* cells thus accords the toxicity. Sugio et al. (1981) found that over 85% of accumulated silver was associated with the cell wall and membrane fraction in *T. ferrooxidans*. For uranyl ion, which is an inhibitor of iron oxidation and carbon dioxide fixation, no comparable data are available, yet uranium is of considerable interest because of the involvement of *T. ferrooxidans* in the production of uranium leach liquors and its ability to utilize reduced uranium as an energy substrate (Tuovinen and Kelly, 1974a; 1974b; DiSpirito and Tuovinen, 1982a; 1982b).
For the present work, uranium accumulation into cell material of *Thiobacillus ferrooxidans* was investigated. Cellular distribution of uranium was also analyzed by determining uranium content in each cell fraction. Moreover, electron microscopic evidence is presented in support of the chemical analysis that uranium is largely accumulated in the cell envelope.

**Materials and Methods**

**Bacteria and Culture Conditions.** *Thiobacillus ferrooxidans* strain TFI-35 (DiSpirito and Tuovinen, 1982a) was cultured in 120 mM ferrous sulfate (Tuovinen and Kelly, 1973) at pH 1.5 in 20 l carboys under forced aeration for 4-5 days at room temperature. Tetra-thionate grown *T. ferrooxidans* (strain TFK-35) was derived from TFI-35 as described by Tuovinen and Kelly (1974d) and cultured in 10 l fermentors at room temperature. The cells were harvested and washed as previously described (DiSpirito and Tuovinen, 1981).

**Cellular Accumulation of Uranium.** Accumulation of uranium into intact cells was determined using uranyl
sulfate of natural isotopic composition (Alfa Products, Danvers, MA, USA). The incubations were carried out at 23°C at the pH range of 1.0 to 4.0 adjusted with sulfuric acid. The reaction mixture contained 1.0 mg protein ml⁻¹, 0.01, 0.1, 1.0, 2.5, 5.0, or 10.0 mM UO₂²⁺ with and without 1.0 mM each of Ni²⁺ (added as NiSO₄·6H₂O), Cu²⁺ (CuSO₄·5H₂O), Fe³⁺ (FeCl₃), Fe²⁺ (FeSO₄·7H₂O), Cr³⁺ (CrCl₃), Cd²⁺ (CdCl₂·2.5H₂O), Co²⁺ (CoCl₂·6H₂O), and Zn²⁺ (ZnSO₄·7H₂O). Except for the time course experiments, all samples were incubated for 3 h on a gyratory shaker. For determination of cell-associated uranium, bacteria were collected by filtration (0.45 μm membrane filters), rinsed with 0.1N H₂SO₄, and placed in 1 ml polyethylene snap-top vials for neutron activation analysis (NAA). The samples in vials were irradiated for 6-8 h at an average flux of 3.97·10¹¹ neutrons cm⁻¹ sec⁻¹. The gamma-ray spectra were detected with a Princeton Gamma-Tech Ge(Li) detector and a Canberra model 8180 multichannel analyzer. The spectra were analyzed with a Digital Equipment Corporation model PDP 11/05 minicomputer. The 209.73 gamma-ray from ²³⁹Np was
used for analysis for $^{238}\text{U}$ concentration. To determine the interference due to filters and biomass, standards were also analyzed with NAA. These standards included liquid samples of uranyl sulfate solutions with and without filters and cells as well as aliquots dried on the membrane filters before NAA. No significant difference was observed between the different types of standards and for routine purposes only liquid standards with known amounts of uranium were used. The retention of uranium in the filter material was also examined in each experiment and subtracted from the samples containing filtered cells.

Cell suspensions were standardized by protein determination (Lowry et al., 1951).

**Cell Accumulation and Analysis of Metals.** Nickel, cobalt, iron, cadmium, zinc, and copper uptake was determined at pH 1.5 at 23°C. The reaction mixture contained 1 mg protein·ml$^{-1}$, 1 mM each of the metal ion and either 1 or 10.0 mM $\text{UO}_2^{2+}$ in 0.11 N $\text{H}_2\text{SO}_4$. The cells
were incubated for 3 h on a gyratory shaker. For analysis of the metal uptake (except UO$_2^{2+}$) by T. ferrooxidans, 30 ml of the reaction mixture were centrifuged at 16,000 x g for 20 min at 4°C. The cell pellet was resuspended in 0.11 N H$_2$SO$_4$ and centrifuged again at 16,000 x g for 20 min. This washing procedure was repeated two additional times. The washed cell pellet was resuspended in 2 ml of concentrated fuming sulfuric acid and digested at 23°C for 12 h. The volume of the digested cell suspension was brought up to 10 ml with 0.11 N H$_2$SO$_4$ and the concentration of metal ion determined on a Perkin-Elmer 403 atomic absorption spectrometer.

Cell Fractionation. Cell suspensions of T. ferrooxidans were incubated in 10 mM UO$_2$SO$_4$ in a 0.11 N H$_2$SO$_4$ solution containing 1 mg protein·ml$^{-1}$, either 0 or 60 mM FeSO$_4$, and either 0 or 10 mM KCN. Following the incubation period the cells were harvested and washed as previously described.

The uranium treated cells were resuspended in 0.5 M sucrose and the lipopolysaccharide (LPS) layer and periplasmic space material were removed by the
method of Forsberg et al. (1970). The LPS layer and periplasmic material were separated by centrifugation at 144,000 x g for 4 h at 4°C. The sucrose-treated cells were passed three times through a French press at 140 MPa. The cell homogenate was separated from the remaining cells by filtration (0.45 um membrane filter). The filtrate was centrifuged at 20,000 x g for 40 min at 4°C to pellet the cell wall fraction. The cell wall fraction was resuspended in 0.11 N H₂SO₄ containing 0.1% Tween 100, vortexed for 3 to 5 min, and centrifuged at 20,000 x g for 40 min at 4°C. This washing procedure was repeated three times. The supernatant from the first 20,000 x g centrifugation was centrifuged at 144,000 x g for 4 h at 4°C to separate the cell membrane layer from the cytoplasmic fraction.

Electron Microscopy. Cells of *T. ferrooxidans* were treated with either 0 or 10 mM UO₂SO₄ and either 0 or 60 mM KCN and washed as previously described. The treated cells were fixed with glutaraldehyde and osmium tetroxide by the Luft method (1971). Thin sections were examined and photographed under a Zeiss model 9A electron microscope. No stains other than ruthenium red were used during the preparation for electron microscopy.
Results

Time Course of Uranium Uptake. The time course for uranium uptake by washed cells of *T. ferrooxidans* in 10 mM UO$_2^{2+}$ solution is presented in Table 1. An equilibrium was reached within the first hour of incubation. Viable cells could not be recovered from the reaction mixture following the 6 h incubation period.

Effect of External Uranium Concentration on Uranium Uptake. The uptake of uranium by iron (TFI-35) and tetrathionate (TFK-35) grown *T. ferrooxidans* was examined at several different test concentrations of uranium (Table 2). The uptake of uranium was proportional to the external concentration of uranium in TFI-35, TFK-35, and in dead cells of TFI-35. The uptake of uranium by TFI-35 cells was slightly higher than TFK-35. The addition of iron to the reaction mixture, however, did not increase the uptake of uranium (Table 3). The uptake of uranium by KCN-poisoned cells or by those inactivated by ultraviolet radiation showed a 10-40% increase in uranium uptake compared to cells initially live.
Table 6.1. Time course of uranium uptake by tetra-thionate grown *Thiobacillus ferrooxidans* incubated in the presence of 10 mM uranium.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell-associated uranium (μg·mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>39.73</td>
</tr>
<tr>
<td>1.0</td>
<td>70.14</td>
</tr>
<tr>
<td>3.0</td>
<td>66.15</td>
</tr>
<tr>
<td>6.0</td>
<td>75.36</td>
</tr>
<tr>
<td>12.0</td>
<td>86.43</td>
</tr>
<tr>
<td>24.0</td>
<td>73.76</td>
</tr>
</tbody>
</table>
Table 6.2. Uranium uptake (3 h incubation) by iron (TFI-35) and tetrathionate (TFK-35) grown \( T. \) ferrooxidans. The values are in \( \mu g \) U·mg protein\(^{-1} \).

<table>
<thead>
<tr>
<th>( \text{UO}_2^{2+} ) (mM)</th>
<th>TFK-35 Untreated Cells</th>
<th>TFI-35 UV-Treated Cells</th>
<th>KCN-treated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.66</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>0.10</td>
<td>0.73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>8.32</td>
<td>11.12</td>
<td>13.61</td>
</tr>
<tr>
<td>2.50</td>
<td>21.73</td>
<td>21.89</td>
<td>27.71</td>
</tr>
<tr>
<td>5.00</td>
<td>37.61</td>
<td>66.19</td>
<td>71.88</td>
</tr>
<tr>
<td>10.00</td>
<td>63.28</td>
<td>72.18</td>
<td>102.65</td>
</tr>
</tbody>
</table>

* -, not analyzed.
Cellular Distribution of Uranium. To determine the intracellular distribution of uranium accumulated in *T. ferrooxidans* the cells were fractionated as described in Materials and Methods. In cells incubated in the presence of 10 mM uranium, the major cell fractions of uranium accumulation were the cell wall and cell membrane (Table 3). On a percentage basis little uranium was detected in the LPS, periplasmic material, or cytoplasmic fractions. Cells inactivated by KCN showed an 8-fold increase in the amount of uranium in the cytoplasmic fraction. However, the difference in the total uptake by live and poisoned cells can be accounted for by the increased uranium content of the cytoplasmic fraction (Tables 2 and 3).

The addition of ferrous iron did not alter the amount or distribution of uranium. Thus, the exclusion of uranium from the cytoplasmic fraction may be a passive mechanism whereby an intact cell membrane provides a diffusion barrier. An alternate explanation is that the cells contain adequate intracellular energy reserves to exclude UO$_2^{2+}$ for 3 h from the cells by for example, maintaining membrane energization. At the present
Table 6.3. Distribution of uranium in cell fractions of TFI-35. After 3 h incubation in the presence of 10 mM uranium, cells were fractionated and the values given as µg U are adjusted to represent the incorporation in each cell fraction obtained from 1 mg cell protein.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\text{UO}_2^{2+}$-Treated</th>
<th>$\text{UO}_2^{2+}$ + 10 mM KCN Treated</th>
<th>$\text{UO}_2^{2+}$ + 60 mM Fe$^{2+}$ Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cells</td>
<td>74.60</td>
<td>110.80</td>
<td>71.35</td>
</tr>
<tr>
<td>Cells minus LPS</td>
<td>68.74</td>
<td>98.30</td>
<td>65.30</td>
</tr>
<tr>
<td>LPS</td>
<td>0.21</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>Periplasmic Space</td>
<td>0.66</td>
<td>0.97</td>
<td>0.10</td>
</tr>
<tr>
<td>Cell Wall</td>
<td>21.03</td>
<td>29.60</td>
<td>27.59</td>
</tr>
<tr>
<td>Cell Membrane</td>
<td>13.03</td>
<td>16.41</td>
<td>14.18</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2.92</td>
<td>23.94</td>
<td>2.09</td>
</tr>
</tbody>
</table>
time, there is no known biological transport system specific for UO\textsubscript{2}\textsuperscript{2+}. Biological systems are not known to have a requirement for uranium and therefore, the likelihood of a specific transport system for this metal is remote.

**Effect of pH on Uranium Uptake.** The effect of pH on the uptake of uranium by *T. ferrooxidans* (TFK-35) was investigated at pH 1-4 (Table 4), which is the broad range suitable for growth. The decline in uranium uptake at or above pH 2 may be the result of the lessened solubility of uranium at these pH values. However, the similarity in the uptake of uranium at pH 2-4 indicates that the solubility of uranium was not the only factor involved in this pH effect. In addition, the relationship between the extracellular uranium concentration and uranium uptake by TFK-35 was not observed at or above pH 3.0. The elevated uranium content of cell mass at pH 1 and 1.5 agrees with previous demonstrations that uranium toxicity is enhanced below pH 2 (Tuovinen and Kelly, 1974c; DiSpirito and Tuovinen, 1982c).

**Effect of Transition Metals on Uranium Uptake.**
Table 6.4. Effect of pH on the uranium uptake by tetrathionate grown *T.* ferrooxidans incubated in 5.0 or 10.0 mM uranium.

<table>
<thead>
<tr>
<th>pH</th>
<th>5.0 mM UO$_2^{2+}$ (µg U/mg protein)</th>
<th>10.0 mM UO$_2^{2+}$ (µg U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>50.50</td>
<td>62.11</td>
</tr>
<tr>
<td>1.5</td>
<td>37.61*</td>
<td>63.28*</td>
</tr>
<tr>
<td>2.0</td>
<td>24.45</td>
<td>35.01</td>
</tr>
<tr>
<td>3.0</td>
<td>35.47</td>
<td>27.83</td>
</tr>
<tr>
<td>4.0</td>
<td>36.75</td>
<td>33.26</td>
</tr>
</tbody>
</table>

*Values taken from table 2.*
The presence of 1 mM concentrations of Fe$^{2+}$, Fe$^{3+}$, Ni$^{2+}$, Co$^{3+}$, Zn$^{2+}$, or Cd$^{3+}$ did not significantly alter the uptake of equimolar concentrations of uranium by TFK-35 (Table 5). In addition, the presence of uranium had no effect on the uptake of the metal cations listed above.

Except for uranium, nickel and cobalt were incorporated into the cells more than the other metal ions tested (Table 5). The amount of cell associated chromium observed in this study was approximately 1000 times higher than that observed by Wong et al. (1982). However, the values presented by Wong et al. (1982) are erroneous and should be corrected by multiplying by a factor of 1000 (M. Silver, personal communication, 1983).

Electron Microscopic Examination of Uranium Uptake. Uranium, being the heaviest metal used as an electron stain, is ideally suited to examination by electron microscopy. Figures 1-2 show ruthenium red fixed, thin sectioned (approximately 70 nm) and unstained cells incubated in the presence of 0 or 10.0 mM uranium and 0 or 10.0 mM KCN for 3 h. The distribution of uranium in cells could be visualized as electron dense areas and indicated accumulation on the cell wall. In cells treated
Table 6.5. Uptake of metals by *T. ferrooxidans* TFK-35.

The cells were incubated in the presence of 1 mM metals for 3 h.

<table>
<thead>
<tr>
<th>Addition (1 mM each)</th>
<th>Cell-associated ( \text{UO}_2^{2+} ) (µg·mg protein(^{-1}))</th>
<th>Cell-associated metal(^*) (µg·mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{UO}_2^{2+} )</td>
<td>8.32</td>
<td>-</td>
</tr>
<tr>
<td>( \text{Fe}^{2+} )</td>
<td>-</td>
<td>0.45</td>
</tr>
<tr>
<td>( \text{Fe}^{2+} + \text{UO}_2^{2+} )</td>
<td>6.55</td>
<td>0.43</td>
</tr>
<tr>
<td>( \text{Fe}^{3+} )</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>( \text{Fe}^{3+} + \text{UO}_2^{2+} )</td>
<td>8.55</td>
<td>0.38</td>
</tr>
<tr>
<td>( \text{Ni}^{2+} )</td>
<td>-</td>
<td>3.82</td>
</tr>
<tr>
<td>( \text{Ni}^{2+} + \text{UO}_2^{2+} )</td>
<td>8.51</td>
<td>3.63</td>
</tr>
<tr>
<td>( \text{Cu}^{2+} )</td>
<td>-</td>
<td>&lt;0.10(^\circ)</td>
</tr>
<tr>
<td>( \text{Cu}^{2+} + \text{UO}_2^{2+} )</td>
<td>10.34</td>
<td>&lt;0.10(^\circ)</td>
</tr>
<tr>
<td>( \text{Co}^{2+} )</td>
<td>-</td>
<td>3.38</td>
</tr>
<tr>
<td>( \text{Co}^{2+} + \text{UO}_2^{2+} )</td>
<td>6.49</td>
<td>2.83</td>
</tr>
<tr>
<td>( \text{Zn}^{2+} )</td>
<td>-</td>
<td>0.11</td>
</tr>
<tr>
<td>( \text{Zn}^{2+} + \text{UO}_2^{2+} )</td>
<td>7.25</td>
<td>0.10</td>
</tr>
<tr>
<td>( \text{Cd}^{2+} )</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>( \text{Cd}^{2+} + \text{UO}_2^{2+} )</td>
<td>7.31</td>
<td>0.09</td>
</tr>
<tr>
<td>( \text{Cr}^{3+} )</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>( \text{Cr}^{3+} + \text{UO}_2^{2+} )</td>
<td>Nd(^a)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\(^*\)Listed metal ions other than \( \text{UO}_2^{2+} \).

\(^\circ\)Below the level of detection.

\(^a\)Nd, not determined.
Fig. 6.1. Electron micrographs of *T. ferrooxidans* control cells (not exposed to uranium).

Fig. 6.2. Electron micrographs of cells of *T. ferrooxidans* incubated in the presence of 10 mM uranium for 3 h.
with KCN the cytoplasmic region was more heavily stained as a result of exposure to uranium. In addition, the cells showed a similar staining pattern to that obtained using uranyl acetate as a stain for thin sections (Beveridge, 1978; Hayat, 1981). The electron dense areas in figures 2 and 4 were also the different cell wall fractions, the nuclear regions, and the carboxysomes.

Thick sections (approximately 240 nm) of the same samples used in figures 1-4 were also examined by scanning-transmission electron microscopy equipped with energy dispersive X-ray analysis (EDAX). Although the presence of uranium was readily detected in the energy spectrum obtained with EDAX (results not shown), the distribution of uranium within an individual cell could not be mapped. The small amount of uranium within a single cell and its even distribution throughout the cell wall fractions made mapping impossible because of limits in lower levels of detection.
Discussion

The rate of uranium uptake by *T. ferrooxidans* was similar to that observed in *Saccharomyces cerevisiae* and *Synechococcus elongatus*, but was much slower than *Actinomyces levoris*, *Streptomyces viridochromogens*, and *Pseudomonas aeruginosa* (Horikoshi et al., 1979; 1981; Strandberg et al., 1981). An equilibrium in uranium uptake is achieved by microorganisms such as *A. levoris* within the first few minutes while *T. ferrooxidans* requires at least 1 h exposure before an equilibrium is achieved.

The uptake of uranium by *T. ferrooxidans* was influenced by the external uranium concentration. A similar relationship between uranium uptake and the external uranium concentration has been observed in other microorganisms (Horikoshi et al., 1979; 1981; Tsezos and Volesky, 1981). This relation in *T. ferrooxidans* was not affected by the previous growth history of the cells or by cell inactivation by KCN or by UV-radiation showed an increase of up to 40% in uranium accumulation depending on the external uranium concentration.
The cell wall and cell membrane fractions of *T. ferrooxidans* were the major sites of uranium accumulation. The cellular distribution of uranium was unaffected by the presence of an oxidizable substrate (Fe$^{2+}$) but was affected when the cells were poisoned with KCN. Cells inactivated with KCN showed an increased concentration of uranium in the cell wall and cell membrane fractions and an 8-fold increase in the cytoplasmic fraction. Whether uranium was binding to the carbohydrate, DNA, lipid, or protein components of the cell fraction cannot be determined from the present data. Previous studies have demonstrated that uranium binds to all major components of the cell (Beveridge, 1978; Hayat, 1981; Nakajima et al., 1981; Sukharev et al., 1982). The increase in uranium uptake in the cytoplasmic fraction of KCN- and UV-inactivated cells may result from an increase in membrane permeability. However, studies on the membrane potential of *T. ferrooxidans* and a related bacterium *T. acidophilus* have shown that there is only a small decline in the pH of the cell membrane when the cells are inactivated with azide or cyanide (Cox et al., 1979; Matin et
al., 1982). A second possibility for this increased uranium uptake in UV- and KCN-inactivated cells is an energy-dependent efflux of uranium by a similar mechanism to that described for cadmium in Staphylococcus aureus (Tynecka et al., 1981). However, there is no direct evidence for the presence of a uranium transporter in T. ferrooxidans or in any other microorganism studied. In addition, UO$_2^{2+}$ is a very reactive species with biological material and little would be present as a free cation.

Electron micrographs of unstained cells incubated in the presence of uranium showed an even staining pattern throughout the cell wall fractions. The KCN poisoned and UO$_2^{2+}$ treated cells were similar to cells treated only with UO$_2^{2+}$ although osmotic effects and cell lysis was observed. The electron dense inclusion bodies observed in similar studies with Dunabella salina, a eucaryote, were not observed in T. ferrooxidans in this study (Spry and Bochem, 1981). Electron micrographs presented by Strandberg et al. (1981) shows that uranium forms fibriles on the surface of Saccharomyces cerevisiae. This may be an artifact because (i) similar deposits are formed when uranyl acetate solutions containing microcrystals of uranium
(improperly dissolved) are used as an electron stain, and (ii) microcrystals of cells not treated with uranium were presented for comparison. Moreover, intracellular uranium deposits in *Pseudomonas aeruginosa* (Strandberg et al., 1981) are difficult to evaluate because, again, no control cells were presented.

The uptake of uranium by *T. ferrooxidans* was pH dependent but independent of the presence of other metal cations. The pH dependence on uranium uptake has been observed in other studies near neutral pH values (Horikoshi et al., 1979; 1981; Tsezos and Volesky, 1981) but for the acidic pH range no previous data are available.
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