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TO MY FAMILY

ABSTRACT

The effects of Cr^{3+} and Mn^{2+} on growth and oxidation of Fe^{2+} by growing cultures of Thiobacillus ferrooxidans were studied. Growth of the organism at pH 2.5 was inhibited by concentrations of $Cr_2(SO_4)_3$ greater than 1.5×10^{-2}M or by concentrations of $MnSO_4$ greater than 0.6M. Ferrous iron oxidaton was less sensitive than growth to the inhibitory effects of $Cr_2(SO_4)_3$ and $MnSO_4$, and these inhibitions were greater at the higher pH value of 3.5. Small amounts of cell-associated Cr^{3+} were found in cells growing at pH 2.5. These amounts increased at pH 3.5, up to an estimated 12 μ mol/g dry weight. The effects of Cr^{3+} and Mn^{2+} on oxidation of Fe^{2+} by non-growing cell suspensions of Thiobacillus ferrooxidans was studied manometrically at two different $FeSO_4$ concentrations (33.8 mM and 180mM). The rate of $FeSO_4$ oxidation was greater at the lower concentration but was more severely affected by Cr^{3+} at this concentration. The inhibition of growth and iron oxidation by $MnSO_4$ appeared to be due to general osmotic effects rather than to the specific ionic concentration. Growth of Escherichia coli was inhibited by 10^{-4}M $K_2Cr_2O_7$ or by 2.4×10^{-2}M $MnSO_4$.

Chromium concentrate from the Manitoba Bird River Deposit was leached using Thiobacillus ferrooxidans. Chromium solubilization was enhanced in the presence of added elemental sulfur. A maximum chromium concentration of 140 mg/L, which exceeded more than a thousand times that permissible in natural

potable waters, was solubilized by the bacteria by an indirect process, through the action of H_2SO_4. Although this action may not be sufficient for commercial exploitation, it may be sufficient for environmental concern.

The effects of some heavy metals on the oxidation of thiosulfate by Thiobacillus thiooxidans (ATCC 8085) were studied. The rate of thiosulfate oxidation at 30°C and an initial pH of 4.5 by the organism was 55 ± 3 mg/L/h. Heavy metal cations caused normal inhibition kinetics in the oxidation of thiosulfate by T. thiooxidans. K_i values were calculated for copper (0.46 mg/L), lead (2 mg/L), chromium (Cr^{6+}, 130 mg/L; Cr^{3+}, 187.50 mg/L), manganese (182.81 mg/L) and iron (Fe^{3+}, 369 mg/L). Of the metals tested, only lead is found in tailing pond systems in concentrations that would cause substantial inhibition of thiosalt oxidation. The feasibility of using the organism as an aid to treat mill effluents so as to render them environmentally acceptable requires further studies under sub-optimal conditions resembling those of operating plants.

CHAPTER I

INTRODUCTION

The thiobacilli are a group of Gram negative bacteria capable of oxidizing elemental sulfur and reduced sulfur compounds to fulfill their energy requirements. They are short rod-shaped, non-sporulating bacteria, motile (with a single flagellum), occurring singly and occasionally in pairs. Many of them do not grow well, if at all, on solid media.

Two species of the thiobacilli are represented in this investigation - the acidophilic autotrophic bacteria, Thiobacillus thiooxidans and T. ferrooxidans.

T. thiooxidans was originally isolated from soil enriched with flowers of sulfur (Waksman and Joffee, 1922). It is remarkable for its ability to rapidly oxidize elemental sulfur and other partially reduced sulfur compounds in a pH range of 1-5 (London and Wittenberg, 1964).

T. ferrooxidans, also previously known as Ferrobacillus ferrooxidans (Kinsel, 1960) and Ferrobacillus sulfooxidans (Leathen and Barley, 1954), was originally isolated from an acid mine drainage (Colmer and Hinkle, 1947). In addition to its abilities to oxidize sulfur and sulfur compounds, this organism oxidizes reduced iron at an optimal pH of approximately 2. The bacterium oxidizes iron more rapidly than sulfur (Beck, 1960; Margalith et al, 1966). Rates of sulfur oxidation were comparable with those of T. thiooxidans (Beck, 1960). Thiosulfate is generally oxidized by T. ferrooxidans less

rapidly than sulfur (Silverman and Lundgren, 1959). However at pH 5, Silver (1970) obtained faster rates of oxidation of thiosulfate, tetrathionate and sulfide than of sulfur by sulfur-grown T. ferrooxidans. The metabolism of iron, sulfur and various sulfur compounds by the thiobacilli depends upon oxygen tension, pH, rate of growth, phosphate concentration, metal ions concentration and possibly other unidentified factors. Both T. thiooxidans and T. ferrooxidans are strictly autotrophic, in that they require an inorganic electron donor, an inorganic electron acceptor and use carbon dioxide as their major carbon source. Organic carbon compounds are inhibitory (Borichewski, 1967; Margalith et al, 1966). Carbon dioxide is fixed through the Calvin (reductive pentose phosphate) cycle and the secondary carboxylation of phospho-enolpyruvate (PEP) derived from the Calvin cycle (Aleem et al, 1963; Din et al, 1967; Eldsen, 1962, Kelly, 1971; Maciag and Lundgren, 1964).

Some species of thiobacilli, commonly assumed to be strict autotrophs, may be adapted to assimilate certain organic compounds under specific conditions (Kelly, 1971). Adaptation to growth on glucose has been suggested to result in an apparent loss of iron-oxidizing activity of T. ferrooxidans (Shafia and Wilkinson, 1969); but Guay and Silver (1975) have shown this to be due to the presence of a facultative autotrophic contaminant, Thiobacillus acidophilus. Although a process of metabolic repression has been suggested (Tuovinen et al, 1978), studies on the G + C ratio from such cultures strongly suggest that cultures of T. ferrooxidans are heterogenous (Guay and Silver, 1975; Guay et al, 1976).

The thiobacilli are known to be more tolerant to heavy metals than most other microbes. For instance, a concentration of 2×10^{-7}M of iron is required for growth of most bacteria, whereas 10^{-3}M is inhibitory. Zinc (10^{-3}M) is required for growth of most fungi and 5×10^{-3}M is inhibitory.

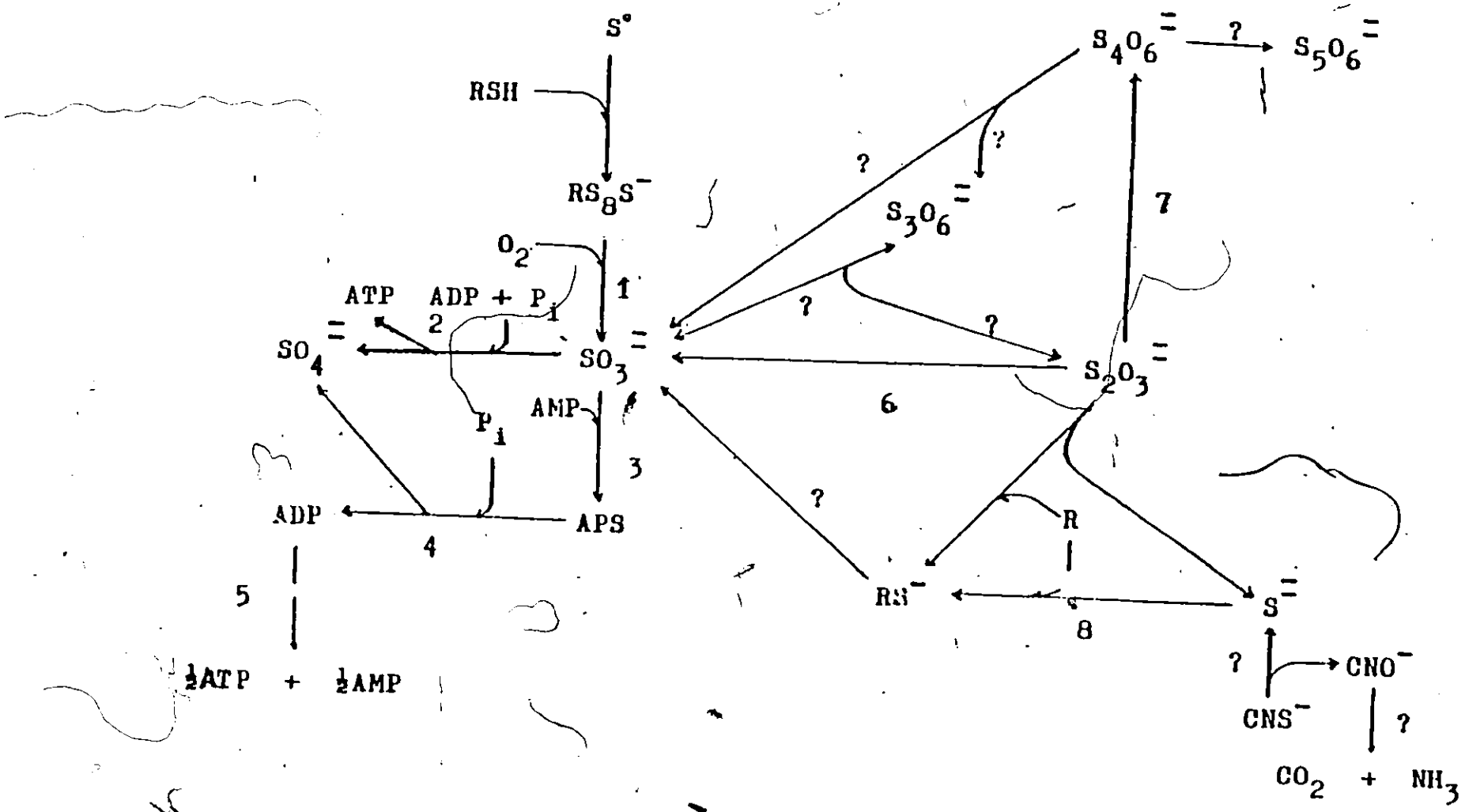
These values are far exceeded in cultures of the thiobacilli (Sadler and Trudinger, 1967; Cobet et al, 1971; Weinberger et al, 1977). Table I shows the tolerance levels of the more common metals as exhibited by T. ferrooxidans during ferrous iron oxidation.

The ability of the thiobacilli to tolerate high concentrations of heavy metals is not well understood. Tolerance to many metals has been shown to be substantially increased by selective sub-culture (Duncan et al, 1967; Duncan and Bruynesteyn, 1971) and the tolerance is retained after sub-culturing in the absence of the heavy metals (Tuovinen and Kelly, 1972) which probably indicates selection for resistant strains. Analysis of the cell wall components of these organisms revealed no essential structural differences to most autotrophic Gram negative bacteria (Lundgren et al, 1964; Remsen and Lundgren, 1966; Wang and Lundgren 1968; VenCaesseele and Lees, 1969; Wang et al, 1970). Resistance of various heterotrophic bacteria to silver, mercury, arsenic and tellurium has been shown to be plasmid borne (Hedge and Baumberg, 1973; McHugh et al, 1975; Smith, 1967; Summers and Jacoby, 1977). It is likely that acidophilic thiobacilli carry resistance factors for metals on transmissible plasmids (Davidson et al, 1983; Dispirito et al, 1981; Martin et al, 1981; Mao et al, 1980). A plasmid of a uranium-resistant strain of iron-oxidizing bacteria has, in fact, been suggested to confer resistance to uranium (Tuovinen et al, 1981).

TABLE I

Heavy metal tolerance by *Thiobacillus ferrooxidans* during iron oxidation.

Metal	Max. Concentration	Reference
Silver	1×10^{-7}M	Norris and Kelly 1978 Hoffman and Hendrix, 1974
Uranium	5×10^{-3}M	Keenan, 1969; Duncan *et al*, 1967
Mercury	5×10^{-7}M	Le Roux, N.W. Unpublished data (Quoted from Brierley, 1978)
Molybdenum	3×10^{-5}M	Duncan *et al*, 1967; Bhappu *et al*, 1965; Wyckoff, 1970
Cobalt	1.7×10^{-3}M	
Copper	1.6×10^{-1}M	Tuovinen and Kelly, 1974b
Nickel	1.7×10^{-1}M	Tuovinen *et al*, 1971
Zinc	1.5×10^{-1}M	Tuovinen and Kelly, 1972
Aluminum	3.7×10^{-1}M	
Manganese	1.8×10^{-1}M	
Arsenic		Tuovinen *et al*, 1971
Tellurium	2×10^{-4}M to	
Selenium	9×10^{-3}M	


S°
RSH
RS8S⁻
O2
1
ATP
ADP + P1
2
SO4 =
SO3 =
Pi
AMP
3
ADP
APS
4
5
½ATP + ½AMP
S4O6 =
?
S5O6 =
?
?
S3O6 =
7
?
?
S2O3 =
6
?
R
RS⁻
S =
8
?
CNO⁻
CNS⁻
?
CO2 + NH3

The effects of heavy metals on T. ferrooxidans have been extensively studied (Tuovinen and Kelly, 1974 a,b; Tuovinen et al, 1971), but very much less so on T. thiooxidans (Silver and Dinardo, 1980). Inhibitions by heavy metals of iron and thiosulfate oxidations of T. ferrooxidans (Tuovinen and Kelly, 1971) and of both iron oxidation and carbon dioxide fixation by this organism (Tuovinen and Kelly, 1974) were reported, suggesting that metals affected enzymes and/or production of ATP or NADH in the thiobacilli.

The manner by which the heavy metals exert these effects on the thiobacilli is not clear. Studies with other micro-organisms indicated that heavy metals generally exert their toxic effects by binding onto cellular surfaces and/or by accumulating inside the cells. The binding onto cellular surfaces may be specific or non-specific. Non-specific binding is typically rapid, reversible and independent of temperature and energy metabolism (Babich and Stotzky , 1978). Tolerance of the thiobacilli to high concentrations of metals suggests that the sites for the binding of ferrous iron and other metal oxidizing systems of the thiobacilli are probably cation specific. Thus these sites may not be readily available to other cations; this may protect the internal enzymic systems from other metals in the environment (Tuovinen et al, 1971). Studies on the thiobacilli cell wall suggested that the lipopolysaccharides function as the main binding sites for ferrous iron (Remsen and Lundgren, 1966; Wang et al, 1970), while sulfhydryl groups of the membrane proteins are the major binding sites for thiosulfate (Lees, 1960;Vishniac and Trudinger, 1962, Karavaiko, 1977). Studies with other bacteria on the anionic ligands for cation absorption include phosphoryl, carboxyl, sulphydryl and hydroxyl groups of membrane proteins, lipids and cell structural components such as bacterial peptidoglycan and teichoic acids. The electrophoretic

behaviour of these organisms at various pH values indicated that carboxyl groups were the main anionic sites at the surfaces of Escherichia coli, Micrococcus lysodeikticus and, together with some phosphoryl groups, of Bacillus megaterium (Neihof and Echols, 1973).

How metals binding on the cellular surface exert their toxic effects on the organism remains unclear. The following events have been suggested:

(A) Inhibition of enzymes that are present on the cellular surfaces

(B) Changes in membrane permeability and the passive entry of metals into cells, thus allowing interaction of the metals with the internal components of the cell

(C) Active uptake of the metals through transport systems

(D) Inhibition of cellular uptake or metabolism of essential nutrients on the cellular surfaces

Events (B) and (C) will undoubtedly assist, at least in part, to bring about the accumulation of heavy metals inside the cell. It is possible that following passive entry, metal ions may accumulate inside the cell against a concentration gradient by their new associations, due to a change in the pH. The metal ions can then interact with cellular components such as enzymes, nucleic acids, ribosomes, etc. A detailed account of the interaction of metals and microorganisms is given by Weinberg (1977).

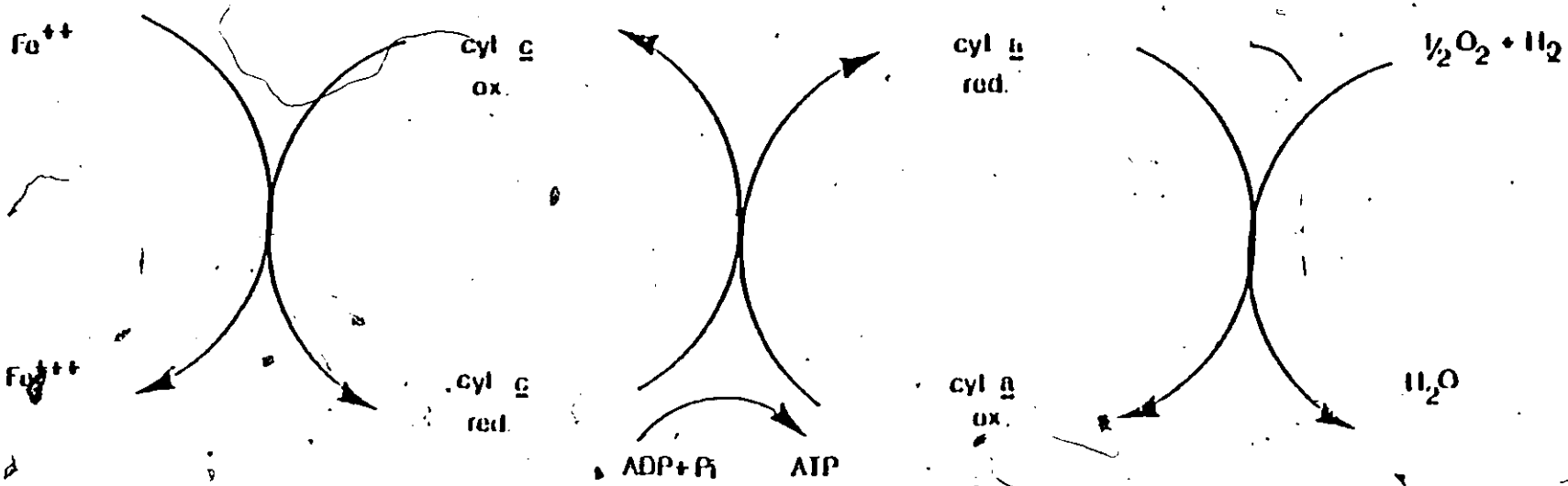
The enzymes controlling energy generation of the thiobacilli have been extensively studied. Based on currently available data, a general scheme for the oxidation of sulfur and reduced sulfur compounds has been reviewed by Silver (1978) and is represented in Figure 1. Most of the enzymes indicated in the figure have been purified and characterized from T. thiooxidans (Beck,

1960; Suzuki, 1965; Moriarty and Nicholas, 1967; 1970; Kodama et al, 1975). Only a few of the enzymes have been reported from studies of T. ferrooxidans (Silver, 1978). However, it is likely that many, if not all, of the enzymes are also present in T. thiooxidans.

FIGURE 2

Oxidation of ferrous iron by *Thiobacillus ferrooxidans*

via the cytochrome system


Fe^{++}
cyl c
ox.
Fe^{+++}
cyl c
red.
ADP + Pi
ATP
cyl a
red.
cyl a
ox.
$\frac{1}{2}O_2 + H_2$
H_2O

Sulfite is the central intermediate of the metabolic pathway for the oxidation of sulfur and reduced sulfur compounds; it is oxdized to sulfate, either by cyctochrome-c-mediated oxidation or via adenosine phosphosulfate (APS) for the production of ATP. A sulfur oxidizing enzyme, in a particulate system, catalyses the oxygenation of elemental sulfur to sulfite (SO_3^{2-}). Thiosulfate ($S_2O_3^{2-}$), produced during sulfur oxidation, may be cleaved either by the thiosulfate oxidizing enzyme or by rhodanase to sulfite (SO_3^{2-}) and a sulfide moiety (RS^-), or oxidized sequentially to tetrathionate ($S_4O_6^{2-}$), trithionate ($S_3O_6^{2-}$) and thiosulfate ($S_2O_3^{2-}$) with the concomitant formation of sulfite (SO_3^{2-}).

The oxidation of ferrous iron by the thiobacilli is described by the following equation:

$$4\ Fe^{2+} + O_2 + 4H^+ \longrightarrow 4\ Fe^{3+} + H_2O \qquad (1)$$

Studies indicate a simple electron transport system for the oxidation of iron in T. ferrooxidans (Figure II). Co-enzyme Q has been detected by Dugan and Lundgren (1964). Lazaroff (1963) and Less et al (1969) found that sulfate was required for the oxidation of ferrous iron. It is postulated that sulfate controls the entry of ferrous iron into the cell or that sulfate is required for the transfer of electrons for the iron oxidase system with Co-enzyme Q, which acts as an intermediary electron carrier between the ferrous iron-sulfate-organic complex and the cytochrome system in the cell envelope (Dungan and Lundgren, 1964).

A membrane-associated enzyme which is responsible for the oxidation of ferrous iron, ferrous-cytochrome c-oxidoreductase, has been purified and characterized (Yates and Nanson, 1966; Din and Suzuki, 1967; Din et al, 1967). The mechanism of the reaction has been described as the "Ping Pong Bi

Bi" type (Din and Suzuki, 1967) and is represented in Figure 3. An atom of ferrous iron becomes oxidized by binding onto the enzyme containing one atom of ferric iron. The enzyme-bound iron becomes reduced and the oxidized iron is released. One molecule of the oxidized cytochrome c then becomes bound by the enzyme whose ferrous iron is oxidized while the ferric iron of the cytochrome c is reduced. Another ferric-enzyme complex is subsequently formed and the reduced cytochrome c is released.

With the exception of the crude preparation of the cell envelope-associated iron oxidase systems of Imai et al (1972) and Bodo and Lundgren (1974), which showed an optimal pH range of 3.5 - 4, all other internal enzymes of the thiobacilli, including the same but more purified iron oxidase system, showed pH optima ranging from 5-9 (Blaylock and Nanson, 1963; Din et al, 1967; Silver and Lundgren, 1968a,b; Tabita et al, 1969; Howard and Lundgren, 1970; Vestal and Lundgren, 1971; Adapoe and Silver, 1975). This is significantly higher than the organism's external pH. Dewey and Beecher (1966) maintained that the acidophilic thiobacilli possess a cell envelope which is selectively impermeable to high concentration of H^+ ions. The nature of this selectivity is not clear. Beck (1960) suggested that this proton barrier on the thiobacilli is of a passive nature, due to the fact that resting cells of T. ferrooxidans were able to tolerate long period of storage at low pH values. Current studies suggest that the barrier is more active. Membrane-associated ATPase was isolated and characterized (Marunouchi and Mori, 1967; Adapoe and Silver, 1974). The role of such enzymes in maintaining an alkaline pH inside the cell of the thiobacilli is still highly speculative. Inglew et al. (1977) and Apal et al, (1978) suggested that a transmembrane pH gradient of T. ferrooxidans in acid medium, with an internal pH close to neutrality to allow normal metabolism to function, could be used to conserve

energy with the coupling of the gradient to ATP synthesis via a chemiosmotic ATPase reaction of H^+ ions with oxygen inside the cell matrix (equation 2) with the other half-reaction (equation 3) occuring outside the cell matrix.

$$2\ e^- + 1/2\ O_2 + 2H^+ \longrightarrow 2\ H_2O \qquad (2)$$

$$2\ Fe^{2+} \longrightarrow 2\ Fe^{3+} + 2e^- \qquad (3)$$

Thus H^+ ions are drawn into the cell matrix to maintain electric neutrality, as a result of electrons generated through the oxidation of ferrous iron. The high concentration of ferric iron bound in the cell membrane is thought to contribute to the proton barrier due to high electrostatic charge.

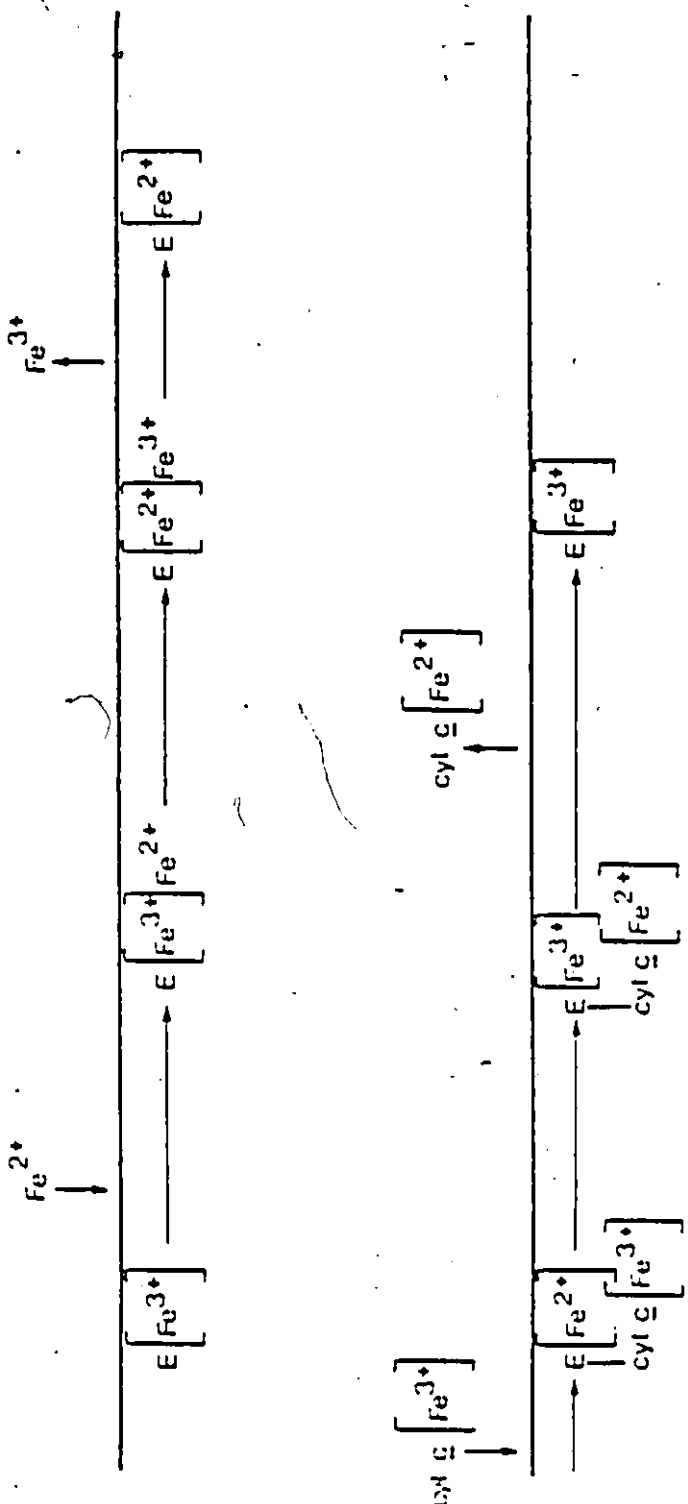
The abilities of the thiobacilli to tolerate high concentrations of H^+ ions and metallic cations have lent themselves to the application of the bacterial leaching of minerals, a process widely practiced since the early history of the Romans who were, of course, not aware of the role of bacteria. It was not until the last 30 years that the essential role of the bacteria has been proved and large scale operations developed (Shaffer and Evans, 1968; Fletcher, 1970; Sascon, 1975; Brierley, 1978). Much of the present interest in bacterial leaching has been generated in part by a gradual depletion of high-grade ore deposits and in part by the enormous quantities of industrial metallic wastes that are accumulating. The industries, with the ever increasing demands for metals, and stringent government regulations on environmental pollution control, are forced to examine more economical and efficient methods to deal with their metal extraction problems. Bacterial leaching of many low-grade ores and industrial wastes have been considered practical and beneficial. Not only may valuable quantities of metals be recovered, but toxic substances may also be removed from the industrial wastes. Sulfur dioxide emission normally associated in the conventional smelting of sulfides is also prevented.

An extensive range of mineral sulfides are involved in the leaching of minerals by the thiobacilli (Brierley, 1978; Silver and Lundgren, 1980). The more important of these include pyrite (FeS_2), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), covellite (CuS), galena (PbS) and sphalerite (ZnS). Oxides of minerals similarly leached by the thiobacilli include those of uranium [e.g. uraninite(UO_2)], manganese [e.g. pyrolucite (MnO_2)] and antimony Sb_2O_3. The potential commercial significance of these mineral leachings rests primarily in the extraction of soluble metal values. The desired elements are recovered through the dissolution of the metals from the mineral. At times, insoluble precious metals, dispersed within the mineral lattice, such as gold, may also be freed from the mineral (Livesey-Goldblatt, 1983) while the microorganism extract sulfide from the mineral lattice. The leaching of sulfide of copper and uranium is of major industrial and economic significance. Some 11.5% to 15% (Burkin, 1971; Brierley, 1978) of the total United States copper production comes from the leaching of low grade copper waste materials. Bacterial-assisted recovery of uranium was practiced in Canada at the Stanrock Mine (MacGregor, 1966; 1969) and Milliken Mines, Elliot Lake District (Fisher, 1966). The former mine commercially produced more than 10,000 lb of U_3O_8 per month during 1966. The Agnew Lake uranium mine was operated for seven years using iron-oxidizing bacteria for the recovery of uranium (Guay and Silver, 1981).

The principles, practices and applications of bacterial leachings have been extensively discussed (Duncan and Trussell, 1964; LeRoux, 1969; Fletcher, 1970; Duncan and Bruyns, 1971; Tuovinen and Kelly, 1972; 1974; Dutrizac and MacDonald, 1974; Kelly, 1976; Karaviako, Kunzneststov and Golomzik, 1977; Schwartz, 1977; Torma, 1977; Murr, Torma and Brierley, 1979; Kelly, Norris and Brierley, 1979; Lundgren and Silver, 1980). Essentially, bacterial action on the minerals is either direct or indirect. Direct action on the mineral

FIGURE 3

Enzymatic mechanism of ferrous-cytochrome c oxidoreductase of *Thiobacillus ferrooxidans*


Fe^{2+}
Fe^{3+}
E $[Fe^{3+}]$
E $[Fe^{3+}]$ Fe^{2+}
E $[Fe^{2+}]$ Fe^{3+}
E $[Fe^{2+}]$
cyl ⊆ $[Fe^{3+}]$
E $[Fe^{2+}]$
cyl ⊆ $[Fe^{3+}]$
E $[Fe^{3+}]$
cyl ⊆ $[Fe^{2+}]$
cyl ⊆ $[Fe^{2+}]$
E $[Fe^{3+}]$

occurs mainly through the biological oxidations of iron and sulfide moities of the mineral lattice. The indirect mechanism involves the generation of ferric iron and/or sulfuric acid through the oxidations of external iron, sulfur or reduced sulfur compounds and the chemical interaction of ferric iron or sulfuric acid with the minerals. Some authorities speculated that bacterial leaching may be explained by an indirect attack on the crystal lattice by ferric iron and sulfuric acid (Stumm-Zallinger, 1972). However, insoluble sulfide minerals have been shown to be degraded in the absence of ferric iron (Mizoguchi et al, 1976; Silver, 1970; Kingma and Silver, 1979). Present evidence by scanning electron microscopy also strongly indicates that bacteria are capable of direct oxidative attack by attaching themselves onto the surfaces of crystals (Brierley and Murr, 1978). The production of metabolites that assist the dissolution of mineral sulfides was also suggested (Bennett and Tributchi, 1978; Agate et al, 1969). Lundgren and Tano (1978) have presented a model to explain iron and sulfide oxidations where two membrane systems, utilizing a chemiosmotic ATPase reaction, are needed for the oxidation of iron and sulfur in an acid environment.

In general, bacterial leaching of mineral sulfides may be represented by the following simplified equation:

$$MS + 2\ O_2 \longrightarrow MSO_4 \qquad (4)$$

Where M is a bivalent metal. A typical example of direct bacterial attack of this type is the leaching of chalcocite (Cu_2S) which is oxdized to copper sulfate with digenite (Cu_9S_5) and covellite (CuS) as intermedites:

$$10\ Cu_2S + 2\ H_2SO_4 + O_2 \xrightarrow{\text{bacteria}} 2\ Cu_9S_5 + 2\ CuSO_4 + 2\ H_2O \quad (5)$$

$$2\ Cu_9S_5 + 8\ H_2SO_4 + 4\ O_2 \xrightarrow{\text{bacteria}} 10\ CuS + 8\ CuSO_4 + 2\ H_2O \quad (6)$$

$$10\ CuS + 20\ O_2 \xrightarrow{\text{bacteria}} 10\ CuSO_4 \quad (7)$$

$$10\ Cu_2S + 10\ H_2SO_4 + 25\ O_2 \longrightarrow 20\ CuSO_4 + 10\ H_2O \quad (8)$$

The indirect leaching of mineral sulfides involving ferric iron is generally represented as:

$$FeS + 2\ Fe^{3+} + H_2 + 2\ O_2 \xrightarrow{\text{aerobic}} Me^{2+} + 2\ Fe^{2+} + SO_4^{2-} + 2\ H^+ \quad (9)$$

$$Fe_2(SO_4)_3 + FeS_2 \xrightarrow{\text{anaerobic}} 3\ FeSO_4 + 2\ S \quad (10)$$

In a leaching system in which iron is present, a combination of direct and indirect attack generally occurs. The oxidation of chalcopyrite for instance:

$$48\ CuFeS_2 + 204\ O_2 + 24\ H_2SO_4 \xrightarrow{\text{bacteria}} 48\ CuSO_4 + 24\ Fe_2(SO_4)_3 + 24\ H_2O \quad (11)$$

$$12\ CuFeS + 24\ Fe_2(SO_4)_3 \longrightarrow 60\ FeSO_4 + 12\ CuSO_4 + 24\ S \quad (12)$$

$$60\ FeSO_4 + 30\ H_2SO_4 + 15\ O_2 \xrightarrow{\text{bacteria}} 30\ Fe_2(SO_4)_3 + 30\ H_2O \quad (13)$$

$$24\ S + 24\ H_2O + 36\ O_2 \xrightarrow{\text{bacteria}} 24\ H_2SO_4 \quad (14)$$

$$30\ Fe_2(SO_4)_3 + 120\ H_2O \longrightarrow 20\ [H[Fe(SO_4)_2 \cdot 2Fe(OH)_3]] + 50\ H_2SO_4 \quad (15)$$

$$60\ CuFeS + 255\ O_2 + 90\ H_2O \longrightarrow 60\ CuSO_4 + 20\ H_2SO_4 + 20[H[Fe(SO_4)_3 \cdot 2Fe(OH)]] \quad (16)$$

The oxidation of uraninite is mainly an indirect process, ferric iron being generated from the oxidation of pyrite normally present in the uranium ore. Thus:

$$4\,FeS_2 + 15\,O_2 + 2\,H_2O \xrightarrow{\text{bacteria}} 2\,Fe_2(SO_4)_3 + 2\,H_2SO_4 \quad (17)$$

$$UO_2 + Fe_2(SO_4)_3 \dashrightarrow UO_2SO_4 + 2FeSO_4 \quad (18)$$

Additional ferric iron is generated from bacterial oxidation of ferrous sulfate (equation 1). Recent investigations by colorimetric (Soljanto and Tuovinen, 1979) and respirometric (Ivarson, 1980) techniques indicated the oxidation of insoluble tetravalent uranium to the soluble hexavalent state by T. ferrooxidans according to the following equation:

$$2\,UO_2 + O_2 + H_2SO_4 \xrightarrow{\text{bacteria}} 2\,UO_2SO_4 + 2\,H_2O \quad (19)$$

Applications in the area of mineral extraction, particularly from low-grade nonrenewable waste-ore deposits were generally investigated in laboratories and small scale pilot plant experiments. Small scale laboratory experiments were primarily geared towards the establishment of the optimal leaching conditions. This encompasses such parameters as temperature, pH, pulp density, composition of leaching medium and metal toxicity. Each of these factors has recently been discussed (Lundgren and Silver, 1980) and the last of these factors forms part of this investigation. Laboratory experiments, to determine the rate and extent of alteration of mineral in ore, are best performed with vessels agitated by stirring (Duncan et al, 1964) or shaking (Duncan et al, 1967) and respirometer vessels (Razzel and Trussel, 1963). Heap and dump leaching are the predominant commercial operations used in the bacterial extractions of mineral ores. They are generally simulated with filled columns with or without solution percolations (Bryner et al, 1954); Malouf and Prater, 1961; Fletcher, 1970). Large scale tanks and

fermentors are also used (Corrick and Sutton, 1965; McCreedy et al, 1969). In dump leaching, solutions are allowed to percolate through large quantities of low grade ores or waste rocks, deposited on impermeable ground, usually in valleys. The pregnant solution containing the soluble metal values, is then collected for concentration of metal values. Heap leaching is practiced on a smaller scale with finer and higher grade ores, to justify the cost of the operation, mounted on prepared drainage pads. Leaching solutions are brought in contact with the ores by percolation (Gow et al, 1971), alternate flooding and dewatering (Wadsworth, 1975), spraying and hosing (Sheffer and Evans, 1968; MacGregor, 1966; 1969). Metals of value are also extracted from underground mines by injection of leach solutions (Wadsworth, 1975).

Two important aspects of bacterial leaching are investigated in this study. The first, forming the major section of the study, was related to the leaching problems of crude ores, particularly that of manganese (pyrolucite) and chromium (chromite). The second was related to work undertaken at the CANMET laboratories, Ottawa and dealt mainly with industrial waste treatments and their environmental considerations.

Attempts were first made to examine in detail the tolerance to manganese and chromium of a strain of T. ferrooxidans in both growing cultures and resting cell suspensions. Although the effects of Cr^{6+} on the growth of T. thiooxidans has been studied by Zajic et al, (1980), the sensitivity of T. ferrooxidans to chromium has not yet been established. The tolerance of growing cultures of T. ferrooxidans to soluble manganese has been examined previously, but with conflicting results. Zimmerly et al, (1958) found that 3 g/litre was inhibitory, whereas Tuovinen et al, (1971) found that as much as 10 g/litre did not inhibit growth. Sensitivity to the above two metals

becomes of practical concern in connection with the possibility that thiobacilli might be used in the treatment of manganese and chromium ores.

Most manganese ores are oxides. The most common is pyrolucite (MnO_2). Although MnO_2 is not soluble in sulfuric acid, thiobacilli are able to dissolve this compound through the action of the intermediate compounds of sulfur metabolism (Imai, 1978). Chromium ores usually exist in nature as impure chromite mineral $(Fe,Mg)O(Cr,Al,Fe)_2O_3$, instead of pure chromite ($FeOCr_2O_3$), which contains 68% of Cr_2O_3 and 32% FeO. Sometimes calcium, phosphorous and sulfur are also present in the crystal structure (Raicevic, 1979; 1977). An important factor in the mineral chromite of metallurgical and chemical grades is the chromium to iron ratio (Cr : Fe). Generally Canadian chromite deposits have a low Cr:Fe ratio which is unfavourable for commercial exploitation. Metallurgical grade chromite requires a Cr:Fe ratio of 2-3 : 1. Between 1971 - 1977, the Ontario Research Foundation and CANMET reported a combined physical and chemical method of producing chromium additives suitable for the steel industry in the production of chromium metal and sodium dichromate, from the Manitoba Bird River chromite deposit. The method is, nevertheless, economically unfavourable in competition with natural chromite ores of foreign sources on which Canada now wholly depends (Raicevic, 1977). It would thus be of prime economic and industrial advantage, especially when foreign sources become unavailable during times of political instability, to examine the possibility of a biohydrometallurgical method of producing metallurgical grade chromite concentrate. To determine the extent to which both Fe and Cr could be brought into solution through direct or indirect bacterial action, samples from the same Bird River chromite deposit were used in this study.

The effects of manganese and chromium were also studied on the other bacteria, namely Escherichia coli and Thiobacillus thiooxidans. E. coli can accumulate high concentration of manganese through a transport system (Silver et al, 1970). Since the thiobacilli tolerate high concentrations of manganese, this raises the question whether manganese might similarly accumulate in the thiobacilli.

During the milling and flotation of sulfide ores, part of the sulfide undergoes oxidation to yield sulfate ion, elemental sulfur and partly oxidized sulfur anions such as thiosulfate, trithionate and tetrathionate (Rolia and Barbeau, 1978). Under alkaline conditions frequently present, sulfide is formed from sulfide minerals, such as pyrite, according to the following equation (Scott and Bragg, 1975):

$$2\ FeS_2 + 4\ OH^- \longrightarrow 2\ FeS + 2S^{2-} + H_2O + O_2 \qquad (20)$$

The sulfide and residual sulfite from various ore flotation operations exert a nucleophilic attack on the elemental sulfur molecules (Schmidt, 1965), which result in the formation of linear polysulfides; these hydrolize spontanously to thiosulfate ($S\text{-}SO_3^{2-}$), trithionate ($O_3S\text{-}S\text{-}SO_3^{2-}$), tetrathionate ($O_3S\text{-}S\text{-}S\text{-}SO_3^{2-}$), and other polythionates ($O_3S\text{-}S_n\text{-}SO_3^{2-}$). A portion of these partly oxidized anions, collectively known as thiosalts, pass through conventional tailings ponds and liming operations, which is then discharged to the natural water systems (Schmidt and Conn, 1971).

In most tailing pond systems, the initial thiosalt concentrations are low and the retention times are long, with the result that the thiosalt levels are reduced to negligible values before liming and subsequent discharge. In some operations, however, thiosalt concentrations are higher. These high

thiosalt levels, combined with tailings ponds with a low retention time, can give rise to environmental problems associated with the oxidation of the thiosalts to sulfuric acid after being discharged to the natural water systems. The presence of dissolved heavy metal ions in the tailings ponds could exacerbate this problem by inhibiting the bacterial oxidation of thiosalts. Silver and Dinardo (1981) investigated the inhibition of this process by the metallic cations cadmium, copper, lead, silver and zinc. As an extension of this study, the inhibitory effects of Mn^{2+}, Cr^{3+}, Cr^{6+}, Fe^{2+} and Fe^{3+} on this process were investigated.

Several questions are thus investigated in this thesis. With respect to the inhibitory effects of chromium and manganese on T. ferrooxidans, these are:

1. What concentration of chromium or manganese will T. ferrooxidans tolerate during iron oxidation?

   This is especially important, if the feasibility of using the organism for the leaching of chromium or manganese ores is to be studied. It could be very desirable to select a strain of T. ferrooxidans that could tolerate high concentrations of chromium or manganese.

2. Is chromium or manganese accumulated by T. ferrooxidans during growth?

   This question is of interest both for the physiology of transport in these bacteria and also in relation to the relative toxicity of chromium and manganese: presumably, toxic effects are caused by ions that enter or are closely associated with the cells.

3. What effects will different pH and substrate concentrations have on the oxidation of ferrous iron by T. ferrooxidans in presence of chromium and manganese?

   This is important if different leaching conditions are to be employed for chromium and manganese ores.

Questions investigated with respect to bacterial leaching of chromium ores are:

1. Will chromium ores be successfully leached by T. ferrooxidans?

   The objective here is to use the organism either to bring the chromium into solution or to upgrade the chromium ores to the desired Cr : Fe ratio of 2-3 : 1.

2. Is the leaching process due to the bacterial oxidation of the chromium and/or iron moities of the mineral lattice or to an indirect bacterial activity?

   It is important to determine, during leaching, if bacterial growth occurs, as well as the extent to which the ore's body is being altered.

3. Does the addition of oxidizable substrates increase the rate of leaching by the organism?

Finally, with respect to the feasibility of using T. thiooxidans for the removal of thiosalts from biostabilization ponds, the question as to what metal cations are likely to limit the oxidation of thiosulfate and other thiosalts is investigated.

CHAPTER II

MATERIALS AND METHODS

(A) Maintenance of Culture

Thiobacillus ferrooxidans

Thiobacillus ferrooxidans was isolated from acid mine waters in Northern Quebec. The bacterium was a generous gift of A.E. Torma, formerly of the Centre de Recherche Mineraux, Complexe Scientifique du Quebec, Ste-Foy, Quebec, Canada. Physiological and morphological characteristics are similar to those of T. ferrooxidans described in the eighth edition of Bergey's Manual (Vishniac, 1974).

The organism was propagated at 30°C in 12 litres glass carboys using ferrous sulfate-9K medium of Silverman and Lundgren [(1959) 9000 ppm Fe^{2+}, pH 2.5, with or without 10^{-3}M $MnSO_4.H_2O$ or $K_2Cr_2O_7$] under forced aeration and harvested after 38-42 hours. Harvested cells were washed twice and resuspended with 9K basal salt solution (pH 2.5) to obtain a 10% (w/v) cell suspension. Yields per litre were usually 120-130 mg (wet weight).

Thiobacillus thiooxidans

Thiobacillus thiooxidans was a generous gift of Dr. I Suzuki presently in the Department of Microbiology, University of Manitoba, Winnipeg,

Manitoba. Physiological and morphological characteristics are similar to those described in the eighth editions of Bergey's Manual (Vishniac, 1974).

T. thiooxidans was propagated at 30°C in 500 ml Erlenmeyer flasks containing 200 ml of the following growth medium.

Ingredients	Concentration (g/litre)	
$Na_2S_2O_3$	4.4 (2000 ppm)	..... Solution (A)
KH_2PO_4	0.0079 (5 ppm)	)
$CaCl_2$	0.20	)
$MgSO_4$	0.002	) ..... Solution B
$(NH_4)_2SO_4$	0.094 (16 ppm)	)
pH 4.5		

Generally five fold concentrations of solution (A) and (B) were prepared and autoclaved separately as stock solutions.

Escherichia coli.

E. coli K-12 was a generous gift of Mrs. Rebecca Wallace formerly of the Laboratory Centre for Disease Control, Ottawa. The organism was maintained in nutrient agar slants and subcultured twice into appropriate growth medium prior to each experiment.

(B) Effects of Chromium and Manganese on Iron Oxidation by T. ferrooxidans

T. ferrooxidans oxidation of ferrous iron and its growth in the absence and presence of chromium or manganese were investigated by inoculating 20 µl of a 10% cell suspension to each of 500 ml Erlenmyer flasks containing 125 ml of ferrous sulfate-9K medium (pH 2.5 and 3.5). Control flasks contained 1% (w/v) thymol in 2% (v/v) methanol which completely inhibit

growth. Stock solutions of chromium and manganese were prepared in 9K basal medium at pH 3.5. The solutions were filtered through a milipore membrane filter (0.45 μm pore size) and the concentrations of chromium and manganese were determined colorimetrically. Flasks were agitated on a reciprocating shaker (120 cycles/min) at 30°C. Periodically, 1 ml aliquot of growth medium was removed for ferric iron determination and microscopic cell count. Where indicated (e.g., Fig. 4) 3 gm of solid ferrous sulfate was added to each flask. The experiments were performed in duplicates and repeated at least once with similar results.

(C) Effects of Chromium and Manganese on The Growth of *Escherichia coli* K-12

The effects of chromium and manganese on this organism were investigated by inoculating 1 ml of an actively growing culture (late log phase) into 500 ml side arm flasks containing 125 ml of the growth medium with or without addition of these metals. The medium used to study the effect of manganese on the growth contained:

Ingredients	Concentration
KCl	2.4×10^{-3}M
NaCl	1.3×10^{-3}M
$FeSO_4.7H_2O$	0.005g/litre
$MgSO_4.7H_2O$	0.05g/litre
NH_4NO_3	1.0g/litre
Bacto peptone	0.5%
Glucose	0.5%
pH 6.5	

For experiments on the effect of chromium, this medium was supplemented with 0.42 g/litre K_2HPO_4 and 0.18 g/litre KH_2PO_4.

Flasks were shaken at 45 cycles/min and incubated at 37°C. The optical density of the medium was monitored at 600nm with a Coleman Junior II spectrophotometer. The experiments were performed in duplicate and repeated at least once with similar results.

(D) Bacterial Action on Chromite Ores

A chromite ore concentrate of particle size minus 10 mesh (less than 1.68 mm) obtained from the Bird River deposit, Manitoba, Canada, was washed extensively with distilled-deionized water and dried. The effect of T. ferrooxidans on these low grade ores at various pulp densities (weight in grams of ore per 100 ml of medium) in presence and absence of elemental sulfur and ferrous sulfate was investigated by inoculating 0.5 ml of a 10% cell suspension, previously grown in ferrous sulfate-9K medium in presence of 10^{-3}M of Cr^{6+}, into 2L flasks containing 1L of 9K basal medium. Control flasks contained the same ingredients as other experimental flasks plus 5 drops of KCN (1%) added from a Pasteur pipette. The surface levels of the solutions on these flasks were marked and the flasks were agitated on a reciprocating shaker (120 cycles/min) at 30°C. Distilled-deionized water was added to each flask to compensate for the loss of water through evaporation prior to each sampling. The initial pH of the culture's medium were adjusted to 3.2 with concentrated H_2SO_4. In both experimental and control flasks, pH values increased and were routinely adjusted to approximately 3.2 with concentrated H_2SO_4 (see Fig. 11). At various time intervals, the flasks were removed from the shaker and the suspended materials allowed to settle before pH was measured and samples taken. A 10 ml aliquot in an acid washed 15 ml glass centrifuge tube was centrifuged at 20,000 x g for 15 min. and the supernatant used for chromium determination. The pellets obtained from the 12% pulp density

flasks were washed twice with 5 ml HCl-acidified distilled water (pH 2) and resuspended in 5 ml distilled water for protein determination. In order to stop bacterial action on the ores, 5 ml of 10% (w/v) thymol in 2% (v/v) methanol was added at times shown (see Fig. 12).

At the end of the experiments the residual ores were extensively washed with distilled-deionized water and dried before analyzing for ferrous iron, chromium and carbon.

Leaching experiments on the chromite concentrate with or without T. ferrooxidans were done in duplicate. The chromium analysis values from the two flasks were found to vary not more than 5% from the mean value.

(E) Effects of Heavy Metals on Thiosulfate Oxidation

The bacterial oxidation of sodium thiosulfate in the presence and absence of heavy metal was investigated by inoculating 5 ml of an actively growing culture (48-54 hours) which was previously adjusted asceptically to pH 4.5 with 1N NaOH, into each of 500 ml Erlenmeyer flask containing 125 ml of T. thiooxidans growth medium (pH 4.5). Control flasks contained no bacteria; instead 5 ml of sterilized distilled water was added. Flasks were agitated on a reciprocating shaker (120 cycles/min) at 30°C. At various time intervals, a 3 ml aliquot of growth medium was removed from each flask for pH and thiosalt determinations.

(F) Techniques Utilized

(I) Ferric Iron Determination

The method of Schnaitman, Korczynski and Lundgren (1969) was adapted for the determination of ferric iron. Fifty µl of the growth medium was added directly to test tubes containing 5 ml of 2N HCl which was then made up to 10

ml with H_2SO_4-acidified distilled water (pH 2.5), and the optical density was determined at 410nm. Ferric sulfate was used as the standard. Appropriate blanks consisting of 50 µl of ferrous sulfate-9K medium, with or without chromium or manganese were prepared. All readings were taken at 30°C.

(II) Manganese and Chromium Determinations

Manganese and chromium were determined colorimetrically by the periodate and permanganate methods respectively (Rand et al, 1975). Each determination of cell associated manganese and chromium consisted of cells from two flasks (total volume of sample - 250 ml) which were removed from the shaker at various time intervals. Samples were immediately filtered into an acid washed glass centrifuge tube through a Whatman No. 1 filter paper. In this process more than 50% of the cells passed through the filter paper. Cells from the filtrates were separated by centrifugation at 20,000 x g for 15 min. with 5 ml 9K-basal solution (pH 2.5) and treated with concentrated sulfuric acid at fuming temperature for at least 60 mins. prior to metal analysis.

(III) Thiosalts Determination

During the investigation of the effects of various heavy metals on the oxidation of thiosulfate by T. thiooxidans, the disappearance of thiosalts was determined by acidometric titrations (Makhija and Hitchen, 1978) with pH 8.2 as the end point. Duplicate results which do not deviate more than 2.5% from the mean were obtained for each determination consisting of a 0.5 - 2 ml sample. Samples were mixed with 2 ml of formaldehyde before each titration. The thiosulfate concentrations are expressed as mg/litre. This method can detect total thiosalts in samples containing as little as 0.10 mg in as much

as 30 ml. All rates of thiosulfate oxidation were calculated with a regression analysis computer program. The same program was used to fit the lines of the semi-reciprocal plots and to calculate the V_{max} and K_i values.

(IV) Tyndallization of Elemental Sulfur

Tyndallization was accomplished by placing 10-20 g of elemental sulfur in aluminium foil packets and exposing them to flowing steam at atmospheric pressure for approximately one hour on three successive days.

(V) Cellular Protein and Cell Count

Cellular protein was determined using the colorimetric method of Lowry et al (1951) with bovine serum albumin as the standard. During the investigation on the actions of T. ferrooxidans on chromite (12% pulp density) with 1% elemental sulfur (Tyndallized) the method of Proteau and Silver (1977) was employed. Benzene (2 ml) was added to the 5 ml cell suspension and vigorously shaken. Cellular protein was determined from the water phase after hydrolyzing with IN NaOH.

Microscopic cell counts were obtained with a Petroff-Hausser hemocytometer. Cells were diluted with methylene blue solution (1%) to obtain 10-15 organisms per field.

Generation times during growth on ferrous iron were calculated from the exponential phase of the growth curve using the expression: 1 / K

Where $K = \dfrac{Log_{10}N_t - Log_{10}N_0}{0.301 t}$

t = elapsed time

N_t and N_o = number of cells at time t and o

(VI) Respiratory Studies

Conventional manometric techniques (Umbreit et al, 1964) were employed to measure oxygen utilization. Each vessel contained the following in 3.7 ml: 10 µmole of β-alanine-SO_4 buffer (pH 2.5) prepared by adjusting a solution of the amino acid with IN H_2SO_4, a cell suspension (0.1 ml) containing 375 ± 10 µg of protein, ferrous iron, and chromium sulfate or manganese sulfate as indicated. The centre well contained 0.2 ml of 10% (w/v) KOH. The vessels were shaken at 148 oscillations/min in a 30°C water bath. After 15 min of equilibration the cells were tipped into the main compartment from the side arm. The rate of oxygen consumption was calculated from the linear portion of the curve (between 5 and 25 min). The experiments were performed in duplicate and the mean values obtained. The two values were found to vary not more than 2% from the mean.

(G) Chemicals

All chemicals used were of the highest purity grade commercially available. Bovine serum was obtained from Sigma Chemicals Ltd., St. Louis, Mo. Chromite ore from the Bird River of Manitoba, was supplied by CANMET, Ottawa, Canada.

CHAPTER III

RESULTS

(A) Effects of Chromium and Manganese on Growth and Iron Oxidation of Thiobacillus ferrooxidans

Figures 4 and 5 show the effects of $Cr_2(SO_4)_3$, $K_2Cr_2O_7$ and $MnSO_4$ on the autotrophic growth of T. ferrooxidans at pH 2.5 with $FeSO_4$ as an energy source. Growth was measured both by cell counts and Fe^{3+} formation; in most cultures there was a close correlation between these two indices of growth. Attempts were also made, but without success, to follow the growth of bacteria during iron oxidation by analyzing for cellular protein, using the Lowry (1951) and Bradford (1977) methods; and DNA content, using Setaro and Morley (1977) method. Ferric iron was found to interfere with all these methods. Growth of single colonies on solid media was also not successful.

Concentrations of $Cr_2(SO_4)_3$ and $K_2Cr_2O_7$ of 1.5×10^{-2}M and 10^{-3}M, respectively, did not affect growth and ferrous iron oxidation although higher concentrations caused partial or complete inhibition. Growth and ferrous iron oxidation were not inhibited by 0.5M $MnSO_4$, but higher concentrations (0.6M and above) did cause inhibition. This inhibition may not be due to Mn^{2+} ion, but rather to the high osmality since 0.6M K_2SO_4 inhibited growth and ferrous iron oxidation more than 0.6M $MnSO_4$. Growth and ferrous iron oxidation were

completely inhibited by 1M $MnSO_4$. The concentration of Cr^{3+} ions which completely inhibited growth could not be determined, due to the limit of solubility of $Cr_2(SO_4)_3$. A 0.1M concentration of $Cr_2(SO_4)_3$ inhibited growth strongly or completely. It should be noted that $K_2Cr_2O_7$ can oxidize $FeSO_4$ according to the reaction:

$$K_2Cr_2O_7 + 6FeSO_4 + 7H_2O \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 7H_2O \qquad (21)$$

This reaction is probably responsible for the appearance of Fe^{3+}, when Fe^{2+} is added to culture at 48 hours (Fig. 4a), even though cell growth does not always continue (Fig. 4b). Changes in pH before and after the addition of ferrous iron were between 2.45 - 2.74 in flasks containing 1M $K_2Cr_2O_7$ and 2.13 -2.18 in all other flasks.

Cr^{3+} and Mn^{2+} inhibited growth (as measured by Fe^{2+} oxidation) more strongly at pH 3.5 than pH 2.5 (Figs. 6 and 7, compare to Figs. 4 and 5). Cells may be more sensitive to these ions because they bind considerably more Cr^{3+} when growing at pH 3.5 than at pH 2.5 (Table II). Slight, if any, oxidation of Fe^{2+} was caused by any of the concentrations of Cr^{3+} or Mn^{2+} used, at either pH 2.5 or 3.5.

The effect of Cr^{3+} and Mn^{2+} on oxidation of Fe^{2+} by non-growing cell suspension of T. ferrooxidans was studied manometrically at two different $FeSO_4$ concentrations (Table III). The rate of oxidation was slightly lower at the higher concentration. Oxidation was not strongly inhibited by the metals studied, only slight inhibition being caused by 7.4×10^{-2}M Cr^{3+}. Mn^{2+} had relatively little effect; even 1M $MnSO_4$ inhibited oxidation only 60%. Little change in the pattern of inhibition was caused by changes in the $FeSO_4$ concentration, or by previous growth in the presence of Cr^{3+} or Mn^{2+}.

Morphological changes were observed on exposure to chromium. The cells were found mostly in pairs and were both longer and wider than untreated cells.

(B) Inhibition of Growth of Escherichia coli

The effect of Cr^{6+} ($K_2Cr_2O_7$) and Mn^{2+} ($MnSO_4$)$_3$ on the growth of E. coli were examined. The effects of $Cr_2(SO_4)_3$ on growth could not be measured, since on adjusting the pH values of the medium in this compound to those necessary for growth of E. coli, precipitation occurred. As Figs. 8 and 9 show, growth of E. coli was inhibited by 10^{-4}M $K_2Cr_2O_7$ and by 2.4×10^{-2} $MnSO_4$. These inhibitions were observed in a complex medium, which probably had considerable binding power for the inhibitors studied, at least for Mn^{2+}. Thus E. coli, for reason still unknown, seems much more sensitive to these compounds than T. ferrooxidans

(C) Accumulation of Cr and Mn by T. ferrooxidans

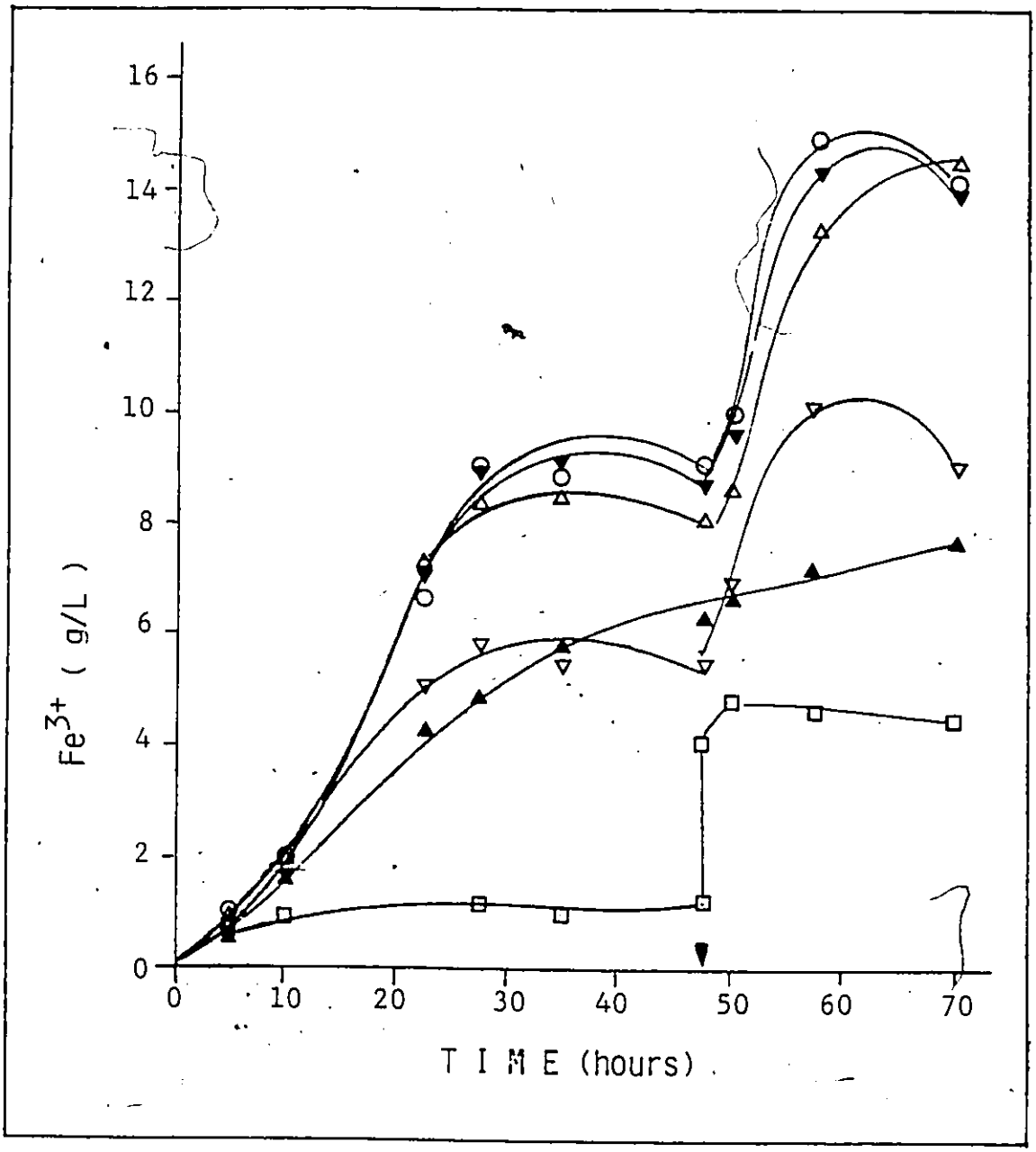
T. ferrooxidans growing on Fe^{2+}-9K medium in presence of 7.35×10^{-2}M $Cr_2(SO_4)_3$ was found to accumulate Cr^{3+} (Table II). More Cr^{3+} (about 10 times) was accumulated by cells growing at pH 3.5 than at pH 2.5. Ferrous iron oxidation was also inhibited to a greater extent by Cr^{3+} at the higher pH.

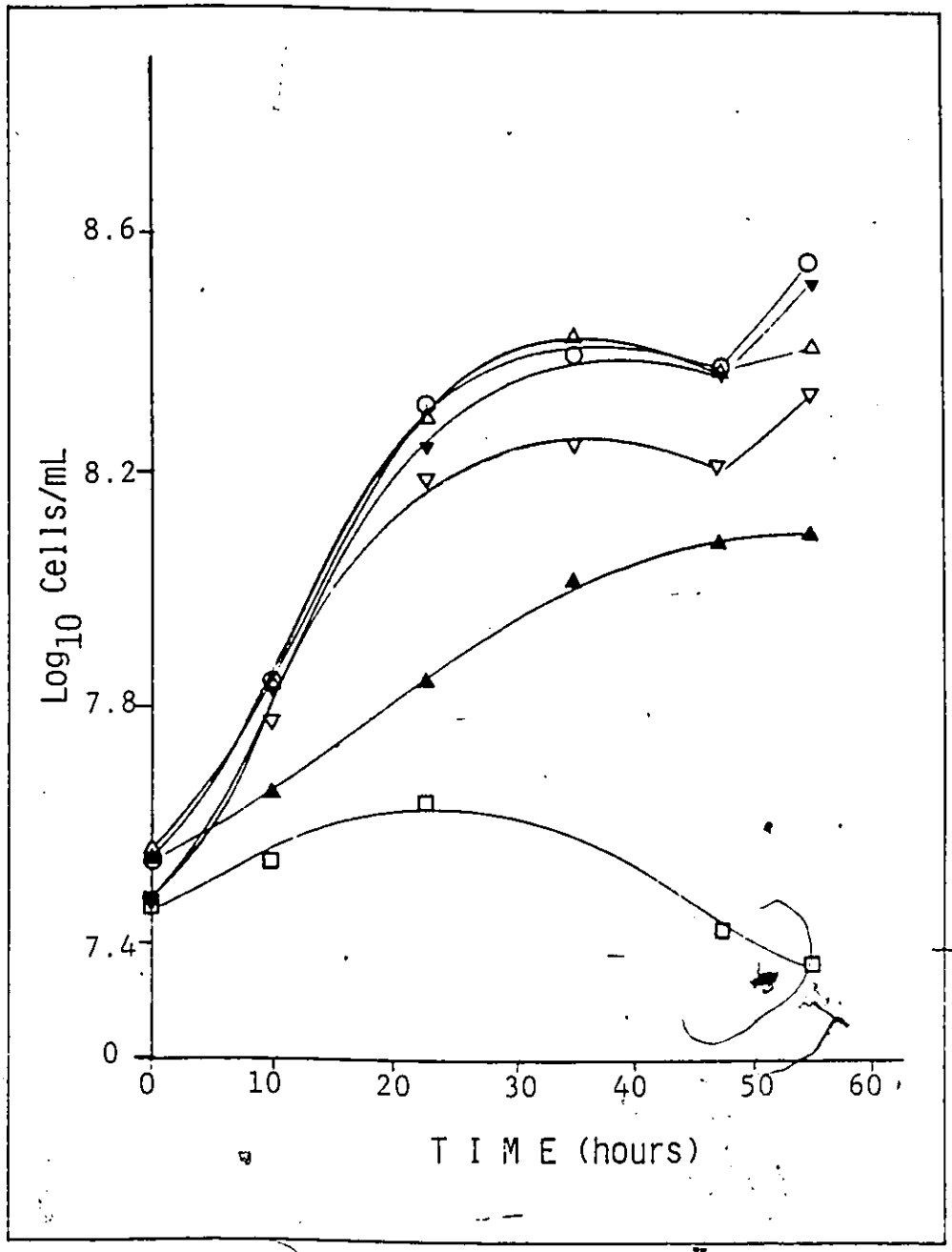
No cell-associated Mn was detectable during ferrous iron oxidation by T. ferrooxidans growing in the presence of 0.1M, 0.6M and 0.8M of $MnSO_4$.

FIGURES 4a AND 4b

Effects of chromium on the oxidation of Fe^{2+} (9,000 ppm, pH 2.5) and cell growth at 30^0C with T. ferrooxidans. At 48 hours. (arrow) 3g solid $FeSO_4$ was added.

(0) inoculated control
(△) in presence of 10^{-3}M $K_2Cr_2O_7$
(▽) in presence of 10^{-2}M $K_2Cr_2O_7$
(□) in presence of 10^{-1}M $K_2Cr_2O_7$
(■) in presence of 1.5×10^{-2}M $Cr_2(SO_4)_3$.
(▲) in presence of 7.35×10^{-2}M $Cr_2(SO_4)_3$.

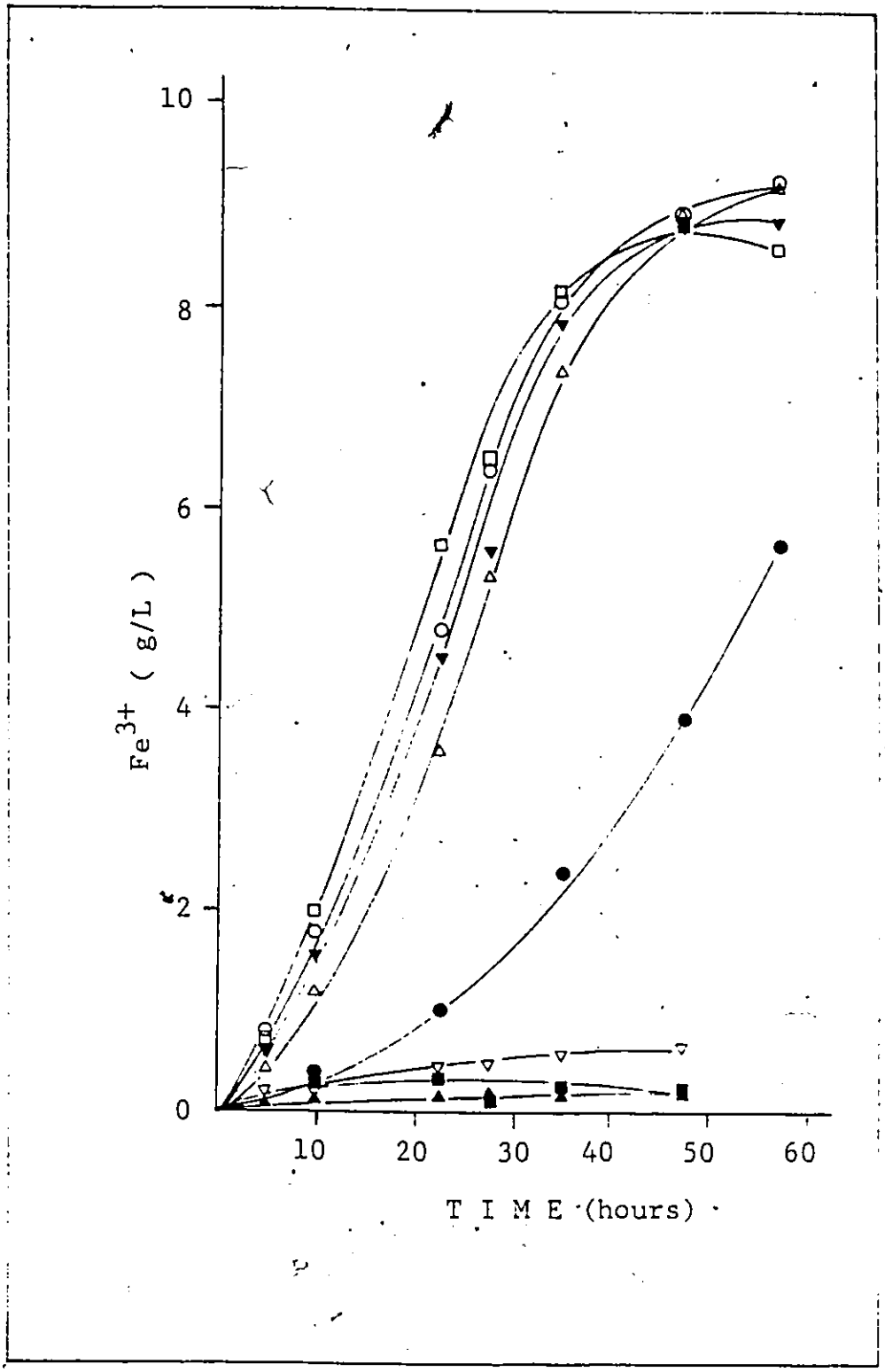

16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
Fe^{3+} (g/L)
T I M E (hours)

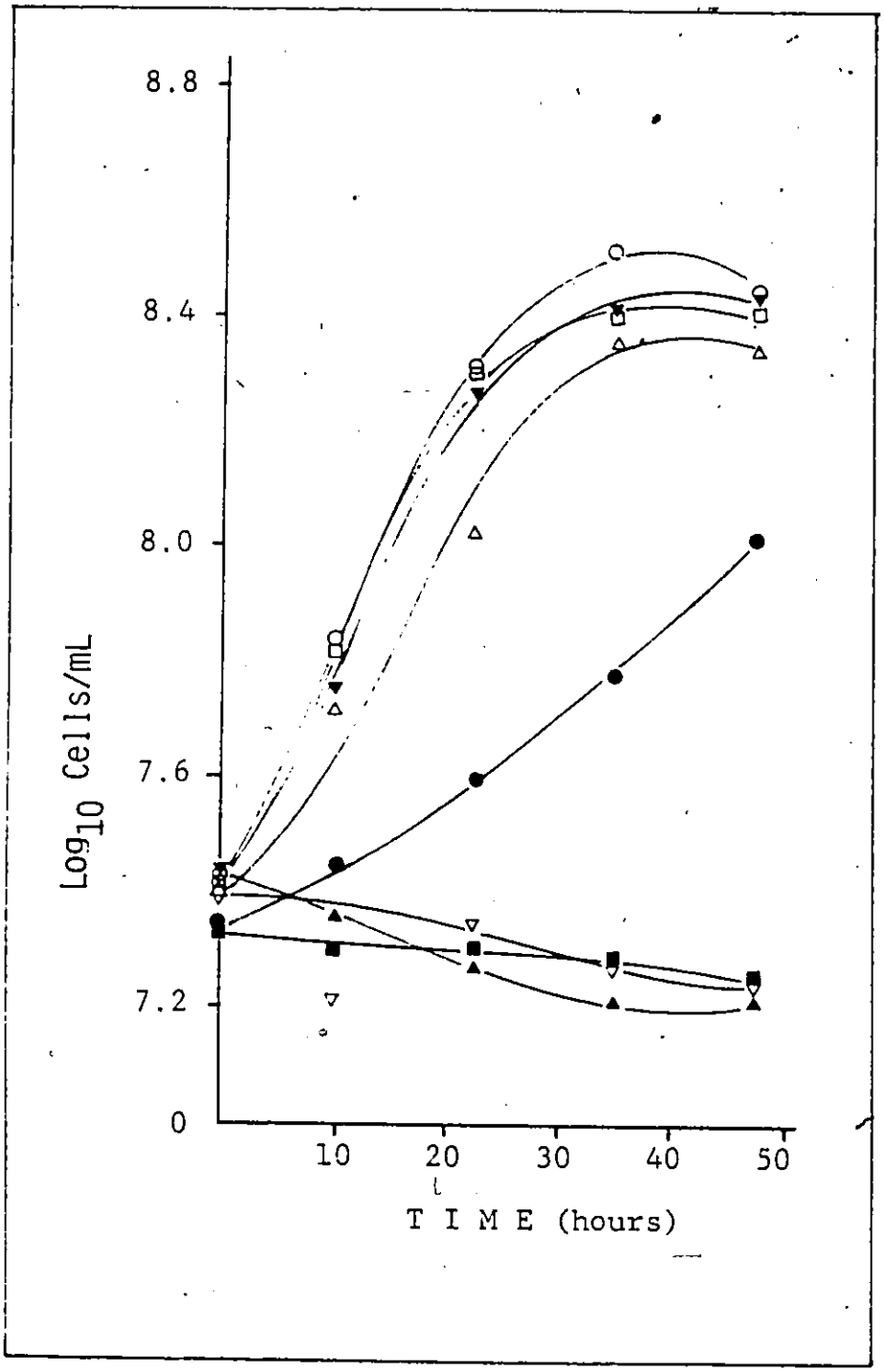

8.6
8.2
7.8
7.4
0
Log10 Cells/mL
0
10
20
30
40
50
60
T I M E (hours)

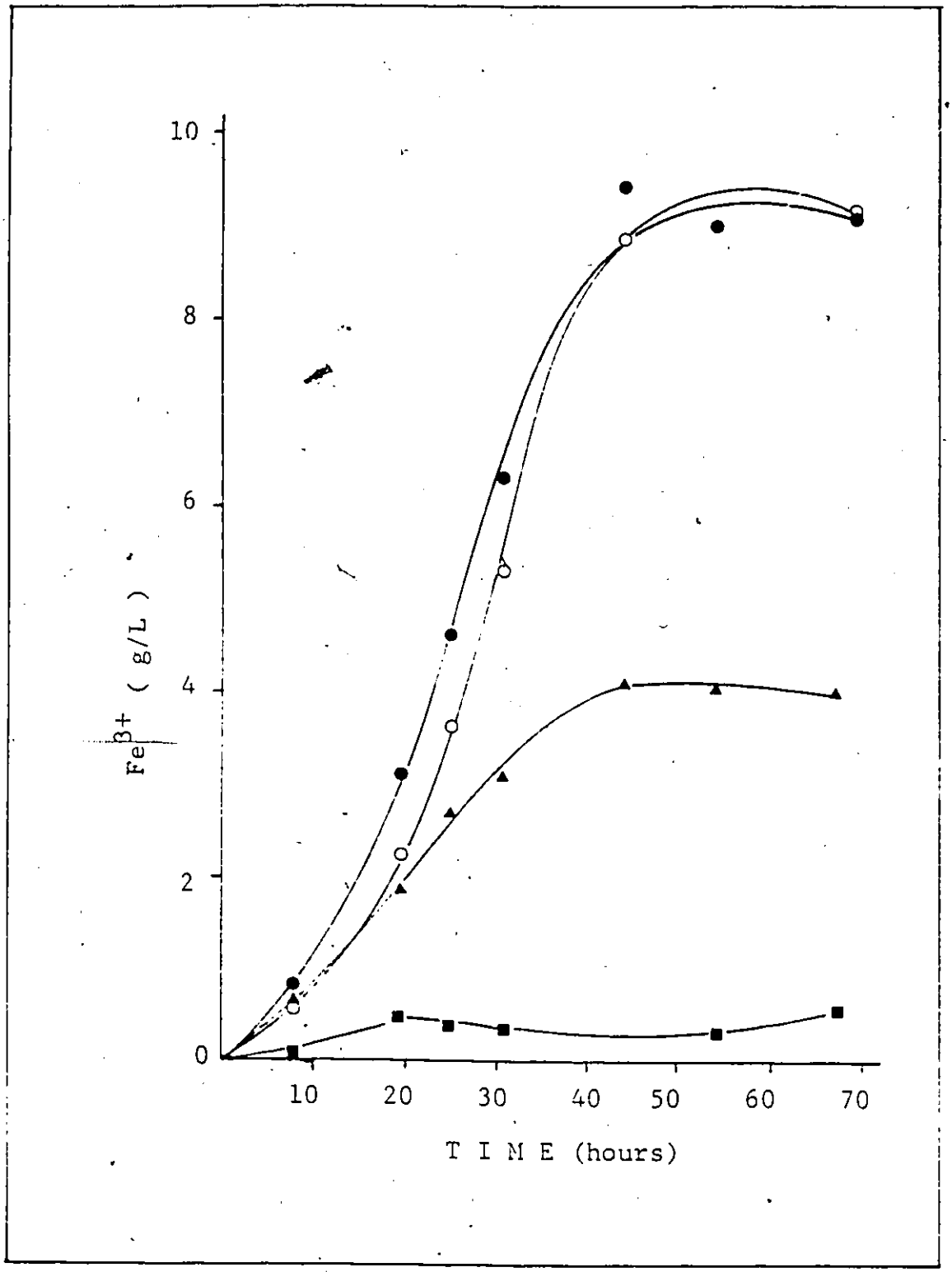
FIGURES 5a AND 5b

Effects of Mn^{2+} on the oxidation of Fe^{2+} (9,000 ppm, pH 2.5) and cell growth at 30°C with Thiobacillus ferrooxidans.

(○) inoculated control
(▽) uninoculated control
(□) in presence of 10^{-1}M of $MnSO_4.H_2O$
(△) in presence of 5 x 10^{-1}M of $MnSO_4.H_2O$
(●) in presence of 8 x 10^{-1}M of $MnSO_4.H_2O$
(▲) in presence of 1M of $MnSO_4.H_2O$
(▼) in presence 10^{-1}M of K_2SO_4
(■) in presence of 6 x 10^{-1}M of K_2SO_4


10
8
6
4
2
0
Fe^{3+} (g/L)
10
20
30
40
50
60
T I M E (hours)


8.8
8.4
8.0
7.6
7.2
0
Log10 Cells/mL
10
20
30
40
50
T I M E (hours)


10
8
6
4
2
0
Fe^{3+} (g/L)
10
20
30
40
50
60
70
T I M E (hours)

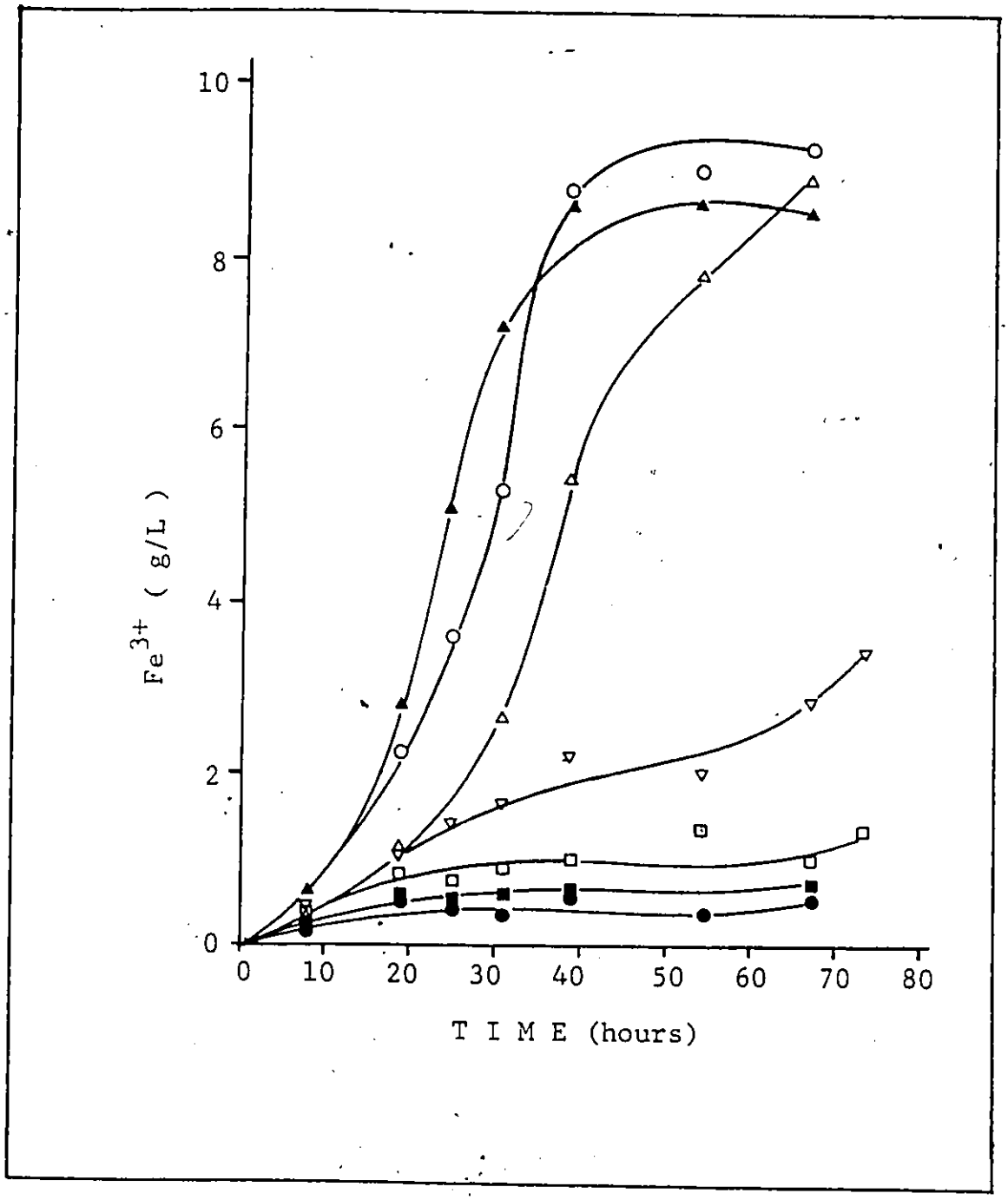

10
8
6
4
2
0
Fe^{3+} (g/L)
0
10
20
30
40
50
60
70
80
T I M E (hours)

TABLE II

Cell associated chromium in Thiobacillus ferrooxidans grown in the presence of 7.35×10^{-2}M $Cr_2(SO_4)_3$ with Fe^{2+}(9000 ppm) as substrate.

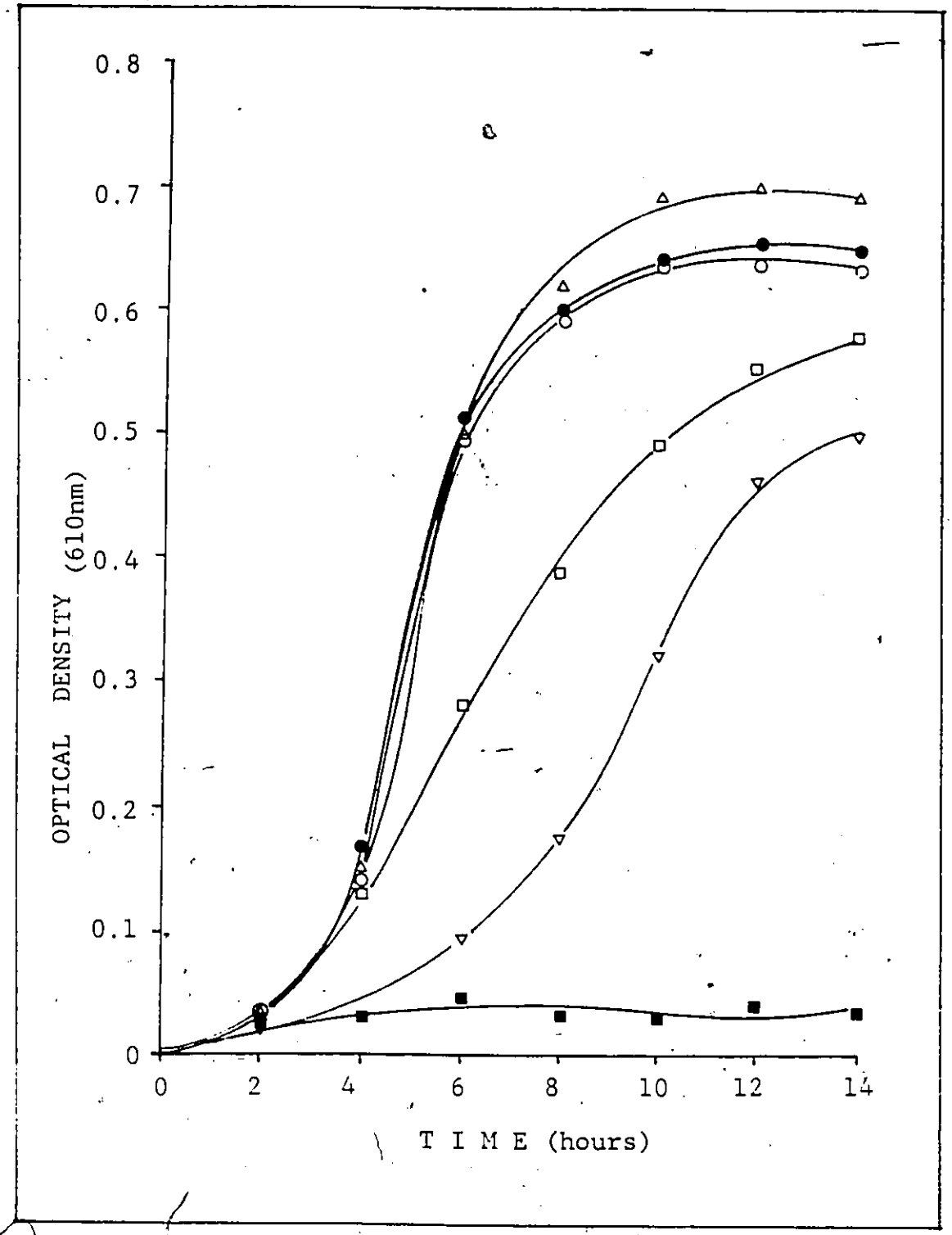
(A)	Time	Prior to filtration		Total recovery after filteration and washing			Cr^{3+} (µg/mg)
pH 3.5	(hr)	µg Fe^{3+}/ml	Cell x 10^8	Cell x 10^8	Protein(µg)	Cr^{3+}(µg/L)	protein
	0	0	68.00	-	250	12.5	0.050
	20.5	9	122.80	72.67	535	178.0	0.333
	41.5	23.82	184.40	98.75	930	245.0	0.263
	65.5	21.47	86.67	19.62	150	185.0	1.233
	88.5	23.55	73.33	19.32	120	160.0	1.333
(B)							
pH 2.5	0	0	60.00	43.00	305	20.0	0.066
	20.5	15.24	130.50	68.24	540	62.5	0.116
	41.5	33.24	195.13	98.16	890	75.0	0.083
	65.5	39.47	236.60	122.28	1180	87.5	0.074
	88.5	49.86	396.10	131.42	1190	117.5	0.099

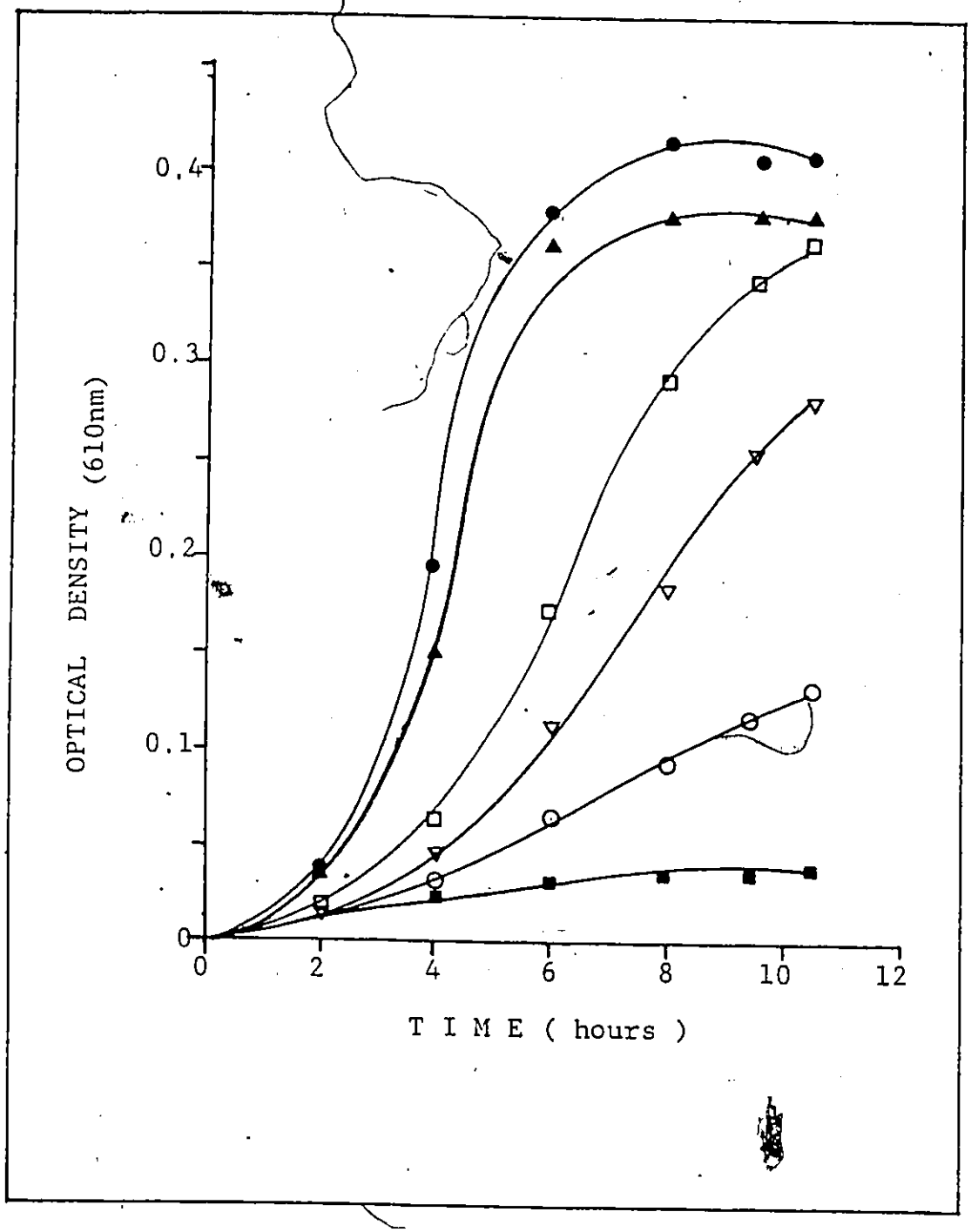
TABLE III

Rate of oxygen consumption by iron, chromium and manganese grown T. ferro-oxidans with ferrous sulfate as substrate in the presence or absence of chromium and manganese.

	33.778mM $FeSO_4$		180mM $FeSO_4$	
Inhibitor	Iron Grown Cells	Inhibitor Grown Cells	Iron Grown Cells	Inhibitor Grown Cells
Chromium (Cr^{3+})				
None (Control)	100(2.63)	100(2.87)	100(2.21)	100(2.44)
1.50×10^{-2}M	100.7	100	97.3	97.6
7.35×10^{-2}M	71.9	56.5	72.8	71.3
Manganese				
none (Control	100(2.63)	100(3.13)	100(2.21)	100(2.57)
1×10^{-3}M	100	106.7	105	102.3
1×10^{-2}M	100	101	94.1	99
1×10^{-1}M	98	98.7	89.1	94
2×10^{-1}M	89.7	74.5	75.6	79.9
5×10^{-1}M	65.7	66.1	60.2	66.9
6×10^{-1}M	64.2	55.2	59.3	60.3
8×10^{-1}M	59.6	43.8	43	47.4
1 Molar	40	36.8	33.9	39
Potassium sulfate				
6.7×10^{-1}M	68.4	-	61.5	-
Sodium sulfate				
1 Molar	36.1	-	34.4	-

Figures show % of control activity. Figures in paranthesis show control activity in µmole min^{-1}.mg $protein^{-1}$. Inhibitor grown cells indicate washed cell suspensions previously grown in the presence of 10^{-3}M Mn^{2+} and Cr^{6+}.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY (610nm)
0
2
4
6
8
10
12
14
T I M E (hours)


OPTICAL DENSITY (610nm)
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
T I M E (hours)

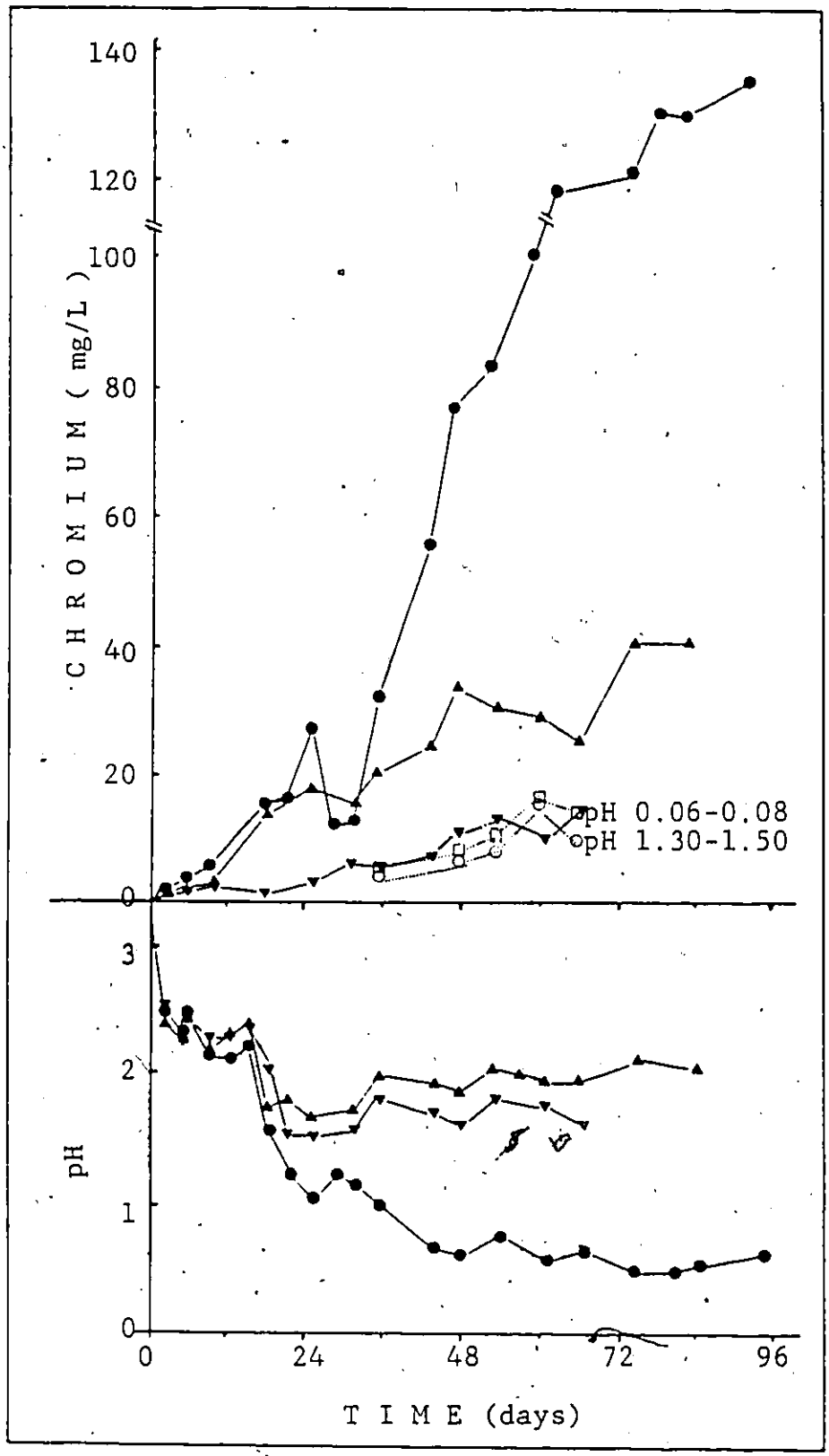
(D) Action of Thiobacillus ferrooxidans On Chromite Ore

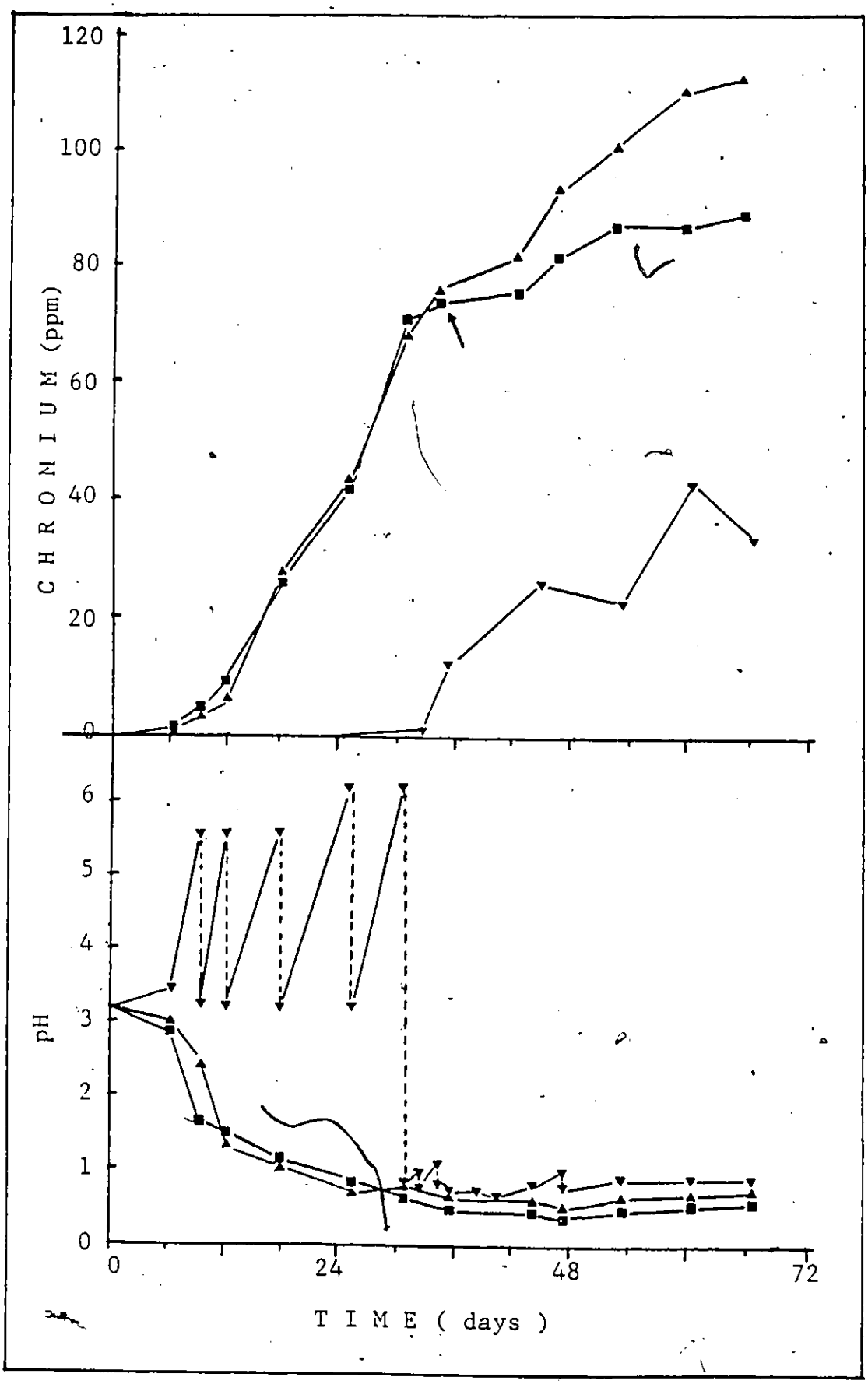
Because oxides of antimony, uranium and manganese were found to be solubilized by T. ferrooxidans (Lyalikova, 1972; Torma and Gabra, 1977; Brierley, 1978; Ivarson, 1980; Imai, 1978), and because chromite ore contain oxidizable ferrous iron and sometimes sulfur (Raicevic, 1976; 1977), it was thought that chromite ores could possibly be leached by this organism. This was made more probable by our finding, described above, that T. ferrooxidans is able to grow in high concentration of chromium.

When T. ferrooxidans was agitated in a flask at an initial pH of 3.2 in the presence of 6% pulp density chromite ore, about 40 mg/litre chromium was released into solution within 48 days (Fig. 10). In the presence of $FeSO_4$ (10 g/litre), only about half this amount of chromium was released into solution. In the presence of 12% pulp density chromite ore, similar amounts of chromium were found in solution after 60 days incubation (Fig. 11). When 1% elemental sulfur was used as substrate for bacterial growth, 130 to 140 mg/litre chromium was found in solution after 84 days in the presence of 6% pulp density chromite ore and after 60 days in the presence of 12% pulp density chromite ore.

The chromite ore contained alkaline gangue material that caused the pH to rise in the absence of T. ferrooxidans. Adjustment of the pH to 3.2 with H_2SO_4 during the first 30 days of the experiment did not result in further solubilization of chromium (Fig. 11). Adjustment to pH values less than one caused an increased chromium solubilization to a maximum of approximately 40 mg/litre. Addition of thymol, a bacteriostatic agent, at 37 days decreased the rate of chromium solubilization.

Analysis of the residue ores at the end of these incubation processes showed that little, if any, changes in their Cr:Fe ratios had occurred (Table IV). Because of the high initial Fe content of the concentrate (approximately 32%) a substantial amount of Fe would have to be released to make a measurable difference in the Cr:Fe ratio of the residue ores. The absolute amount released was probably very small.


CHROMIUM (mg/L)
140
120
100
80
60
40
20
0
pH 0.06-0.08
pH 1.30-1.50
pH
3
2
1
0
0
24
48
72
96
TIME (days)


120
100
80
60
40
20
0
CHROMIUM (ppm)
6
5
4
3
2
1
0
pH
0
24
48
72
TIME (days)

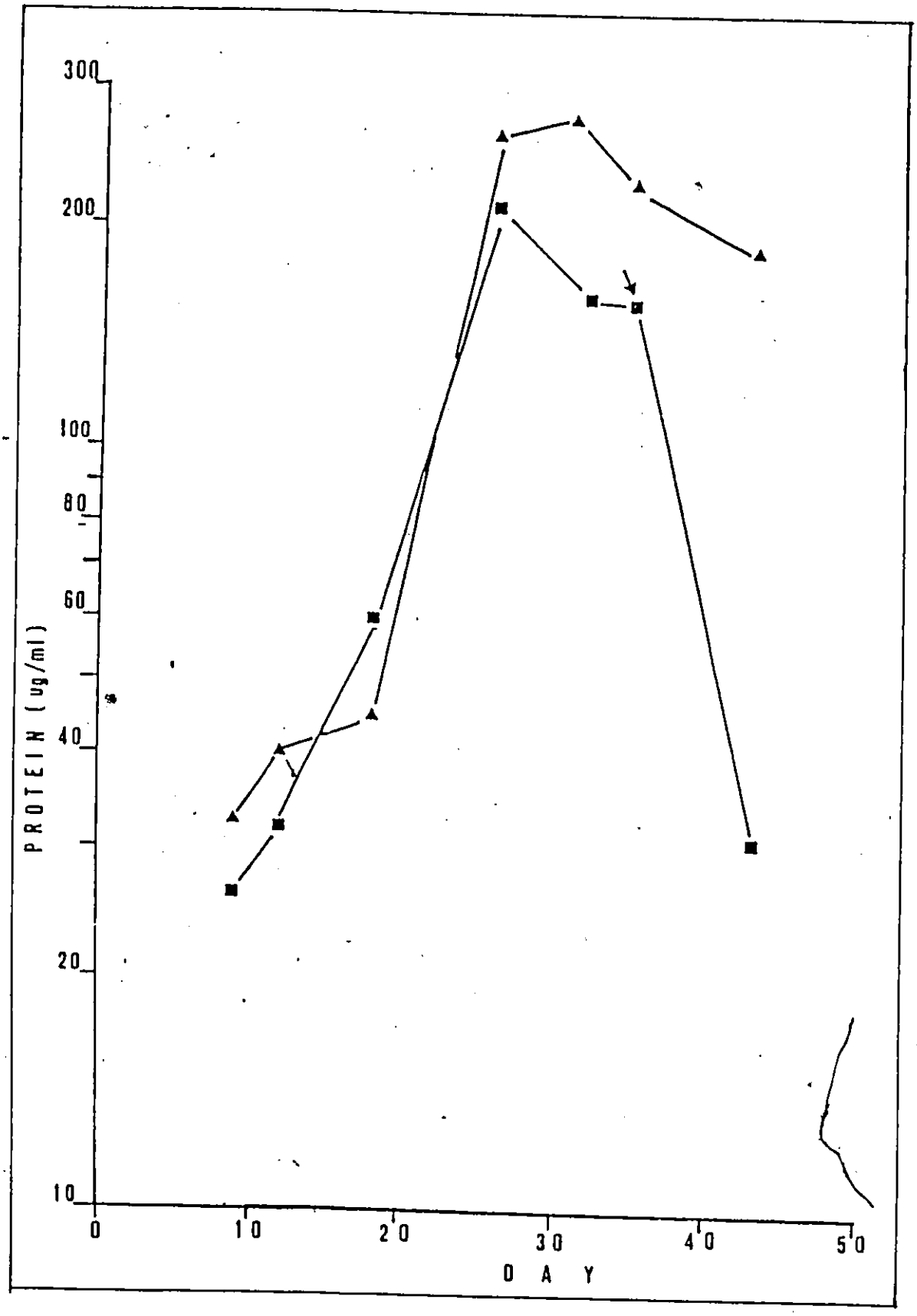

300
200
100
80
60
40
20
10
PROTEIN (ug/ml)
0
10
20
30
40
50
DAY

TABLE IV

Cr and Fe content of Chromite concentrate after treatment with *T. ferrooxidans*.

Expiremental Conditions			Total %			
P.D.	S	$FeSO_4$	Cr	Fe^{2+}	Carbon	Cr:Fe
6%	none	none	25.04	22.16	0.03	1.14 : 1
6%	1%	none	24.69	21.03	0.06	1.18 : 1
6%	none	1%	22.68	21.86	0.06	1.04 : 1
12%	1%	none	24.69	21.92	0.05	1.13 : 1
Unleached Samples			21.86	21.03	0.17	1.04 : 1

(E) Effects of Heavy Metals on Thiosulfate Oxidation by

Thiobacillus thiooxidans

The amounts of thiosulfate and polythionates are generally determined individually. Earlier analytical methods were complicated by the presence of sulfite and sulfide. However, suitable methods for the determinations of thiosulfate and polythionates concentration were developed by Makhuja and Hitchen (1977) and Cotton and Johnson (1976). Using these methods, the rates of oxidation of thiosalts by the thiobacilli in conditions similar to the mill circuits and tailing ponds were determined in the absence and presence of heavy metal cations.

Figure 13 illustrates the oxidation of thiosalts in the presence and absence of manganese ($MnSO_4$). The results are typical of the effect of metal cations on the oxidation of thiosulfate by T. thiooxidans. The pH of the medium was always found to increase initially. This was due to an alkali producing reaction of thiosulfate to tetrathionate (Sinha and Walden, 1966).

$$2\ Na_2S_2O_3 + H_2O + 1/2\ O_2 \dashrightarrow 2\ Na_2S_4O_6 + 2\ NaOH \qquad (22)$$

The above reaction, however, eventually proceeded less rapidly than the acid production by the combined biological and chemical oxidation of thiosalts to sulfate according to the following reactions:

$$S_2O_3^{2-} + 2H^+ \xrightarrow{\text{chemical}} H_2S_2O_3 \qquad (23)$$

$$H_2S_2O_3 \xrightarrow{\text{chemical}} H_2O + SO_2 + S \qquad (24)$$

$$H_2S_2O_3^{2-} \xrightarrow{\text{biological}} H_2S_4O_6 + 2H^+ \qquad (25)$$

$$S_4O_6^{2-} + O_2 + H_2O \xrightarrow{\text{biological}} S_3O_6^{2-} + SO_3^{2-} + 2\ H^+ \qquad (26)$$

$$S_3O_6^{2-} + O_2 + H_2O \xrightarrow{\text{biological}} S_2O_3^{2-} + SO_3^{2-} + 2\ H^+ \qquad (27)$$

$$Na_2S_2O_3 + 2\ O_2 + H_2O \xrightarrow{\text{biological}} Na_2SO_4 + H_2SO_4 \qquad (28)$$

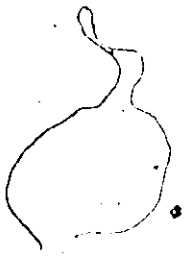
Ferrous iron at concentration up to 0.5M had no inhibitory effect on the oxidation of thiosulfate by T. thiooxidans. Concentration of ferrous iron higher than 0.5M were not investigated since the total concentration of iron in most tailing pond systems where thiosalts are present, is generally no greater than 40 mg/L (Schmidt and Conn, 1969). Under similar conditions, all other metal tested (cadmium, copper, chromium, ferric iron, lead, manganese, silver and zinc) exerted normal inhibition kinetics on the oxidation of thiosulfate by T. thiooxidans. Increasing the concentrations of these metals lengthened the lag period of thiosulfate oxidation from 24 hours to as much as 120 hours. The extended lag period was presumably used for adaptive changes in the cellular structure that would enable the bacteria to tolerate these heavy metals (Sadler and Trudinger, 1967).

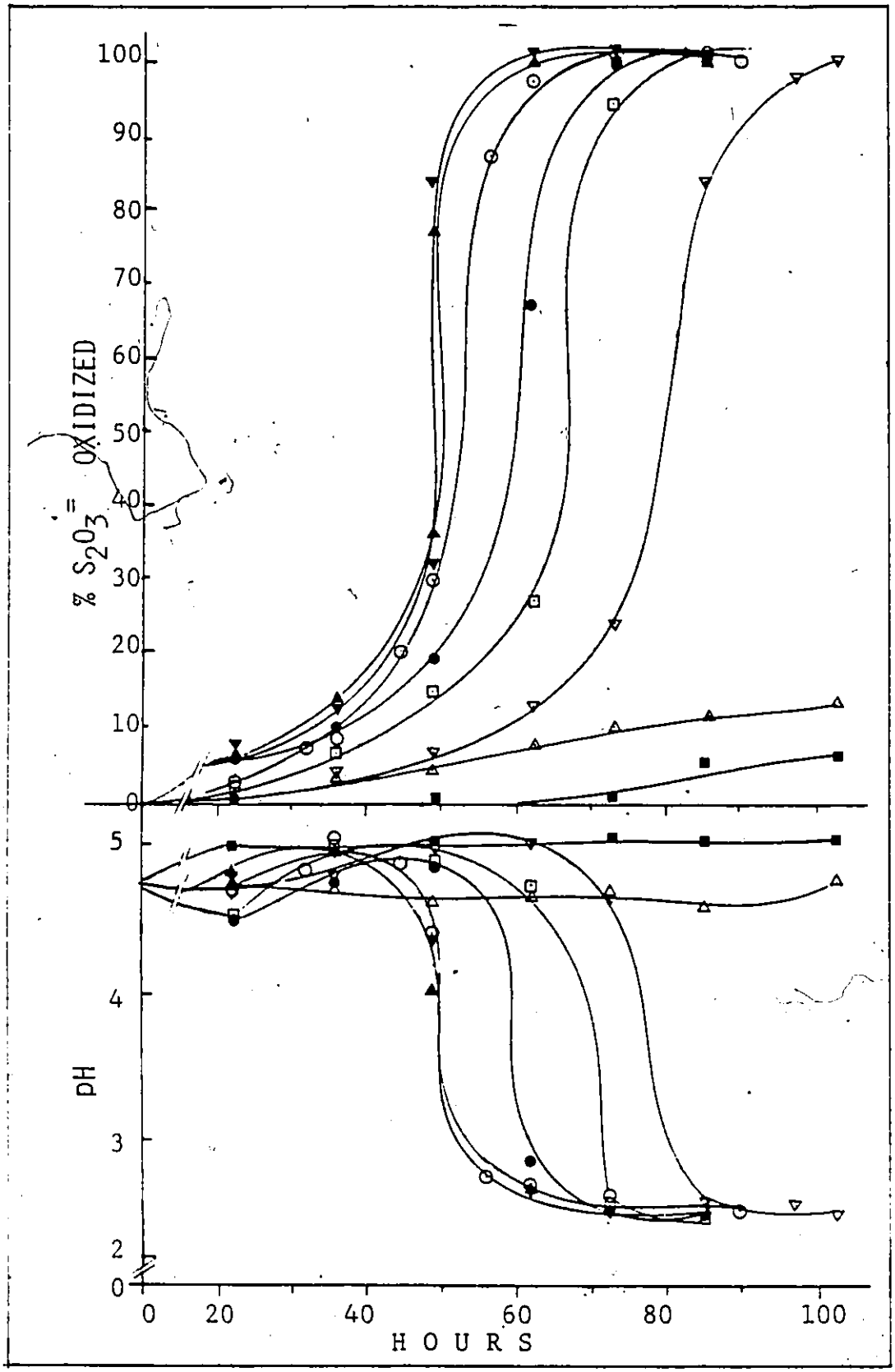
The inhibitor constant (K_i), was calculated by plotting the reciprocal of the oxidation rate versus metal concentration. This constant is the inhibitor concentration at which the reaction would proceed at half the maximum rate (V_{max}). Table V shows the K_i values of the metals studied on the oxidation of thiosulfate by T. thiooxidans, which are obtained from plots shown in Figs. 14 to 17. From these values a metal toxicity series is obtained:

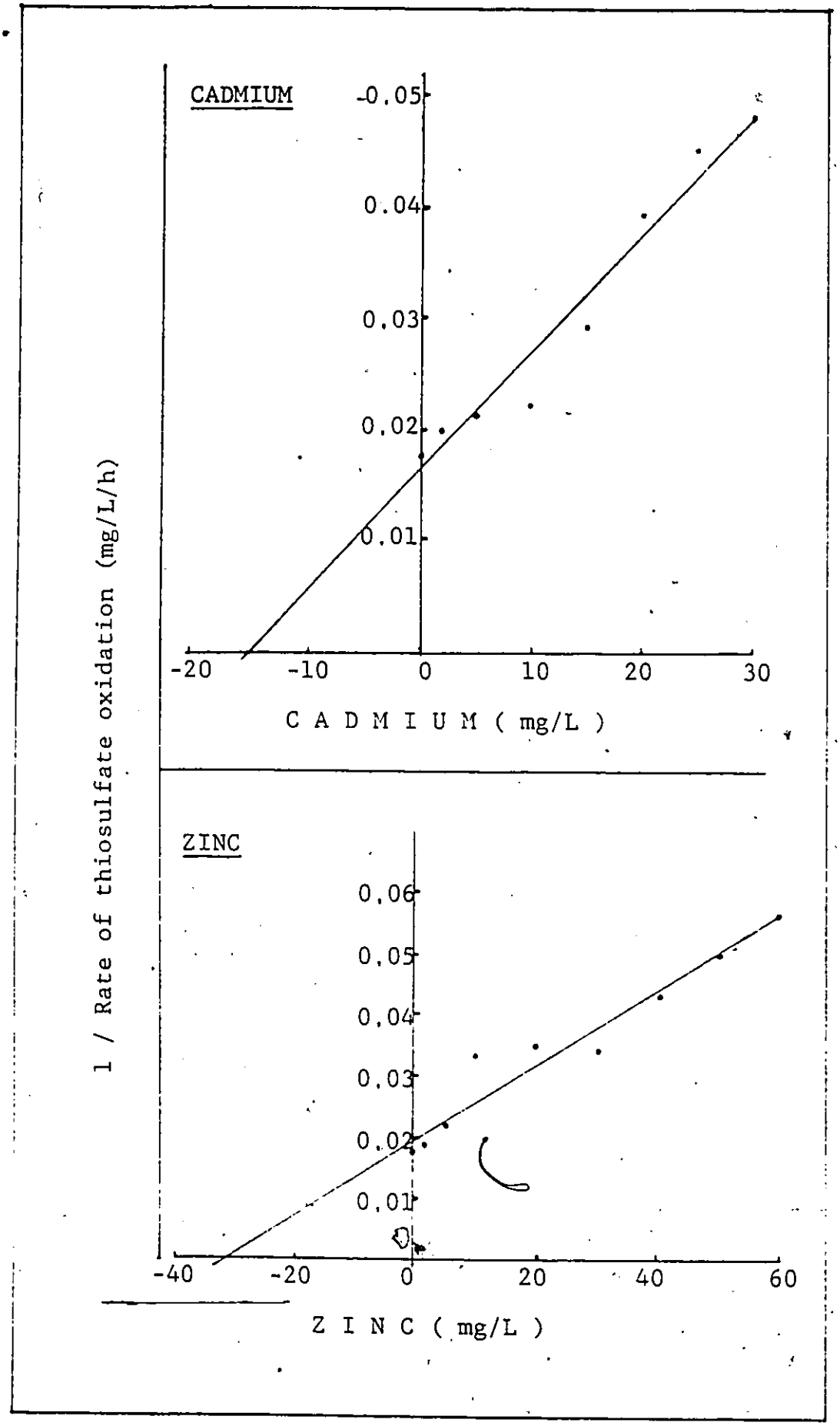
$$Cu^{2+} > Pb^{2+} > Ag^{+} > Cd^{2+} > Zn^{2+} > Cr^{6+} > Mn^{2+} > Cr^{3+} > Fe^{3+} > Fe^{2+}$$

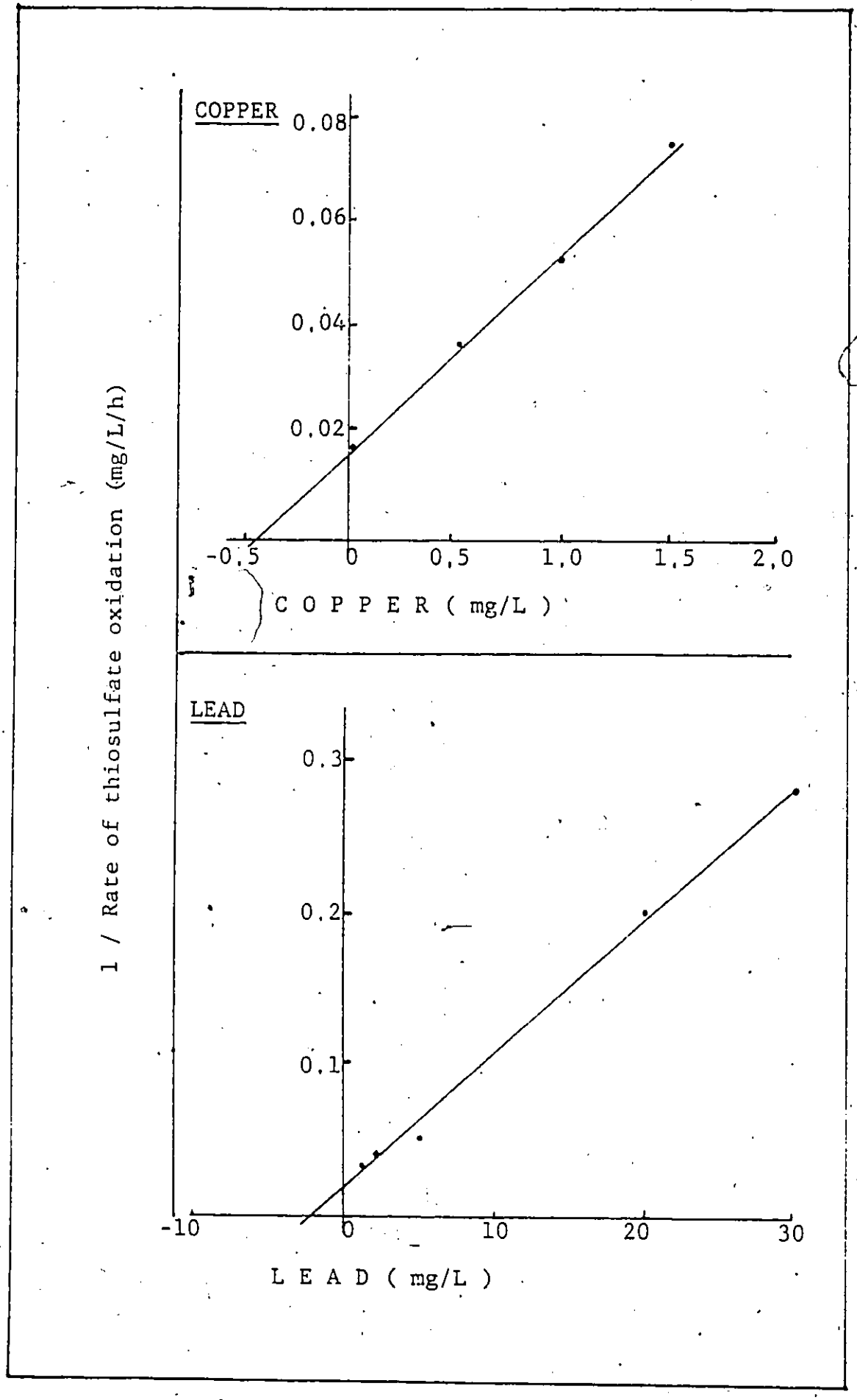
With the exception of copper, the toxicity increases with increasing atomic weight. Silver and Dinardo (1980) have carried out similar experiments under similar conditions and have shown that the inhibitory effects of the metals are not additive. They found that thiosulfate oxidation rates in the presence of combinations of different metals were only slightly lower than would be expected if each of the metals were present individually. The metals that were not included in the Silver and Dinardo study (chromium, manganese and

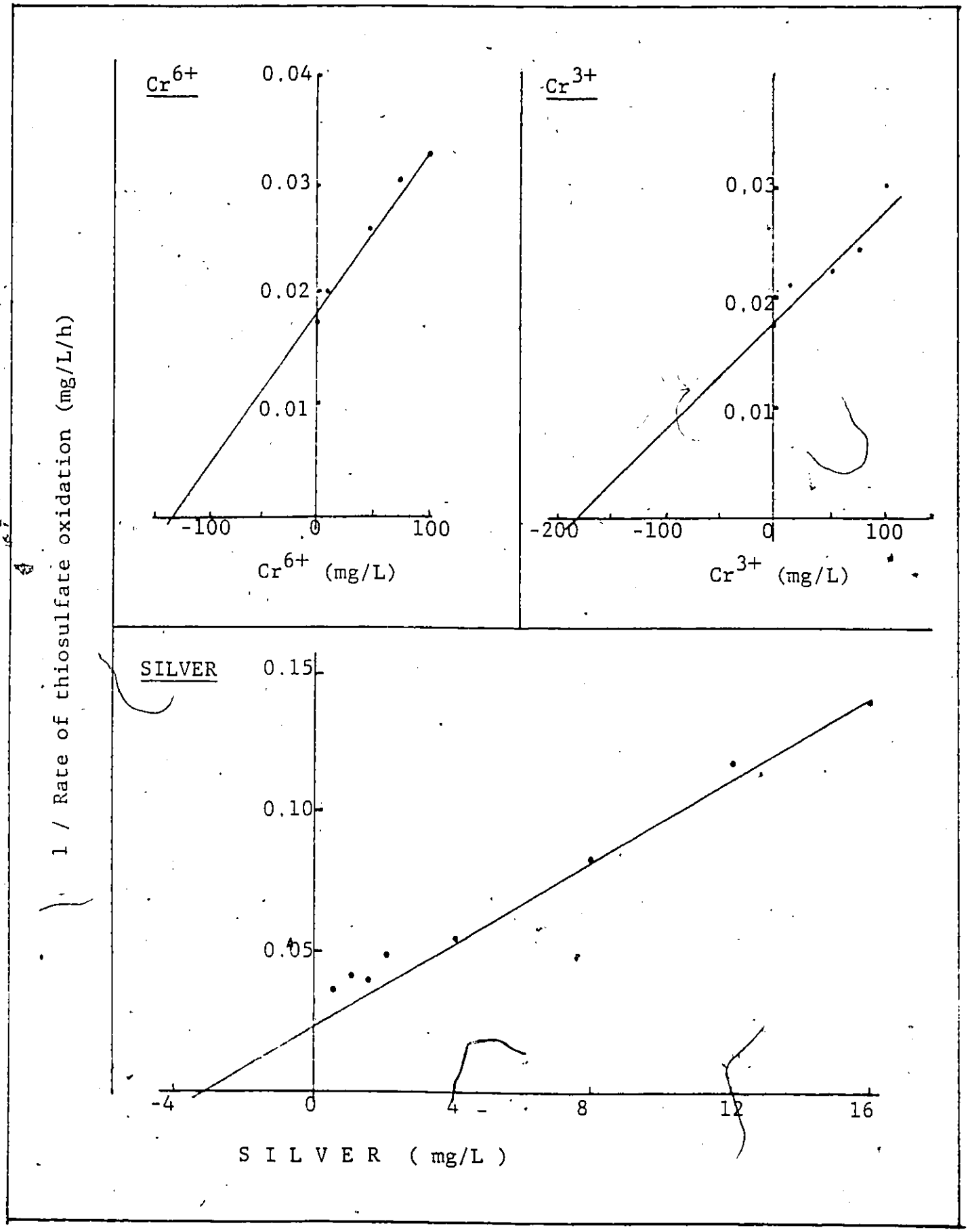
iron) were shown in this study to exert a lower degree of inhibition on thiosulfate oxidation by T. thiooxidans. All metals were shown, in both studies, to have an average V_{max} value of 52.56 mg/L/h for the oxidation of thiosulfate by T. thiooxidans.




% $S_2O_3^=$ OXIDIZED
100
90
80
70
60
50
40
30
20
10
0
pH
5
4
3
2
0
0
20
40
60
80
100
HOURS


CADMIUM
0.05
0.04
0.03
0.02
0.01
-20
-10
0
10
20
30
C A D M I U M (mg/L)
ZINC
0.06
0.05
0.04
0.03
0.02
0.01
-40
-20
0
20
40
60
Z I N C (mg/L)
1 / Rate of thiosulfate oxidation (mg/L/h)


COPPER
0.08
0.06
0.04
0.02
-0.5
0
0.5
1.0
1.5
2.0
C O P P E R (mg/L)
LEAD
0.3
0.2
0.1
-10
0
10
20
30
L E A D (mg/L)
1 / Rate of thiosulfate oxidation (mg/L/h)


Cr6+
0.04
0.03
0.02
0.01
-100
0
100
Cr6+ (mg/L)
Cr3+
0.03
0.02
0.01
-200
-100
0
100
Cr3+ (mg/L)
1 / Rate of thiosulfate oxidation (mg/L/h)
SILVER
0.15
0.10
0.05
-4
0
4
8
12
16
S I L V E R (mg/L)

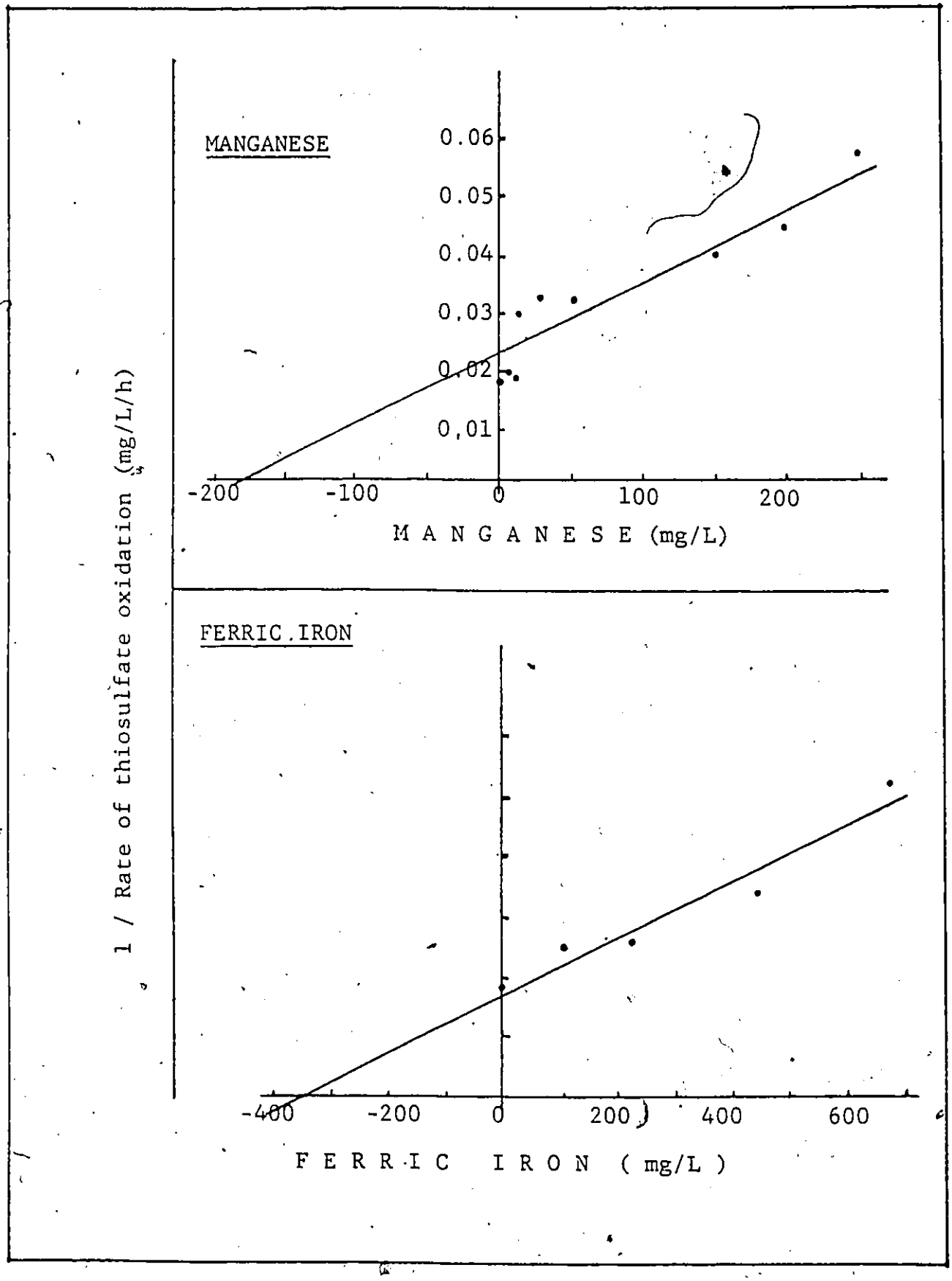

MANGANESE
0.06
0.05
0.04
0.03
0.02
0.01
-200
-100
0
100
200
M A N G A N E S E (mg/L)
FERRIC.IRON
-400
-200
0
200
400
600
F E R R I C I R O N (mg/L)
1 / Rate of thiosulfate oxidation (mg/L/h)

TABLE V

K_i values of metal cations of thiosulfate oxidation by Thiobacillus thiooxidans.

Metal	K_i (mg/L)
Cu^{2+}	0.46
Pb^{2+}	2.00
Ag^{+}	3.10
Cd^{2+}	16.00
Zn^{2+}	33.00
Cr^{6+}	130.00
Mn^{2+}	182.81
Cr^{3+}	187.50
Fe^{3+}	369.00

CHAPTER IV

DISCUSSION

(A) Effect of Chromium and Manganese on Iron Oxidation and Growth of T. ferrooxidans.

Studies on the effects of heavy metals on growing cells of the thiobacilli have often been complicated by lack of specific technique in the enumeration of the organism. Ferric iron produced during oxidation of ferrous iron by T. ferrooxidans was found, in this study, to interfere with the usual methods of measuring bacterial growth by cellular protein or DNA. At best, the activities of growing cultures of the thiobacilli are measured indirectly.

Thiobacillus ferrooxidans derives its energy from the oxidation of Fe^{2+} to Fe^{3+} according to equation 1 (p. 12). The rate of iron oxidation was shown to be directly associated with cell growth (Silverman and Lundgren, 1959). In the present study an adapted method of Schnaitman et al (1969) was conveniently used to follow the Fe^{2+}-oxidizing activity of the bacteria. Again we showed a close correlation existed between ferrous oxidation and cell growth. Generation time (6.5 ± 0.5 hours) during growth on ferrous sulfate was in close agreement with those previously reported (7 hours), with growth similarly measured by cell count (Silverman and Lundgren, 1959). The method is relatively fast and simple, and can be used for growth inhibition studies when the pH value employed is sufficiently low to ensure that ferric iron formed during growth remains in solution.

In contrast to most heterotrophic bacteria, the iron-oxidizing thiobacilli are 10 to 100 times less sensitive to the inhibitory effects of heavy metals (Weinberg et al, 1977; Cobet et al, 1971; Sadler and Trudinger, 1967). T. ferrooxidans is inhibited by 0.1 - 1M concentrations of aluminium, cobalt, nickel and zinc ions (Tuovinen and Kelly, 1974a, b; Tuovinen et al, 1971). The present study showed that growth of T. ferrooxidans was inhibited by manganese ion concentrations of more than 0.5M; respiration of non-growing cell suspensions was inhibited by concentrations of more than 1M. The oxidation of ferrous iron and growth was inhibited 30-40% by 7.35×10^{-2}M of Cu^{3+}. Other metal ions (Norris and Kelly, 1978; Hoffman et al, 1967; Tuovinen and Kelly, 1974b; Bhappu et al, 1965) exert a greater inhibition on iron-oxidizing thiobacilli; these ions include Ag^{+} (inhibitory at 10^{-7}M), Hg^{3+} (5×10^{-7}M), MoO^{2-} (3×10^{-5}M) and UO^{2+} (5×10^{-3}M).

In the present study, a small amount of Cr^{3+} was found to be cell associated. This amount increased at higher pH levels and was thought to be responsible for greater chromium sensitivity under the latter conditions (Table II). Norris and Kelly (1978) found that T. ferrooxidans accumulated 50 µmole Ag^{+} per gram dry weight. Calculations from Table II, assuming that cells are about 50% protein, gives 12 µmole per gram dry weight as the highest amount observed. The mechanism of chromium accumulation in the thiobacilli is not clear. Accumulation of chromic ions and growth inhibition by these ions have been studied in other microorganisms (Schroll, 1978; Nelson and Evans, 1969; Crowin et al, 1965). In aqueous solutions, chromium probably exists usually in the oxyanion configuration (CrO_4^{2-}), whose binding and uptake may be quite different from that of heavy metal cations. An energy-dependent

uptake of CrO_4^{2-} in Neurospora crassa via a sulfate (SO_4^{2-}) permease system was reported by Robert and Marzluf (1971). A similar sulfate permease system has not been reported in the thiobacilli.

The degree of inhibition of Fe^{2+} oxidation by Cr^{3+} is less at higher Fe^{2+} ion concentration (Table II), suggesting that Cr^{3+} ions may bind to the cell surface in competition with Fe^{2+} ions, thus affecting the iron-oxidizing systems of the cell. Yates and Nason (1966) found that 10^{-3}M chromium inhibited the activity of iron-cytochrome c reductase of T. ferrooxidans by 18%. This enzyme, comprising part of the iron oxidase system, is thought to be membrane associated. Therefore, it could be inhibited by Cr^{3+} without this cation entering the cytoplasm. Inhibition of the iron oxidase system might also inhibit respiratory mechanism of eliminating the protons from the cells via the transmembrane pH gradient coupled to a chemiosmotic ATPase reaction (Ingledew et al, 1977).

The morphological changes of the cells exposed to high concentrations of Cr^{3+} may be due to an interference by this cation with the normal processes of cell wall synthesis and cell division. Alternatively, these changes could be due to a response to reduced oxygen tension in the environment (Silverman and Rogoff, 1961).

Mutants of E. coli with damaged iron transport systems were relatively sensitive to chromic ions (Wang et al, 1969); the addition of iron during growth appreciably relieved this inhibition. The effects of Cr^{3+} on the iron oxidizing thiobacilli were not relieved by the addition of iron during growth (Figs. 4a and 4b). However, these damaged cells, when subcultured to chromium-free 9K medium, were observed (result not shown) to resume their normal rate of iron oxidation and revert to their normal cell morphology.

The effects of Cr^{6+} and Mn^{2+} are unclear. Much of the ferrous iron will be oxidized according to the following equation:

$$K_2Cr_2O_7 + 6FeSO_4 + 7H_2O \longrightarrow Cr_2(SO_4)_3 + 7H_2O \qquad (29)$$

when the concentration of $K_2Cr_2O_7$ reached 10^{-2}M. Morphological changes were not observed in the presence of either Cr^{6+} or Mn^{2+}.

In E. coli, manganese was shown to be actively transported and accumulated by the cells without any adverse effects on the growth of the organism and the manganese uptake system. This manganese transport system is, nevertheless, competitively inhibited by iron (Wang et al, 1969).

The presence of a similar transport system in T. ferrooxidans was not demonstrated in this study. Essentially no accumulation of manganese by the growing iron-oxidizing thiobacilli was detected. In acidic media, tolerance of the acidophlies to most metals probably results from the effective competition of H^+ ions for negatively-charged sites at the cell surface. Oxyanions of arsenate, selenate, tellurate and molybdate are more toxic than most cations of T. ferrooxidans (Touvinen et al, 1971) perhaps through the relative ease of excess, without H^+ ions competition, to cells. The results from the present study indicated that the relatively low toxicity of manganese and chromium on T. ferrooxidans may, in part, be due to the high concentration of H^+ competing for the negatively-charged sites at the cell surfaces. Considering that sodium sulfate (0.1M) and potassium sulfate (6.7×10^{-1}M) inhibited iron oxidation by non-growing cell suspensions of T. ferrooxidans to about the same level (60%) as did manganese sulfate (Table III), the effect may have also been partly due to its high ionic concentration rather than to a specific Mn^{2+} effect. Indeed, sodium sulfate (IN) will reduce CO_2 solubility by about 32% (Glasstone, 1953). This will eventually slow down the process of

biosynthesis and the rate of ferrous iron oxidation as the demand for energy decreases.

(B) Leaching of Chromite Ore by T. ferrooxidans

Chromite has a cubical spinal crystal structure. The main constituents of this mineral ore are FeO (32%) and Cr_2O_3 (68%). Manganese, aluminium, sulfur, phosphorous and large amounts of alkaline gangue materials are normally present in the ore (Raicevic, 1977, 1976). Since the sulfur and iron molecules in the chromite structure are not associated as sulfides, any direct bacterial action on the chromite mineral is questionable. Direct oxidations of the metallic moities of minerals by T. ferrooxidans have been suggested. These moities include: antimony as in Sb_2S_3 and Sb_2O_3 (Lyalikova, 1972; Torma and Graba, 1977); copper in Cu_2S (Beck, 1977; Imai, 1978) and uranium in UO_2 (Ivarson, 1980; Soljanto and Tuovinen, 1979). However, the results here do not suggest that bacteria oxidize chromium ore directly. Rather, they probably act on the ore through H_2SO_4 formation. The following reactions may occur:

$$FeO + H_2SO_4 \longrightarrow FeSO_4 + 3\ H_2O \qquad (30)$$

$$Cr_2O_3 + 3\ H_2SO_4 \longrightarrow Cr_2(SO_4)_3 + 3\ H_2O \qquad (31)$$

Thus in the absence of iron oxidizing bacteria, as observed in the control flasks, solubilization of chromium depends mainly on the added H_2SO_4. In the presence of iron-oxidizing bacteria, the $FeSO_4$ formed (equation 30) was oxidized (equation 1), and additional H_2SO_4 formed (equation 35). Hence, there was a slight increase of chromium solubilized in the inoculated flask. The addition of elemental sulfur increased the metal solubilization process, mainly through the formation of more acid upon its oxidation by iron-oxidizing thiobacilli (equation 32) and possibly through oxidation of H_2S (equation 33)

since H_2S was produced in cultures of thiobacilli (Imai et al, 1962; Starkey, 1937).

$$S^0 + H_2O + O_2 \longrightarrow H_2SO_4 \qquad (32)$$

$$H_2S + 2\ O_2 \longrightarrow H_2SO_4 \qquad (33)$$

Sulfide may be produced by the oxidation of thiosulfate, which is formed during sulfur oxidation (Silver and Lundgren, 1968) by the following reaction:

$S_2O_3^{2-}$ + 2 GSH (reduced glutathione) $\xrightarrow{\text{thiosulfate reductase}}$

$$SO_3^{2-} + H_2S + GSSG \qquad (34)$$

Although ferrous sulfate is readily oxidized by the iron-oxidizing thiobacilli, its addition to the growth medium did not enhance the leaching process. This may be explained by the fact that both secondary ferric sulfate minerals, such as jarosite, and sulfuric acid are formed according to the equation:

$$3Fe_2(SO_4) + 12\ H_2O \longrightarrow 2[H[Fe(SO_4).2Fe(OH)_3]] + 5\ H_2SO_4 \qquad (35)$$

The secondary ferric iron minerals tend to coat the mineral surface and thus impede action of H_2SO_4 on this surface.

That bacterial growth influenced the leaching of chromite ore was demonstrated by the decrease in the rate of chromium solubilization (Figs. 11 and 12) in the presence of thymol, an inhibitor of iron-oxidizing bacteria.

In this work the amount of Cr solubilized by bacterial action (highest concentration measured - 140 mg/L) was not sufficient for commercial exploitation on a large scale, which required approximately 0.3 g/L Cr. The Cr:Fe ratio of the ore was not increased to the required commercial value (2 to 3: 1). However, this bacterial action is certainly of environmental

concern. The recommended upper concentration of chromium in raw waters is 0.1 mg/L (Environment Canada, 1969). Our results showed that the action of iron-oxidizing bacteria on chromite could result in concentrations of chromium to be more than a thousand times that of the permissible level.

(C) Heavy Metal inhibition of Thiosulfate Oxidation by T. thiooxidans.

The inhibition of thiosulfate oxidation in T. thiooxidans by some heavy metals was also studied. During milling and flotation of sulfide ores, high concentrations of thiosulfate and polythionates are formed. Much of the thiosalts will oxidize abiologically to sulfuric acid in the milling circuits or tailing ponds, which can then be neutralized with lime before discharging to rivers (Rolia and Barbeau, 1978; Scott and Bragg, 1975). This oxidation is slow and seldom complete before discharging into the surface water of the receiving streams. Sulfuric acid will continue to be generated resulting in the formation of very acidic conditions deleterious to fish, heterotrophic bacteria and other aquatic life. At Brunswick Mining and Smelting, for instance, pH values of approximately 3 developed in the receiving streams 20 Km from the discharge point, although the pH on the tailing ponds system was adjusted to approximately 10 before their discharge (Schmidt and Conn, 1969). Since T. thiooxidans oxidizes thiosulfate and polythionates to sulfuric acid, this microorganism might be used to accelerate and complete the oxidation of these compounds in the treatment ponds before the neutralization process. Toxic metals that are likely to limit such method are examined in this study.

Under optimal conditions (temperature 32.5°C, pH 3.75), T. thiooxidans ATCC 8085 oxidizes thiosulfate at the rate of 55 ± 3mg/L/h (Silver and

Darnido, 1981). Cadmium, copper, lead, silver and zinc are inhibitory; this inhibition is not cumulative in the presence of combinations of these cations. The almost indiscriminate inhibition of these heavy metal cations on thiosulfate oxidation probably resulted from their competition with thiosulfate for the same active sites, which are located at the cell membrane (Ostrowski and Krawczyk, 1957). These may consist of di-sulfide groups which could react with thiosulfate to form sulfenyl-thiosulfate (Lees, 1966; Visniac and Trudinger, 1962).

$$R\text{-}S\text{-}S\text{-}R + S_2O_3^{2-} \longrightarrow R\text{-}S\text{-}S\text{-}SO_3 + RS^- \qquad (36)$$

Sulfhydryl groups may also participate by a nucleophilic reaction, breaking the R-S-S-R bond. Trudinger (1965) showed that SH binding agents inhibited the oxidation of thiosulfate in T. neapolitanus. Heavy metal cations may combine with SH groups and catalyse the breaking of S-S bonds, thus interfering with thiosulfate oxidation. Among the metals tested in this study, cadmium has the highest affinity towards -SH groups. However, copper, lead and silver are more toxic towards thiosulfate oxidation by T. thiooxidans.

Silver (Norris and Kelly, 1978) and chromium are accumulated by cells of T. ferrooxidans. Lead, silver and cadmium also accumulate in cells of E. coli (Ulmer and Vallee, 1969; Bragg and Rainnie, 1974; Mitra and Bernstein, 1977). Similarly, heavy metal accumulation in T. thiooxidans is possible and may be the reason why thiosulfate oxidation in T. thiooxidans is more severely affected by silver and lead despite the higher affinity to -SH groups of cadmium, since accumulated silver and lead have had a more permanent effect on E. coli. Cadmium in E. coli interacts with DNA and causes single strand breaks. These breaks were eventually repaired during a long lag phase and the cells eventually resumed growth at the normal rate (Mitra and Bernstein,

1977). Toxicity due to lead and silver on the same organism was more permanent. Lead inhibited amino acid incorporation into tRNA by inhibiting the synthesizing enzyme and/or by binding to and hydrolyzing tRNA (Ulmer and Vallee, 1969), and silver disrupted the respiratory chain (Bragg and Rainnie, 1974). The most sensitive sites to inhibition were in the oxidative phosphorylation system between b-cytochrome and cytochrome a_2.

The presence of plasmids conferring partial resistance to cadmium toxicity was reported in Staphylococcus aureus (Perry and Silver, 1982). These plasmids carry genes for a cadmium efflux system which decreases cadmium toxicity by decreasing cadmium accumulation. Plasmid-determined resistance to metals in the thiobacilli is also possible (Tuovinen and Kelly, 1974; Dispirito et al, 1981), but proof of its actual presence must await more definitive studies.

Chromates and dichromates were shown to penetrate cell membranes to a much greater degree than Cr (III) compounds (NAS, 1974). This may possibly explain why Cr (VI) was more toxic than Cr (III) to thiosulfate oxidation by T. thiooxidans.

Although mixing and pH are not controlled, the tailings pond system is similar to a large chemostat, with thiosalts, present mostly as thiosulfate, normally being added at concentrations of about 1 g/L and a pH of approximately 11.5 (Schmidt and Conn, 1969; 1971). The usual concentration ranges of various metal cations are: copper (0.01-0.75 mg/L), zinc (0.04-2 mg/L), lead (0.3-11 mg/L) and iron (0.5-4 mg/L). Of all the metals tested, only lead is found in the tailings pond system in concentrations which could cause substantial inhibition of thiosalt oxidation. Silver and Dinardo (1981) estimated that about 65% of the thiosulfate-oxidizing activity would remain at lead concentration of about 11 mg/L.

Concluding Remarks

From the standpoint of this investigation, recommended future studies on the acidophilic thiobacilli may be briefly outlined.

The effects of heavy metals on the thiobacilli are usually investigated with heavy non-growing cell suspensions in Warburg apparatus (Tuovinen et al, 1971, 1972, 1974). Due to the lack of specific techniques for enumerating the organism in the presence of ferric iron, metal toxicity on growing cultures of the thiobacilli was little studied. Since metal toxicity is probably influenced by the physiological state of the organism, much can be accomplished if future studies will include a comprehensive comparations on the response to heavy metals by growing and non-growing cells of the thiobacilli. The present study showed that ferrous iron oxidation in growing cultures of T. ferrooxidans was more sensitive than the growth of the organism to the inhibitory effects of chromium and manganese. This inhibition was greater at higher pH values. This is comparable to the findings on non-growing bacterial suspension of T. ferrooxidans in the Warburg apparatus, in which carbon dioxide production was found to be more sensitive to metals than was ferrous iron oxidation (Tuovinen and Kelly, 1972).

In the present study, the toxicity of chromium on T. ferrooxidans appeared to be related to the accumulation of the chromium by the cells. This accumulation was greater at higher pH values up to an estimated 12 µmole/ g dry weight at pH 3.5. The possibility of T. ferrooxidans having an active manganese transport system similar to that of E. coli was not investigated. No accumulation of manganese by the cells of T. ferrooxidans during iron oxidation has been detected. The mechanisma of chromium accumulation has not been investigated. Since metal accmulation in most microorganisms frequently

occurs through specifie transport pathways, future studies to evaluate the mechanism of accumulation of toxic metal cations in the thiobacilli, should include specific inhibitors or competitors of cation transport.

The key to the leaching mechanisms of minerals by the thiobacilli rests primarily on the availability of sulfur or oxidizable metals and the chemical form and valence state of metals in the mineral lattice. Studies of the susceptibility of mineral sulfides to bacterial attack have been restricted to two main groups of mineral structure - cubic and hexagonal with their subdivisions of a limited number of basic structure (Kelly et al, 1979). The present study suggests that chromite ores, with cubical spinal crystal structures, are leached by T. ferrooxidans by an indirect process, through the action of H_2SO_4. Although chromium concentrations as high as 140 mg/L were solubilized from the chromite ore, the benefication of chromite ore by T. ferrooxidans did not appear to be economically feasible. However, the present economic trends and enviromental restrictions dictate that biohydrometellurgical process will be competitive with other recovery methods.

The acidophilic thiobacilli, like various heterotrophic bacteria, are likely to carry resistant factors for metals on transmissible plasmids. Substantial progress to increase the leaching rates of specific minerals is likely to come through the genetic selection and manipulations of these organisms. The development of solid differential media to obtain growth of single colonies of the thiobacilli will doubtless contribute immensely to the long awaited progress in the biotechnology of the thiobacilli.

The feasibility of using T. thiooxidans for the treatment of thiosulfate and polythionates in controlled biostabilization ponds is very

encouraging because of this organism's insensitivity to heavy metals. Of the metals tested, only lead could be found in concentrations that would cause substantial inhibition of thiosalt oxidation. Further studies under suboptimal conditions likely to be present in operating plants are required. Complex mutalistic relationships of the thiobacilli and other heterotrophic bacteria or fungi are likely to exist which would enhance (or inhibit) the oxidation process. Studies on the interactions of these organisms with the thiobacilli should also be encouraged.

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