THE EFFECT OF METAL IONS ON THE GROWTH AND IRON OXIDATION CHARACTERISTICS OF LABORATORY STRAINS AND NATURALLY OCCURRING MINE STRAINS OF *Thiobacillus ferrooxidans*

By

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ABSTRACT

Eight different Thiobacillus ferrooxidans strains (2 ATCC strains, 6 natural mine isolates) were subjected to analysis for the ability to grow and oxidize ferrous iron in the presence of inhibitory concentrations of metal ions commonly found as constituents of sulphide based ores. Growth, as measured by the lag period and generation time, was usually inhibited by all metal ions (Cu, Zn, As, Mo, Hg, Ag) initially, but most strains did show some to complete adaptation upon sequential growth in metal-containing media. The mine strains generally grew better in the copper or zinc medium but not, as a rule, any better than the ATCC strains in Mo-, As-, Hg-, or Ag-containing medium. Some mine isolates, however, showed higher tolerance to some metals. Arsenite, copper, and zinc were inhibitory to iron oxidation in the millimolar concentration range, while molybdate, silver and mercury were inhibitory in the micromolar concentration range, according to the observed K_i values. The mine strains showed considerable variability towards metal-inhibited iron oxidation whereas the ATCC strains showed less strain variability and were generally more inhibited by all six metal ions. Some mine strains after adaptation to Cu or Zn were activated by Cu, Zn or ferric iron in the oxidation of ferrous iron. The potential does exist, as shown by the results of this thesis, to selectively choose the optimal candidate T. ferrooxidans strain to implement a biological leaching program.

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ABBREVIATIONS

Cu: copper

Zn: zinc

Mo: molybdenum

As: arsenic

Hg: mercury

Ag: silver

Fe^{2+}: ferrous iron

Fe^{3+}: ferric iron

M: Molar

mM: millimolar

μM: micromolar

K_m: Michaelis constant

V_{max}: maximum velocity

[S]: substrate concentration

K_i: inhibitor constant

C: competitive inhibition

Uc: uncompetitive inhibition

Nc: noncompetitve inhibition

[]: concentration

(i): y-intercept determined K_i

(s): slope determined K_i

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INTRODUCTION

INTRODUCTION

Thiobacillus ferrooxidans is a gram negative rod-shaped bacterium, obligate acidophile, able to fix CO_2 as carbon source, fix atmospheric nitrogen as an N-source if necessary and obtain energy from the oxidation of reduced iron or sulfur compounds. It was first taxonomically described by Colmer et al (1950). Ferrobacillus ferrooxidans is considered to be identical to Thiobacillus ferrooxidans following the work by Kelly and Tuovinen (1972).

T. ferrooxidans is the organism most commonly associated with bacterial assisted mineral leaching operations (Lundgren and Silver, 1980; Hutchins et al., 1986). It occurs in various natural locations but has most often been isolated from sulfide mineral drainages where acid concentrations are high. Quite often the metal concentrations are also high, due in part to sulfate and or ferric iron solubilization of the various metals. It is these metals, for example, copper (Cu), zinc (Zn), silver (Ag), mercury (Hg), molybdenum (Mo) or arsenic (As) that may exhibit toxic effects on the iron oxidation related metabolism of T.ferrooxidans.

Six strains of Thiobacillus ferrooxidans have been isolated from the underground mine waters of Hudson's Bay Mining and Smelting Co. Ltd., Flin Flon, Manitoba, based on their ability to grow on sulfur and ferrous iron as inorganic energy source (Lizama and Suzuki, 1988).

In this thesis the effect of toxic metals on the growth and iron oxidation of these T. ferroxidans isolates as well as two laboratory strains has been studied in order to relate to their metal leaching potentials from sulfide ores. The study examined the differences among various strains in growth rates, tolerance and adaptation to metals during growth and finally effects of metals on iron oxidation by non-adapted and adapted cells. In the last phase of study kinetic parameters such as maximal velocities (V_{max}), Michaelis constants (K_m) and inhibition constants (K_i) were obtained and compared.

HISTORICAL

HISTORICAL

Ferrous iron oxidation by T. ferrooxidans is a highly aerobic process using oxygen (O_2) as the terminal electron acceptor. The organism grows optimally at pH 2 where the following half reactions are mediated (Ingledew, 1982).

$$2\ Fe^{2+} \quad 2\ Fe^{3+} + 2\ e^-$$

$$2\ e^- + 1/2\ O_2 + 2\ H^+ \quad H_2O$$

sum: $2\ Fe^{2+} + 1/2\ O_2 + 2\ H^+ \quad 2\ Fe^{3+} + H_2O$

The affinity for ferrous iron (K_m value) as indicated by oxygen uptake kinetics has been estimated to be as high as 9.4 mM using Warburg manometry (Tuovinen & Kelly, 1974). Kelly and Jones (1978), using more sensitive and precise oxygen electrode measurements, obtained K_m values of between 0.4 to 0.9 mM for 0.4 - 100 mM $FeSO_4$ concentration range. At higher $FeSO_4$ concentrations (100-700 mM) the apparent K_m had increased to 123 mM and over 700 mM ($FeSO_4$) substrate inhibition was noted. In a separate study using β-alanine-sulfate as buffer in a pH range 2.4 - 4.4 the apparent K_m was determined to be 2.2 mM (Schnaitman et al., 1969).

Metal inhibition of iron oxidation may occur in different ways. They may inhibit by binding the ferrous iron sites of the iron oxidase system or alternatively by binding a component of the electron transport chain reaction. Either of these interactions could lower the velocity of the reaction, lower the affinity for substrate or both. The anionic and cationic complexities of membrane transport may also be upset. During growth inhibition, the added interference in cell biosynthetic mechanisms should also be considered.

T. ferrooxidans is very tolerant of copper and zinc as evidenced by the elevated concentrations of the metals in which the bacterium has been shown to be able to thrive in the laboratory and the environment, although strain differences should be considered. Concentrations as high as 0.5 M $CuSO_4$ (Pichuantes et al., 1986) and zinc concentrations as high as 0.8 M - 1.0 M $ZnSO_4$ (Torma et al., 1972) have been reported, but generally the organisms are tolerant of approximately 0.2 M in the growth medium (Tuovinen et al., 1971, Imai et al., 1975). Iron oxidation is comparatively unaffected, although few manometric studies have been located. A study by Tuovinen and Kelly (1974) revealed slightly more inhibition of iron oxidation in T. ferrooxidans by 1.0 M concentrations of Zn than Cu. There was also a gradual decrease in the iron oxidation rate with increasing Cu concentrations between 10 mM and 1.0 M. With different strains of T. ferrooxidans, Imai et al. (1975) showed that 10 mM Cu and 1 mM Zn did not affect iron oxidation. An indication that Cu does not bind components of the electron transport chain was given when cytochromes c and a were reoxidized

spectrophotometrically during iron oxidation (mercury prevented reoxidation of both).

Ferric iron (Fe^{3+}) has been shown to competitively inhibit iron oxidation (Kelly and Jones 1978) with a K_i value of 2.5 mM although low ferric concentration caused increased rates of O_2 uptake. Kovalenko et al. (1982) reported K_i values for ferric iron of 3.2 mM as well as a decreased affinity for ferric iron with decreasing temperature.

Molybdenum has usually been assayed for its effects on Thiobacillus sp. in the anionic form of molybdate (MoO_4^{2-}). In determining the specificity of the anionic requirement, Lazaroff (1977) found that 1.0 mM Na_2MoO_4 completely inhibited iron oxidation. An earlier study in anionic replacement of sulphate (SO_4^{2-}) by Schnaitman et al. (1969) indicated that 2.5 mM molybdate completely inhibited iron oxidation. A study of the effect of different metals on T. ferrooxidans (Tuovinen et al., 1974) showed that partial inhibition of iron oxidation (assayed in a Warburg apparatus) was accomplished by as little as 50 micromolar Na_2MoO_4 while near complete inhibition occurred with 100 um. They suggested an intracellular mode of toxicity due to membrane transport of the anion, as well as interference by molybdate in the initial reactions of sulphate - dependent iron oxidation. A strain of T. ferrooxidans isolated by Roy & Mishra (1981) was not able to tolerate 30 ppm [0.185 mM] molybdate but was able to oxidize iron substrate with 20 ppm [0.125 mM] present. A strain of T. ferrooxidans has been tolerant of about

10 mM arsenite ($NaAsO_2$) (Braddock et al., 1984) in the laboratory while most other studies concerning arsenic have used the arsenate anionic form. Schnaitman et al. (1969) observed a slight stimulation of iron oxidation by 2.5 mM arsenate (Na_2HAsO_4) in the presense and absence of sulphate anion, as did Lazaroff (1977) with 1.0 mM arsenate.

Silver and mercury toxicity has been the subject of many reports on Thiobacillus sp. Perhaps the most revealing results were produced by the Japanese workers Imai and Sugio. They demonstrated that 1 μM Hg^{2+} (Imai et al., 1975) or 1 μM Ag^{+} (Sugio et al., 1981) prevented the reoxidation of cytochrome oxidase, a terminal electron acceptor enzyme during iron oxidation (Sugio et al., 1987; 1988). Imai et al. (1975) investigated metal inhibition of iron oxidation and growth, showing both to be severely affected at low concentrations of Ag and Hg. Ag at 10 μM caused a 2 to 3 day lag in growth and iron oxidation while 10 μM Hg reduced the rates by 80% and 40% respectively without a lag. At 100 μM both metals inhibited the growth and iron oxidation almost completely. A strain of T. ferrooxidans isolated from a coal refuse pile (Sugio et al., 1981) developed 100 hour lag periods during growth experiments in 1 μM $AgNO_3$ (9K medium) and growth was completely halted in 100 μM. Iron oxidizing activity was inhibited by 50% and 25% at 100 μM and 10 μM $AgNO_3$, respectively. A particularly sensitive strain of T. ferrooxidans used by Norris & Kelly (1978) would not grow or oxidize iron in the growth medium at 0.1 μM $AgNO_3$, and at 0.01 μM had a 100 hour lag period before growing as well as the control.

Mercury was tolerated at slightly higher concentrations with 1 μM causing a 50 hour lag before growth started. Iron oxidation has been shown (Roy and Mishra, 1981) to not necessarily be coupled to growth, as 0.5 μM Ag caused a 100 hour lag period in cell growth (plate enumeration) but allowed measurable iron oxidation to proceed immediately.

Cell membrane and electron transfer interference by silver were suggested by Norris and Kelly (1978) following silver accumulation and subsequent rapid potassium loss. A loss of the transmembrane pH gradient would result from the inhibited cytochrome oxidase which would normally reduce O_2 to H_2O and removes protons (H^+) from the cell matrix. The increased resistance of T. ferrooxidans to silver in sulfur media may indicate removal of silver from the media by sulfur (sulfide) or thiol groups. Tuovinen et al., (1985) found that yeast extract alleviated Ag toxicity, probably in a chelating mechanism.

Mercury has been noted to cause complete inhibition of iron oxidation during growth at very low concentrations. Mahapatra and Mishra (1984) showed 0.1 ppm to be deleterious but was alleviated by the addition of EDTA, a powerful chelating agent. Olson et al. (1981, 1982) demonstrated mercury volatilization as a detoxifying mechanism in two environmental strains. These strains, isolated from a coal mine and contaminated sediments would not grow in 0.5 μM $HgCl_2$ but would grow normally when successively subcultured in progressively higher $HgCl_2$ concentrations. Volatilization was

evident in these resistant organisims. They also determined that the Hg concentration must drop to near zero before measurable iron oxidation occurs.

The ability of T. ferrooxidans to adapt to various metals has received interest with respect to increased metal resistance of the strains with capacity to leach toxic metal containing sulfidic minerals. Constitutive mercury adaptation has occurred in Olsons' strains (1982) as has adaptation to copper, zinc and silver in other reports. Sugio et al. (1981) were able to develop a Ag-resistant population which could tolerate up to 0.5 μM Ag during growth with tolerance maintained after subculturing in Ag-free media. Resistant cells were shown to concentrate metallic silver in the cell wall and in particular the cell membrane fraction, as opposed to ten times less silver in the sensitive cells. Inhibitory concentrations of silver, however, were as effective on both resistant and sensitive cells for iron oxidation, as observed manometrically. Pooley (1982) noted and described a nucleation of silver metal on the cell wall after growth in silver containing sulfidic mineral media. Norris and Kelly (1978) developed strains with Ag resistance following sub-culturing of the parent strain in Ag media. Ag-resistant cells, while showing similar growth rates as the Ag-sensitive cells, had half as long lag periods at ten times the silver concentration. Curiously the Ag-resistant cells showed an increased sensitivity to mercury in the growth media.

Olson et al. (1981) showed that successive subculturing in mercury containing media resulted in shorter lag periods and tolerance to higher Hg concentrations. The parent coal mine strain BA-4 developed into both a constitutive strain capable of retaining the Hg-volatilization activity through growth in mercury free media and a strain BA-4C which had lost the activity ("been cured of") (Olson et al., 1982).

Tuovinen & Kelly (1974) adapted T. ferrooxidans (ATCC 13611) to zinc and copper and found the adaptation to be more or less specific for that metal. Copper-tolerant cultures could grow in 150 mM Zn but the lag period was extended from near zero to 75 hours. Torma et al. (1970) referred to zinc-adapted cells growing in both 0.5 M and 1.0 M zinc media but did not further investigate.

MATERIALS AND METHODS

Materials

All chemicals used were of reagent grade and obtained commercially. Zinc sulfate, mercuric chloride, magnesium sulfate, sodium hydroxide, hydrochloric acid, sulfuric acid, potassium tartrate, potassium chloride, dibasic potassium phosphate, ferrous sulfate and ammonium sulfate were obtained from Fisher Scientific Co., Fairlawn, N.J., U.S.A. Silver nitrate, sodium molybdate, sodium carbonate and calcium nitrate were obtained from J. T. Baker, Philipsburg, N.J.; U.S.A. Cupric sulfate was obtained from McArthur Chemical Co., Montreal. Sodium arsenite, polyethylene (20) sorbitan monooleate (Tween 80) and ferric nitrate were obtained from Matheson, Coleman, Bell, U.S.A. Phenol reagent was obtained from Harleco, Philadelphia, Pa., U.S.A. Ferric sulphate was obtained from British Drug Houses, Toronto. All reagents were prepared using glass-distilled water.

Bacterial strains

Thiobacillus ferrooxidans ATCC 13661 (Tf-1), and T.ferrooxidans ATCC 19859 (Tf-2) were the laboratory control strains used. SM-1, SM-2, SM-3, SM-4, SM-5 and SM-8 were isolated by Hector Lizama from water samples from various seepage and drainage locations at the Flin Flon mine of the Hudson Bay Mining and Smelting Co. Ltd., Manitoba. The organisms grew on $FeSO_4 \cdot 7H_2O$ as the sole energy source and were maintained in a modified 9K, M9K, (McCready et al., 1986) medium with composition as follows 0.4g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with concentrated H_2SO_4. The ferrous sulfate solution (33.3 g/100 mL) was filter sterilized separately using a 0.45 um filter apparatus. The rest of the medium, M9K salts, was autoclaved. The stock cultures were regrown at least once every month and were stored at both 4^oC and room temperature.

Media and growth of bacteria

The medium used for growth experiments was the modified M9K medium (McCready et al., 1986) (for details see Bacterial strains). To monitor growth characteristics (lag period, generation time) a culture was grown in 100 mL volume (10% inoculum) in a 250 mL Erlenmeyer flask at 25°C on a rotary shaker at 150 rpm. To collect cells for activity determinations, proportionately larger volumes of media and larger flasks (i.e. 200 mL media/500 mL flask) and smaller rpm values were used (i.e. 0.5 L and 1.0 L flasks at 130 rpm; 2 L at 110 rpm). Growth characteristics were also determined using the 9K medium of Silverman and Lundgren (1959): 3.0 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Ca(NO_3)_2$ per liter adjusted to pH 2.3 with concentrated H_2SO_4 and autoclaved plus 44.2 g per liter $FeSO_4 \cdot 7H_2O$ filter sterilized and added separately. This was to determine if the higher salt concentrations could be responsible for any growth characteristics seen with metal salt additions. To study the effects of copper, zinc, mercury, silver, arsenic and molybdenum, the respective metal salts were incorporated into the M9K medium as follows: Cu as $CuSO_4 \cdot 5H_2O$ [50 mM], Zn as $ZnSO_4 \cdot 7H_2O$ [150 mM], both autoclaved in the M9K medium, Hg as $HgCl_2$ [1 μM or 0.1 μM], Ag as $AgNO_3$ [1 μM or 0.1 μM], As as $NaAsO_2$ [1 mM] and Mo as Na_2MoO_4 [1 mM or 0.1 mM], the latter four metal salts being filter sterilized through a 0.45 μM membrane and added separately to the M9K medium. Flask,

volume and shaker conditions remained the same. Where Ag was used, the $FeSO_4 \cdot 7H_2O$ (3.3 g/10 mL) as substrate was first treated with $AgNO_3$ (0.1 mL [0.1 mM] $AgNO_3$ to 10 mL), before filtration and addition to the medium, to remove the 0.001% chloride present as contaminant in ferrous sulfate, which caused precipitation of the silver as AgCl in the medium. However, 0.1 μM AgCl was soluble.

Before growth occurred for SM-1 and SM-2, a stationary phase of one or more days was required before being put on the shaker. During this period there was a slow increase in the ferric iron production. Once on the shaker, these strains would oxidize the ferrous iron much faster. This requirement seemed to disappear with a higher quality batch of $FeSO_4 \cdot 7H_2O$, which was used in all growth experiments with arsenite and molybdate and also in some experiments with silver (SM-2 and SM-8) and mercury (SM-1).

Growth rate

Stock cultures were subcultured three times (each subculture at 75% - 100% oxidation of ferrous iron) before the growth curve study of actively growing cultures was carried out, with or without the metals.

Cell growth was routinely followed by the determination of ferric iron concentration by measuring the yellow color (Schnaitman et al., 1969) developed after 0.5 mL culture sample was mixed with 4.5 mL 1.O N HCl in a Klett-Summerson Photoelectric Colorimeter. A standard curve of 0 - 150 mM $Fe(NO_3)_3$ was prepared for the determination of the ferric concentration. Cell growth was initially checked by protein determination to verify the proportional increase in protein versus ferric iron production. The protein determination was more difficult in that ferric iron probably interfered especially during the later stages of growth. Therefore, the simpler ferric iron determination was used throughout this study as a measurement of growth. The growth curves were plotted on a semi-logarithmic scale to obtain the generation time (g), lag time, and total growth time in hours. Generation times were determined by the formula ln N - ln N_0 = kt and g = 0.693/k (Ingraham et al., 1983); where N = $[Fe^{3+}]$ at time t, N_0 = $[Fe^{3+}]$ at time zero, and k = the growth rate constant. The lag period was determined as the length of time before exponential growth occurred. Growth time was the total

amount of time for the ferrous iron to be completely oxidized to ferric iron.

To observe the effect of various metals on growth, a wide range of metal concentrations was tested. The concentration at which growth was inhibited with a lengthened lag period, generation time or both was chosen as the concentration at which adaptation would be attempted. Adapted cells were determined as such due to the fact that three successive subcultures were grown without much inhibition of growth in the presence of that metal concentration.

Cell harvesting

To obtain quantities of cells sufficient for kinetic analysis, cells were grown in 1.5 to 2.5 liters of medium in 0.5, 1.0 and 2.0 liter Erlenmeyer flasks. Following removal of the majority of insoluble ferric iron by decantation (after 1-2 hours settling), the remainder of the insoluble ferric iron was removed by low speed centrifugation (100 x g, 10 minutes) in 500 mL Nalgene centrifuge bottles. The remaining suspended cells then were collected by high-speed centrifugation (12,000 x g, 10 minutes) and washed twice in 10 mL pH 3.0 H_2O (made with H_2SO_4) in 15 mL corex centrifuge tubes. Finally a cell suspension was made to a concentration of 50 mg wet cells per mL in pH 3.0 H_2O and stored at 4°C until used.

In the course of this work, three different batches of $FeSO_4 \cdot 7H_2O$ were used (all Fisher) with the harvesting of some strains growing on one of the batches with some ferric iron impurities, requiring special solubilization techniques.

These included suspending the ferric iron precipitate after growth in a $FeSO_4 \cdot 7H_2O$ [3.5g/100 mL] mixture to solubilize some ferric iron and perhaps free the cells. Alternatively, Tween 80 (0.05%) was incorporated into the wash mixture (including 3.5 g $FeSO_4 \cdot 7H_2O$/100 mL pH 2.3). Both methods yielded cells which were not obtainable following the normal treatment, as described above. Occasionally, a rubber scraper was used on the sides of the Nalgene centrifuge

bottles if an obvious cell pellet, after high speed centrifugation, was not present. This usually yielded enough cells for experimentation.

Determination of ferrous iron oxidizing activity

The rate of ferrous iron oxidizing activity by the various strains was determined by measuring the rate of oxygen consumption in a Gilson oxygraph at 25°C. The standard reaction mixture in a volume of 1.2 mL contained 10 uL or 25 uL volumes of cell suspension [50 mg/mL], microliter (3, 6, 10, 50, 150) volumes of $FeSO_4 \cdot 7H_2O$ (0.1 M, pH 2.3) as substrate, and M9K salts (no iron) pH 2.3. The ferrous iron oxidizing activity was expressed as n mole O_2 per minute per mg protein. The order of addition of reagents varied occasionally according to the nature of the assay. For assays without any metal present, the M9K salts solution was added first, then bacterial cells, then substrate. In studying the effects of metals on iron-oxidizing activity, the different metals were incorporated (as the metal salts described above) in microliter volumes. Two different approaches were used for the different metals. For Cu, Zn, and ferric iron, the metal was added before the substrate with the cells initiating the reaction. To study the effect of ferric iron, a suspension of 1 M $Fe_2(SO_4)_3$ in 0.1M $FeSO_4 \cdot 7H_2O$ pH 1.0 was used, whereby the quantity of ferrous iron added from the suspension was taken into consideration upon further addition of substrate. In the case of Ag, Hg, As and Mo, there was a five minute incubation of metal and cells (due to a time-dependant effect), the reaction initiated with ferrous iron addition. This incubation also allowed us to determine if the metal tested was being used as oxidative substrate by the organism.

Determination of Kinetic Parameters

The kinetic parameters studied on the Fe^{2+} oxidation were: V_{max} (the maximal velocity of oxidation at saturating Fe^{2+} substrate concentration), K_m (Michaelis constant, the substrate concentration at half the maximal velocity) and K_i (inhibitor constant as defined later).

Results of kinetic experiments were analyzed by plotting velocity of reaction (v) against substrate (Fe^{2+}) concentration [S] according to Michaelis and Menten (1913)

$$v = V_{max}\ [S]/(K_m + [S]) \qquad (1)$$

and also by plotting 1/v against 1/[S] according to the double reciprocal method of Lineweaver and Burke (1934):

$$1/v = (K_m/V_{max})(1/[S])+1/V_{max} \qquad (2)$$

The apparent K_m value was obtained from the Lineweaver and Burke plots of 1/v versus $1/[Fe^{2+}]$ as the reciprocal of the absolute value of the x-intercept while the V_{max} was obtained as the reciprocal of the y-intercept value.

To determine the K_i values, the same plots were obtained in the presence and absence of inhibiting metals and the effect of

inhibitors was evaluated as changes in slope or intercept by the factor $1 + [I]/K_i$, where [I] is the concentration of inhibitor and K_i is the inhibitor constant and is the concentration of inhibitor that doubles the slope or intercept value. In practice, the slope or intercept was replotted against [I], the inhibitor concentration, and the intercept at the x-axis was taken as $-K_i$ value.

Three types of inhibition were considered in this study according to Cleland (1963):

Competitive: $$1/v = (K_m/V_{max})(1 + [I]/K_i)(1/[S]) + 1/V_{max} \quad (3)$$

Uncompetitive: $$1/v = (K_m/V_{max})(1/[S]) + (1/V_{max})(1 + [I]/K_i) \quad (4)$$

Noncompetitive: $$1/v = (K_m/V_{max})(1 + [I]/K_{i\ slope})(1/[S]) + (1/V_{max})(1+[I]/K_{i\ intercept}) \quad (5)$$

Competitive inhibition occurred if only the slope changed and uncompetitive inhibition if only the y-intercept changed. Noncompetitive inhibition occurred if both changed. Non-linear and more complex types of inhibition were not considered because of the requirement for many more data points for each plot than those possible in this study.

Protein determination

Protein determinations were made to calculate the specific iron-oxidizing activity (per mg protein), as well as to follow the proportional increase in cell protein versus ferric iron concentration. The Lowry method (Lowry et al., 1951) as modified by Bagdigian (1986) and our group to accommodate our needs was used. To follow the protein concentration with respect to ferric iron increase, a 10 mL sample was removed from the culture vessel and centrifuged (12,000 x g, 10 minutes) to collect the pellet which was then suspended in 1.0 mL 0.1 N NaOH and boiled for 10 minutes. For the protein determination of the cell suspensions, duplicate 5 or 10 microliter volumes (of 50 mg/mL cell suspensions) were made to 100 uL with distilled water, mixed with 0.9 mL 0.1 N NaOH and boiled for 10 minutes. Both methods continued with the same subsequent treatment after boiling. The samples were then centrifuged (12,000 x g, 10 minutes) to remove cell debris; 0.8 mL supernatent was combined with 4.0 mL Reagent D (50 mL of 2% Na_2CO_3 with 1 mL of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate); mixed well and let stand for 10 minutes at room temperature. After addition of 0.4 mL Phenol Reagent [1.0 N], good mixing, and standing for 30 minutes or more at room temperature, the absorbance was determined on a Klett-Summerson Photoelectric Colorimeter using a red filter (660 nm). A standard curve of 0 - 100 micrograms bovine serum albumin (BSA) was prepared by treating the BSA samples exactly as the cell suspension samples.

RESULTS

Copper

All the strains tested, except SM-2, adapted to the M9K growth medium with 50 mM $CuSO_4$. In all cases growth with 50 mM Cu (sensitive culture) resulted in increased lag periods (lag), increased generation times (g), and increased growth time (Table 1). SM-1 culture used in these experiments showed an unusually long lag period in the control culture due perhaps to the batch of medium used. Upon 3 successive subcultures in the presence of 50 mM Cu all the adapted cultures showed decreases in lag periods, generation times and growth times almost to the levels of control cultures in most cases. (Table 1 and Figs. 1, 1a and 2, 2a).

The two laboratory strains displayed very similar characteristics in response to Cu. A large increase in the lag was noted for the sensitive cells, while upon adaptation, they decreased to the level of the control cells. Tf-2 was different in that the generation time remained relatively constant in the sensitive cells. Both strains had similar growth yields and activity levels in control and Cu-adapted cells.

The mine isolates displayed much variability among strains in the ability to adapt to 50 mM copper during growth. SM-2 did not grow, and SM-1 and SM-3 only partially adapted to growth with copper (generation time twice that of control). SM-4, SM-5 and SM-8

TABLE 1. Growth characteristics of Tf- and SM- cells in the presence/absence of copper ($CuSO_4.5H_2O$)

Strain	[$CuSO_4$] (mM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield mg protein	Growth yield mg wet cells	Activity (V_{max}) nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1	0	0-5	7	15	8.8	83	1250
Sensitive[a]	50	41	18	85			
Adapted[b]	50	0-5	9	30	12	75	1075
Tf-2	0	0-5	8	25	15.4	80	1250
Sensitive	50	65	10	92			
Adapted	50	0-5	10	27	9.6	75	950
SM-1	0	325	20	350	2.6	20	680
Sensitive	50	300	75	475			
Adapted	50	190	40	290	3.7	25	345
SM-2	0	0-5	19	65	3.3	23	360
Sensitive	50						
Adapted	50	NG[c]					
SM-3	0	0-5	12	35	7.2	49	230
Sensitive	50	20	82	145			
Adapted	50	10	25	75	5.7	87	205
SM-4	0	0-5	12	25	3.4	40	1740
Sensitive	50	100	34	150			
Adapted	50	0-5	16	46	9.7	54	510
SM-5	0	40	10	70	49.1	332	800
Sensitive	50	60	20	105			
Adapted	50	0-5	18	50	9.7	110	1100
SM-8	0	20	30	100	5.0	28	690
Sensitive	50	100	56	240			
Adapted	50	0-5	17	50	2.0	37	830

[a]Initial growth in metal containing media.
[b]Final growth in metal containing media.
[c]NG: no growth.

Figure 1

Adaptation of Tf-1 to 50 mM $CuSO_4$

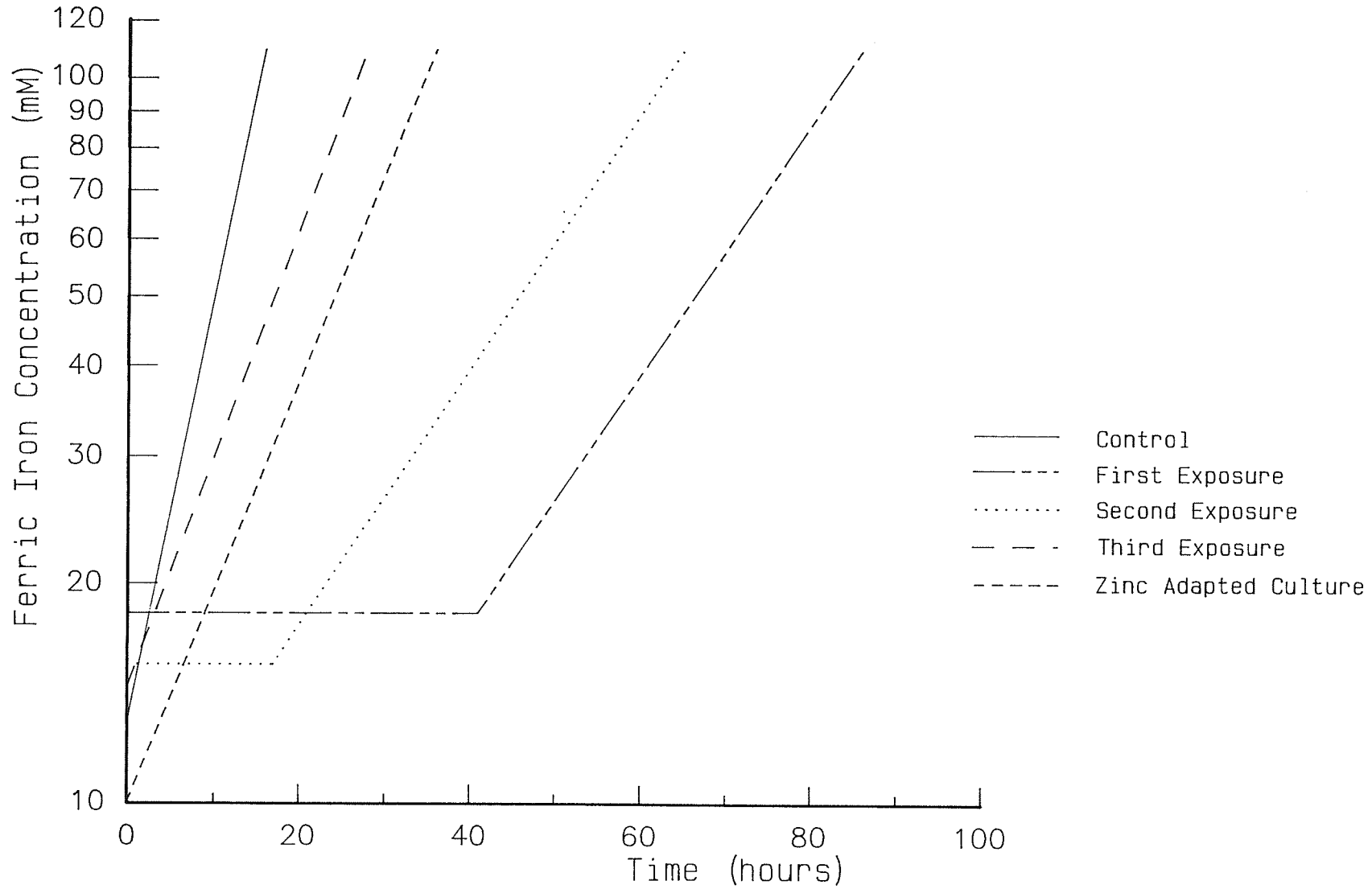

120
100
90
80
70
60
50
40
30
20
10
Ferric Iron Concentration (mM)
0
20
40
60
80
100
Time (hours)
Control
First Exposure
Second Exposure
Third Exposure
Zinc Adapted Culture

Figure 1a

Effect of $CuSO_4$ on Growth of Tf-1

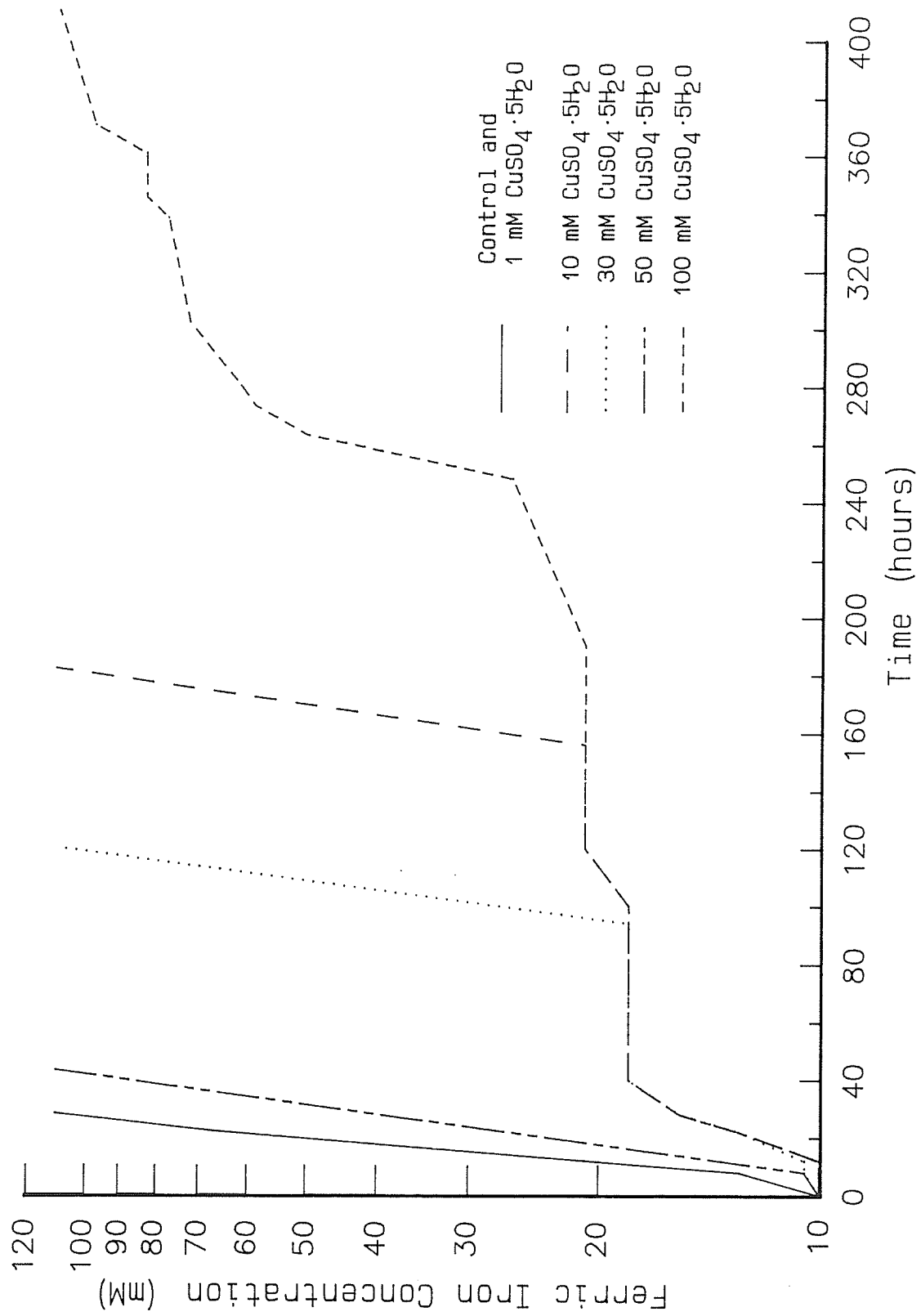

Ferric Iron Concentration (mM)
120
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
280
320
360
400
Time (hours)
Control and
1 mM $CuSO_4 \cdot 5H_2O$
10 mM $CuSO_4 \cdot 5H_2O$
30 mM $CuSO_4 \cdot 5H_2O$
50 mM $CuSO_4 \cdot 5H_2O$
100 mM $CuSO_4 \cdot 5H_2O$

Figure 2

Adaptation of SM-4 to 50 mM $CuSO_4$

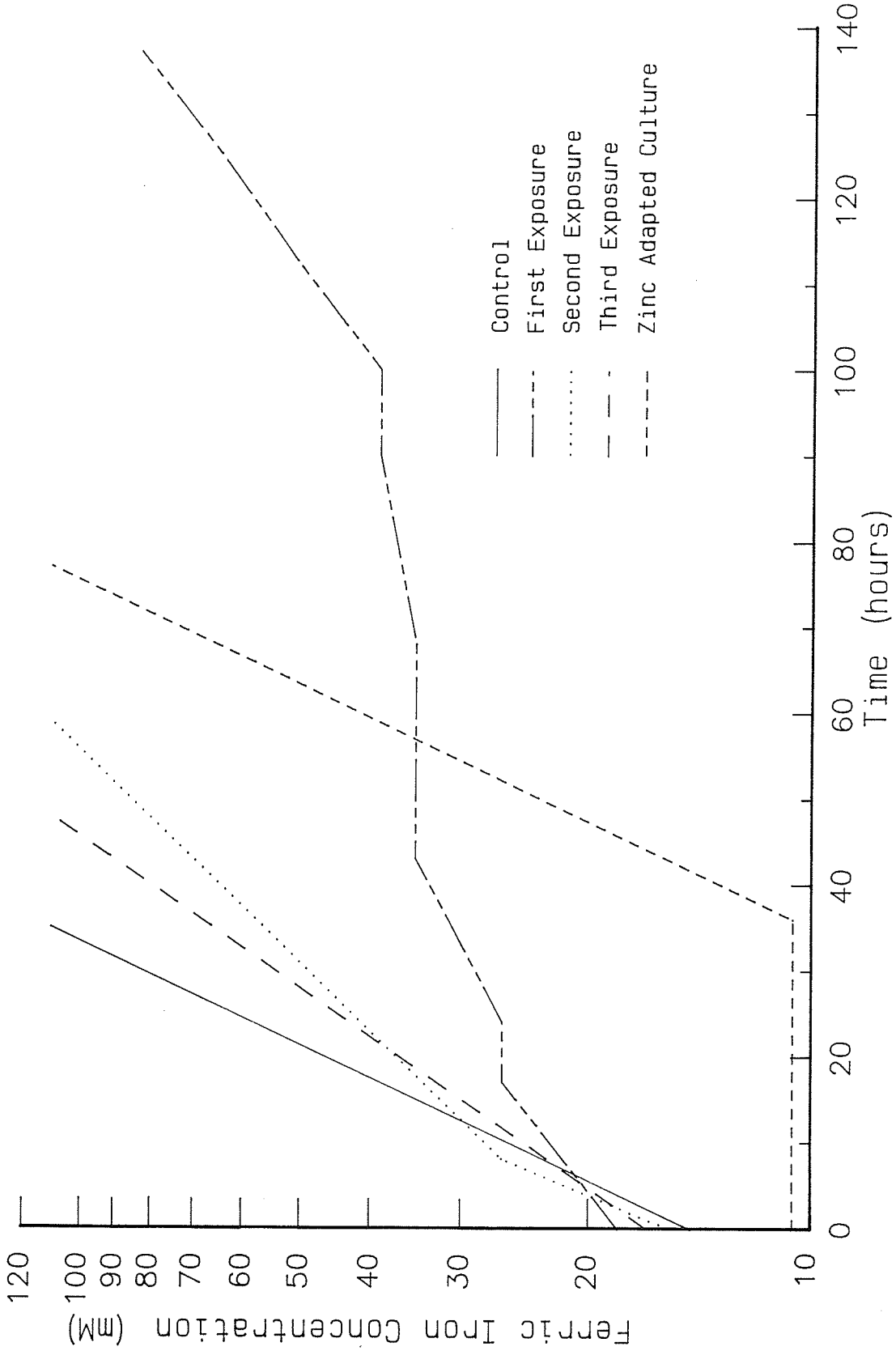
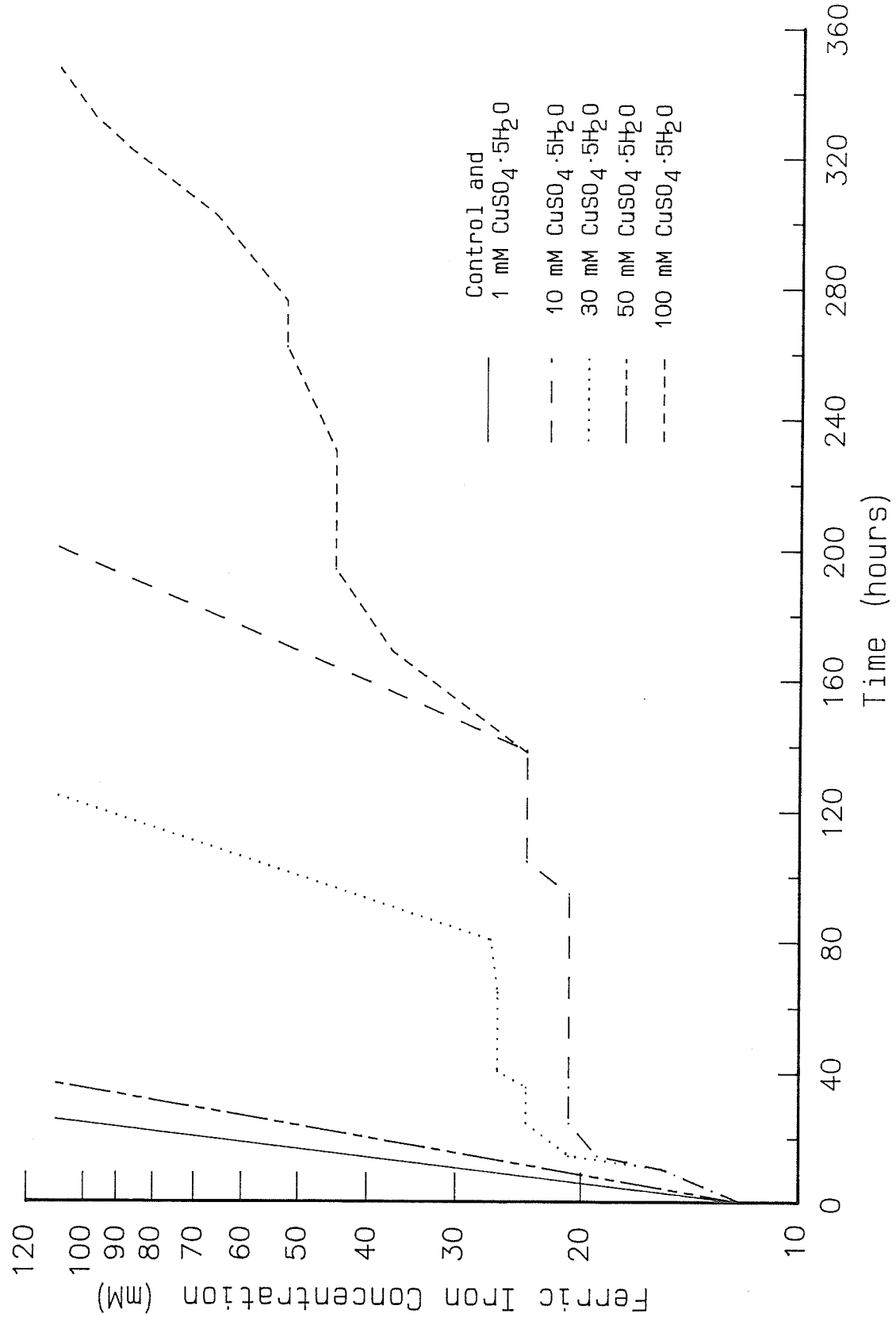

Ferric Iron Concentration (mM)
120
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
Time (hours)
Control
First Exposure
Second Exposure
Third Exposure
Zinc Adapted Culture

Figure 2a

Effect of $CuSO_4$ on Growth of SM-4


Ferric Iron Concentration (mM)
120
100
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
240
280
320
360
Time (hours)
Control and
1 mM $CuSO_4 \cdot 5H_2O$
10 mM $CuSO_4 \cdot 5H_2O$
30 mM $CuSO_4 \cdot 5H_2O$
50 mM $CuSO_4 \cdot 5H_2O$
100 mM $CuSO_4 \cdot 5H_2O$

adapted to the growth with Cu quite effectively. After substantial increases in the lag from 0 - 5 to 100 hours and the generation time from 12 to 34 hours in the presence of Cu, SM-4 adapted cells returned to the 0 - 5 hour lag period and the 12 - 16 hour generation time of the control cells (Table 1 and Fig. 2). A significant decrease of activity (specific activity) observed in the adapted SM-4 cells was balanced by an increase in the growth yield and therefore the total activity per liter of the culture remained fairly constant.

SM-5 and SM-8 both had extended lag periods in the control and sensitive cells but upon adaptation, the lag period nearly disappeared. SM-8 developed a faster generation time upon adaptation than control culture (30 to 17 hours). SM-5 differed from SM-8 in that the generation time was slightly increased over that of the control but both strains showed reduced growth time upon adaptation to Cu.

The effects of copper on iron oxidation (Table 2, Figs. 3-5) clearly differentiated the laboratory strains from the mine strains. High K_m values for Fe^{2+} and biphasic inhibition patterns for Cu (Fig. 3) with both control and Cu-adapted cells characterized the laboratory strains. Both laboratory strains Tf-1 and Tf-2 maintained relatively high constant K_m values of around 1 mM $FeSO_4$ at low Fe^{2+} concentrations with and without adaptation, and the values further increased about three times at high Fe^{2+} concentrations with the adapted cells. A general characteristic of all the control cells (both laboratory strains and mine isolates) except SM-1 was that Cu was a

TABLE 2. Effect of Cu^{2+} on the ferrous iron oxidizing activity of Cu^{2+} -adapted/unadapted Tf and SM cells

Strain	$[CuSO_4]$ in growth medium (mM)	K_m Fe^{2+}(mM)		Type of inhibition[a]		K_i Cu^{2+}(mM)	
		Low[S]	High[S]	Low[Fe^{2+}]	High[Fe^{2+}]	Low[Fe^{2+}]	High[Fe^{2+}]
Tf-1	0	1.20	1.20	Uc	C	50(i)	83(s)
Tf-1 adapted	50	1.25	3.10	Nc	C	135(i)	115(s)
Tf-20	0	0.95	0.95	Nc	C	90(i)	130(s)
Tf-2 adapted	50	0.80	2.90	Nc	C	150(i)	70(s)
SM-1	0	0.35	0.35	C	C	54(s)	54(s)
SM-1 adapted	50	0.23	2.20	A/NI	A/NI	A/NI	A/NI
SM-2	0	1.0	1.0	Uc	C	100(i)	130(s)
SM-3	0	0.20	0.60	Nc	C	150(i)	70(s)
SM-3 adapted	50	0.20	1.10	A	A	A	A
SM-4	0	0.45	0.45	Nc	C	155(i)	30(s)
SM-4 adapted	50	0.20	0.20	C	A	90(s)	A
SM-5	0	0.43	0.43	Nc	C	75(i)	50(s)
SM-5 adapted	50	0.30	0.30	C	A	128(s)	A
SM-8	0	0.33	0.33	N	C	60(i)	68(s)
SM-8 adapted	50	0.17	0.17	C	C	32(s)	32(s)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change);
Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.
(i):y-intercept.
(s):slope.

Figure 3

Effect of Copper on Iron Oxidation Rates by Tf-1

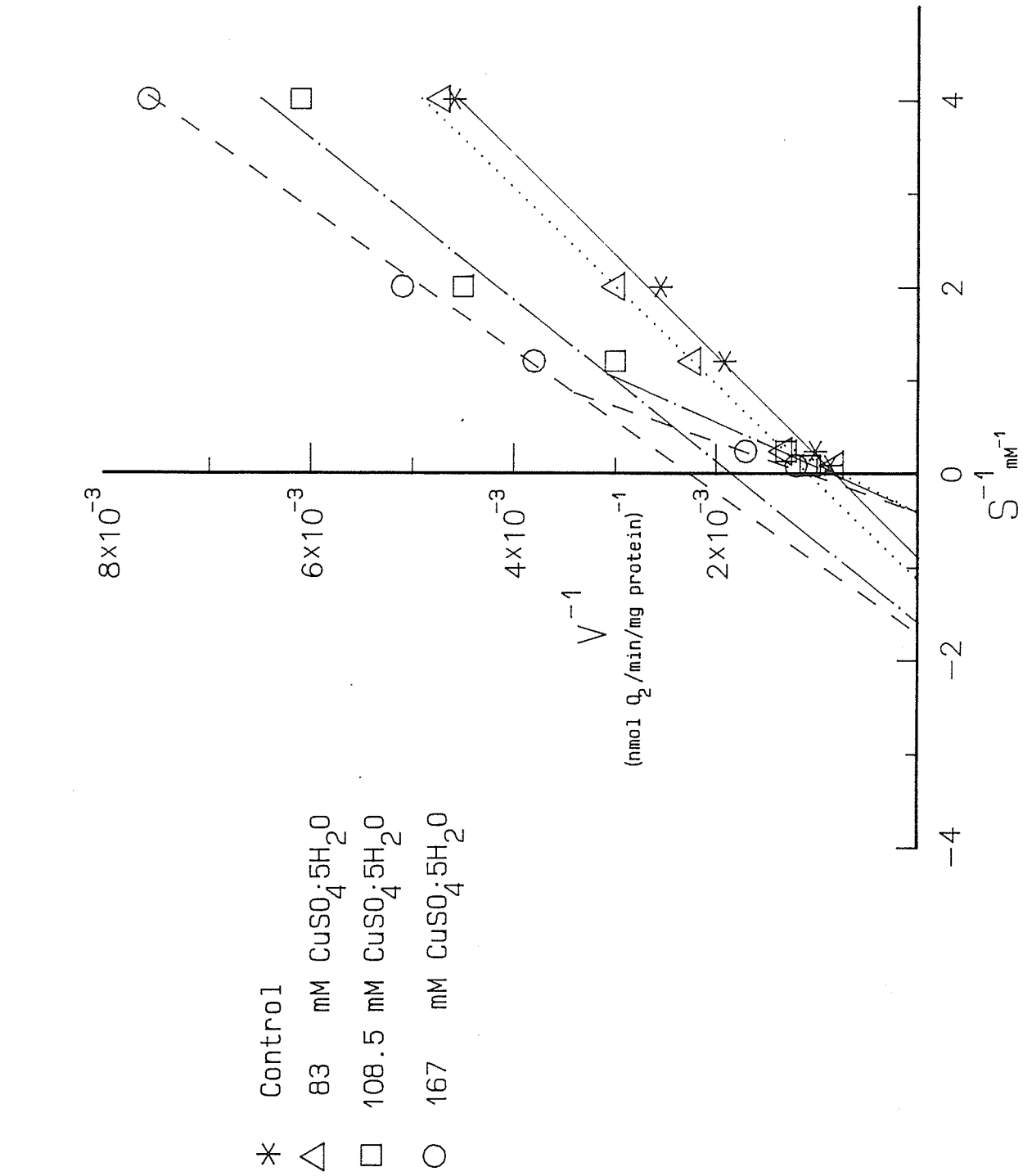

Control
83 mM $CuSO_4 \cdot 5H_2O$
108.5 mM $CuSO_4 \cdot 5H_2O$
167 mM $CuSO_4 \cdot 5H_2O$
8×10^{-3}
6×10^{-3}
4×10^{-3}
2×10^{-3}
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
−4
−2
0
2
4
S^{-1} mM^{-1}

Figure 4

Effect of Copper on Iron Oxidation Rates by SM-3

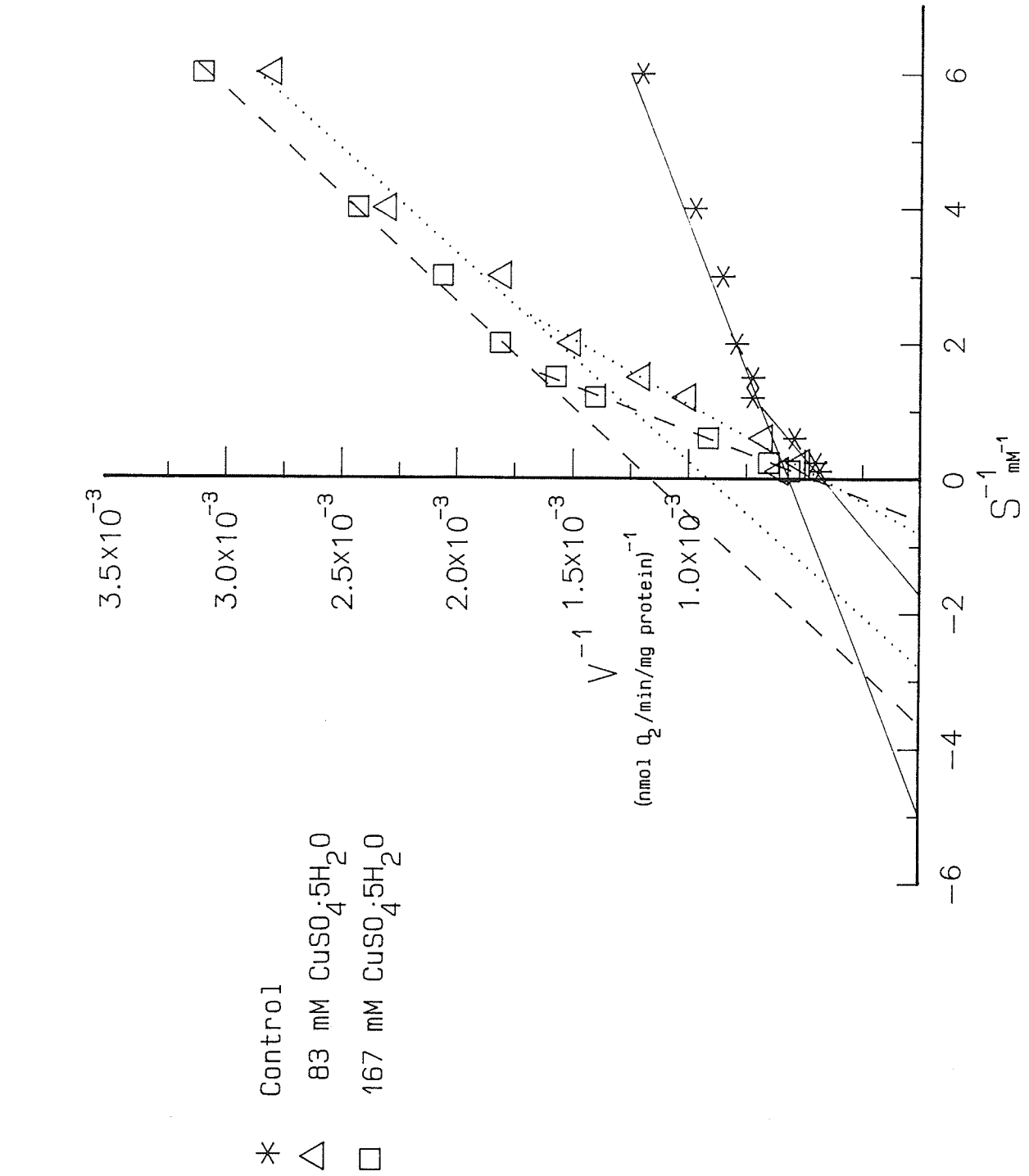

Control
83 mM $CuSO_4 \cdot 5H_2O$
167 mM $CuSO_4 \cdot 5H_2O$
V^{-1} (nmol O_2/min/mg protein)$^{-1}$
$S^{-1}_{mM^{-1}}$
3.5x10^{-3}
3.0x10^{-3}
2.5x10^{-3}
2.0x10^{-3}
1.5x10^{-3}
1.0x10^{-3}
−6
−4
−2
0
2
4
6

Figure 5

Effect of Copper on Iron Oxidation Rates by SM-4

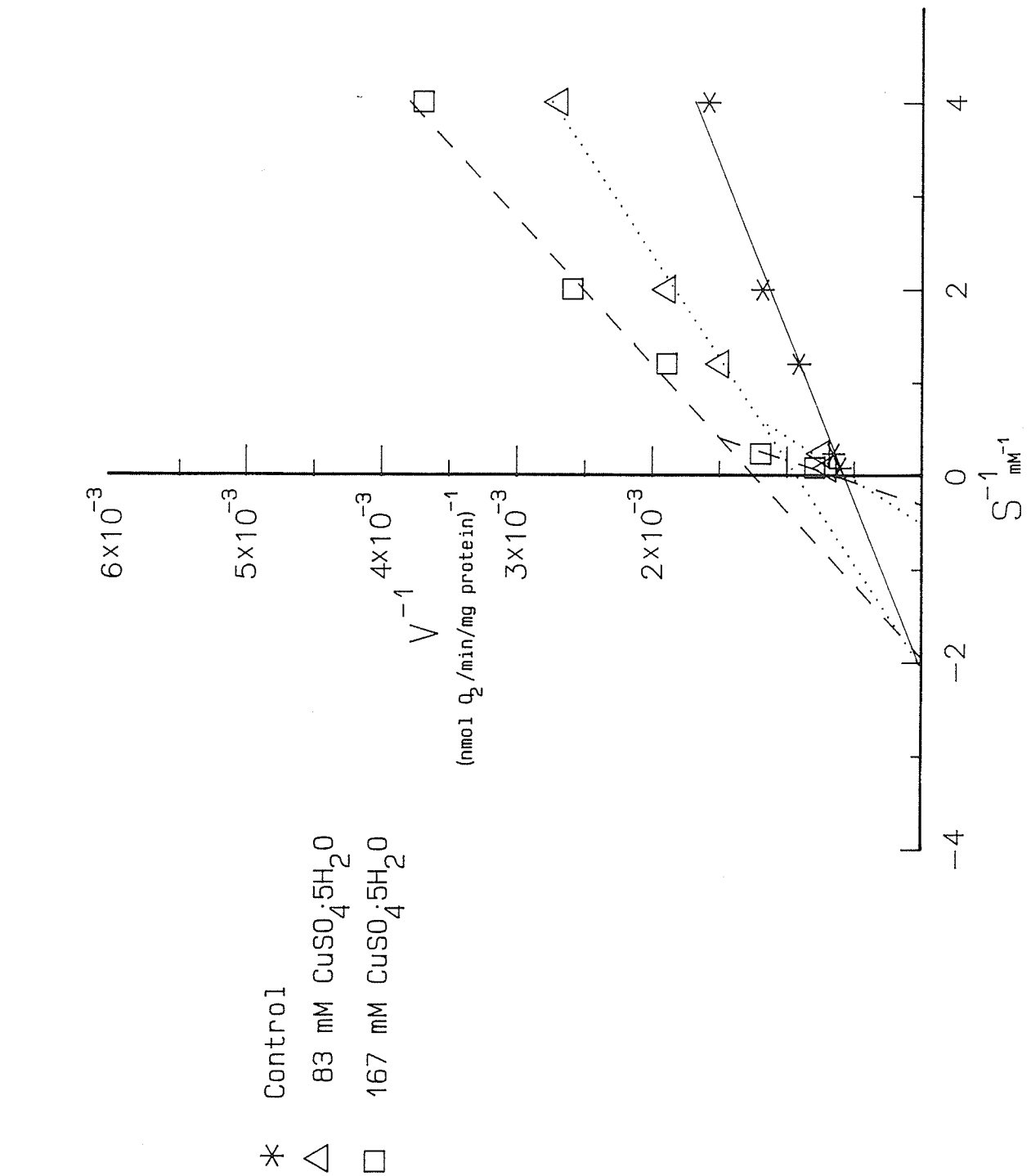
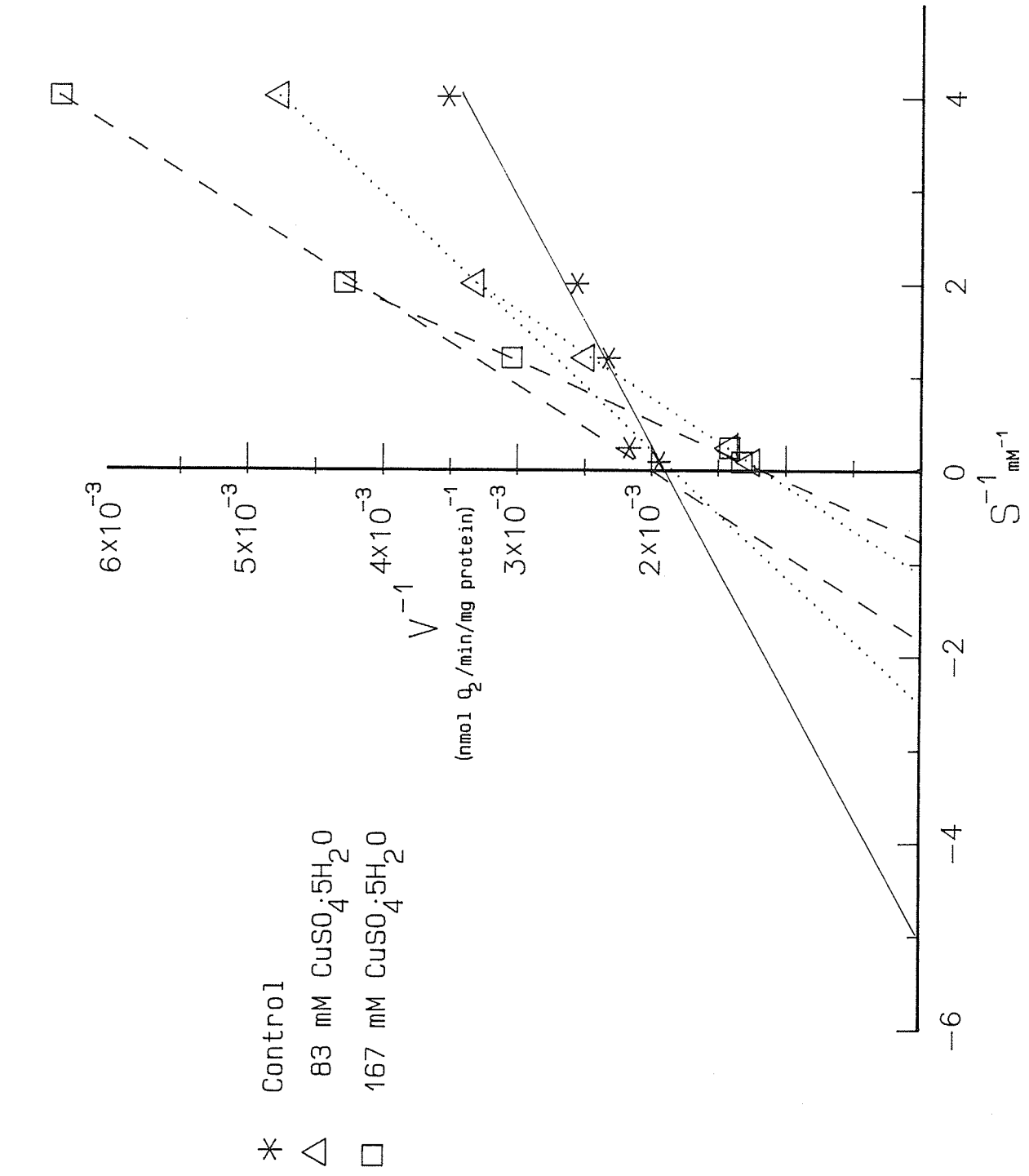

6x10^-3
5x10^-3
4x10^-3
3x10^-3
2x10^-3
V^-1
(nmol O2/min/mg protein)^-1
-4
-2
0
2
4
S^-1 mM^-1
* Control
Δ 83 mM CuSO4·5H2O
□ 167 mM CuSO4·5H2O

Figure 6

Effect of Copper on Iron Oxidation Rates by Copper-Adapted SM-4


6x10^-3
5x10^-3
4x10^-3
3x10^-3
2x10^-3
V^-1
(nmol O2/min/mg protein)^-1
-6
-4
-2
0
2
4
S^-1 mM^-1
Control
83 mM CuSO4·5H2O
167 mM CuSO4·5H2O

noncompetitive or uncompetitive inhibitor of Fe^{2+} oxidation at low Fe^{2+} concentrations, but became a competitive inhibitor at high Fe^{2+} concentrations. This pattern remained unchanged with the laboratory strains upon adaptation to copper while the mine strains developed different and variable patterns. The tolerance to copper, as shown by the K_i values, increased upon adaptation in Tf-1 and Tf-2 at low [Fe^{2+}] and high [Fe^{2+}], except at high [Fe^{2+}] in Tf-2 which became more sensitive. In both strains (distinct from the mine strains) K_i values with unadapted cells increased, while those with adapted cells decreased when the Fe^{2+} concentration was increased.

Contrary to the more uniform characteristics of the laboratory strains, the mine strains showed similarities as well as wide differences (Table 2). K_m values were generally smaller and often much smaller than those of the laboratory strains and with copper adapted cells activation of iron oxidation often took place when copper was present. SM-2 which did not adapt to 50 mM Cu, was the only exception with the K_m and K_i values similar to the laboratory strains. Although SM-1 and SM-3 did not fully adapt in their growth with Cu, they became tolerant of Cu during iron oxidation. Both the K_m and K_i values for SM-1 control cells were quite low at 0.35 mM Fe^{2+} and 54 mM Cu^{2+} respectively and the double reciprocal plots displayed a simple competitive inhibition pattern (no biphasic inhibition/oxidation pattern). With the adapted cells, the double reciprocal plots became biphasic with two K_m values, corresponding to low Fe^{2+} and high Fe^{2+} concentrations. Interestingly Cu activated the Fe^{2+} oxidation (more at 83 mM than at 167 mM Cu and

more at low Fe^{2+} concentrations). A minor inhibition was observed only with 167 mM Cu at the highest Fe^{2+} concentration used (4 and 11 mM). With SM-3 control cells, the Fe^{2+} oxidation activity showed biphasic double reciprocal plots both with and without copper, Cu being a noncompetitive inhibitor at low Fe^{2+} and a competitive inhibitor at high Fe^{2+} concentrations (Fig. 4). In fact all the strains tested (both laboratory and mine isolates) showed a competitive inhibition by Cu at high Fe^{2+} concentrations. With SM-3 Cu-adapted cells, the biphasic nature of the plots was retained, but again Cu activated the Fe^{2+} oxidation (more at 83 mM than at 167 mM Cu).

Control cells of SM-4 (Fig. 5), SM-5, and SM-8 strains showed linear double reciprocal plots which changed to biphasic plots in the presence of Cu, noncompetitive at low Fe^{2+} and competitive at high Fe^{2+} concentrations. The Cu-adapted cells had lower K_m for Fe^{2+} and Cu became a competitive inhibitor at low Fe^{2+} concentrations. At high Fe^{2+} concentrations SM-8 adapted cells retained the competitive inhibition by Cu, but SM-4 (Fig. 6) and SM-5 adapted cells showed biphasic plots leading to Cu activation of Fe^{2+} oxidation at very high Fe^{2+} concentrations (4 and 11 mM for SM-4 and 11 mM for SM-5).

Based on the K_i values for Cu inhibition and activation by Cu, Cu tolerance of cells during Fe^{2+} oxidation follows the order:

At low [Fe^{2+}] unadapted: SM-4, SM-3 > SM-2, Tf-2 > SM-5 > SM-8 > SM-1 > Tf-1

adapted: SM-1, SM-3 > Tf-2 > Tf-1, SM-5 > SM-4 > SM-8

at high [Fe^{2+}] unadapted: Tf-2 > SM-2 > TF-1 > SM-3, SM-8 > SM-1 > SM-5 > SM-4

adapted: SM-1, SM-3, SM-4 > SM-5 > Tf-1 > Tf-2 > SM-8

The effects of zinc and ferric iron on iron oxidation by copper-adapted and control cells were also investigated (Tables 3,4). In the laboratory strains zinc caused a biphasic response in Tf-1 control cells (Uc to C) but a linear competitive inhibition in Tf-2 control cells. Cu-adapted TF-1 cells were not inhibited, but slightly activated by Zn (more at 83 mM than 167 mM Zn) while Tf-2 Cu-adapted cells were competitively inhibited by Zn. Ferric iron competitively inhibited the Fe^{2+} oxidation of control and Cu-adapted cells of Tf-1 and Tf-2. K_i values for Fe^{3+} were much lower than those for copper or zinc, and actually were more similar to the K_m values for ferrous iron substrate. Ferric iron affected the iron oxidizing ability of both Tf-1 and Tf-2 similarly except that Tf-1 was able to tolerate about three times as much ferric iron on the Cu-adapted cells at low Fe^{2+} concentrations.

The Fe^{2+} oxidation by mine isolates showed very similar response to zinc (Table 3) as that to copper (Table 2). The biphasic (Nc to C) nature of the inhibition was noted in SM-3, SM-4, SM-5 and SM-8

control cells as was the activation of Fe^{2+} oxidation by Cu or Zn metal in Cu-adapted SM-1, SM-3, SM-4 (Fig. 7) and SM-5. SM-1 control cells were inhibited competitively by either Cu or Zn at low and high Fe^{2+} concentrations. SM-5 was similarly activated by Cu or Zn at all Fe^{2+} concentrations after adaptation to copper. The Cu-adapted SM-8 was competitively inhibited by either Cu or Zn with a lower K_i value than control cells. Ferric iron (Fe^{3+}) inhibited the Fe^{2+} oxidation of both control and Cu-adapted cells of all mine strains competitively (Table 4 and Fig. 8) with K_i values much lower than those for Cu or Zn and only slightly higher than the K_m for Fe^{2+}, the substrate. Cu-adapted SM-1 cells became more tolerant of Fe^{3+} than control cells. The most dramatic change after Cu-adaptation was observed in SM-4 (Fig. 9) and SM-5 strains. At 0.83 mM Fe^{3+} the slope of the double reciprocal plots increased with higher V_{max} and K_m, resulting in inhibition at low Fe^{2+} and activation at high Fe^{2+} concentrations. A further increase in Fe^{3+} concentration produced a typical competitive inhibition compared to the lowest Fe^{3+} concentration. Thus, compared to the no Fe^{3+} results, (Fe^{3+}) activated at high Fe^{2+} concentrations (more at lower Fe^{3+} than higher Fe^{3+} concentrations). No single trend seems to be associated with these mine strains with respect to K_m values and K_i values indicating again the variability of strains.

The dramatic effect of metals on the ferrous iron oxidation by many copper-adapted mine isolates is shown in Figs. 10 and 11. All the three metals, Cu^{2+}, Zn^{2+} and Fe^{3+}, inhibited the iron oxidation of copper-adapted Tf-2 cells at all the ferrous iron concentrations tested, while they inhibited the oxidation of copper-adapted SM-4 cells only at low ferrous iron concentrations and activated it at high ferrous iron concentrations.

Table 3. Effect of Zn^{2+} on the ferrous iron oxidizing activity of copper-adapted/unadapted Tf and SM cells

Strain	$[CuSO_4]$ in growth medium (mM)	Type of inhibition[a]		K_i Zn^{2+}(mM)	
		Low$[Fe^{2+}]$	High$[Fe^{2+}]$	Low[S]	High[S]
Tf-1	0	Uc	C	80(i)	75(i)
Tf-1 adapted	50	NI	NI	NI	NI
Tf-2	0	C	C	140(s)	140(s)
Tf-2 adapted	50	C	C	130(s)	170(s)
SM-1	50	C	C	77(s)	77(s)
SM-1 adapted	50	A	A		A
SM-2	0	C	C	300(s)	300(s)
SM-3	0	Nc	C	180(s)	180(i)
SM-3 adapted	50	A	A	A	A
SM-4	0	Nc	C	215(i)	46(s)
SM-4 adapted	50	C	A	125(s)	A
SM-5	0	Nc	C	160(i)	55(s)
SM-5 adapted	50	C	A	85(s)	A
SM-8	0	Nc	C	120(i)	116(s)
SM-8 adapted	50	C	C	60(s)	60(s)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.

(i):y-intercept.

(s):slope.

Table 4. Effect of Fe^{3+} on the ferrous iron oxidizing activity ofcopper-adapted/unadapted Tf and SM cells

Strain	$[CuSO_4]$ in growth medium (mM)	Type of inhibition[a]		K_i Fe^{3+} (mM)	
		Low[S]	High[S]	Low[S]	High[S]
Tf-1	0	Uc	C	80(i)	75(i)
Tf-1	0	C	C	1.5(s)	1.5(s)
Tf-1 adapted	50	C	C	4.3(s)	2.7(s)
Tf-2	0	C	C	1.8(s)	1.8(s)
Tf-2 adapted	50	C	C	1.5(s)	2.4(s)
SM-1	0	C	C	1.9(s)	1.9(s)
SM-1 adapted	50	C	C	7.0(s)	7.0(s)
SM-2	0	C	C	2.0(s)	2.0(s)
SM-3	0	C	C	1.2(s)	10.0(s)
SM-3 adapted	50	C	C	2.1(s)	2.2(s)
SM-4	0	C	C	2.1(s)	2.1(s)
SM-4 adapted	50	C	A	2.6(s)	A
SM-5	0	C	C	1.4(s)	1.4(s)
SM-5 adaption	50	C	A	1.0(s)	A
SM-8	0	C	C	3.2(s)	3.2(s)
SM-8 adapted	50	C	C	0.6(s)	0.6(s)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.

(i):y-intercept.

(s):slope.

Figure 7

Effect of Zinc on Iron Oxidation Rates by Copper-Adapted SM-4

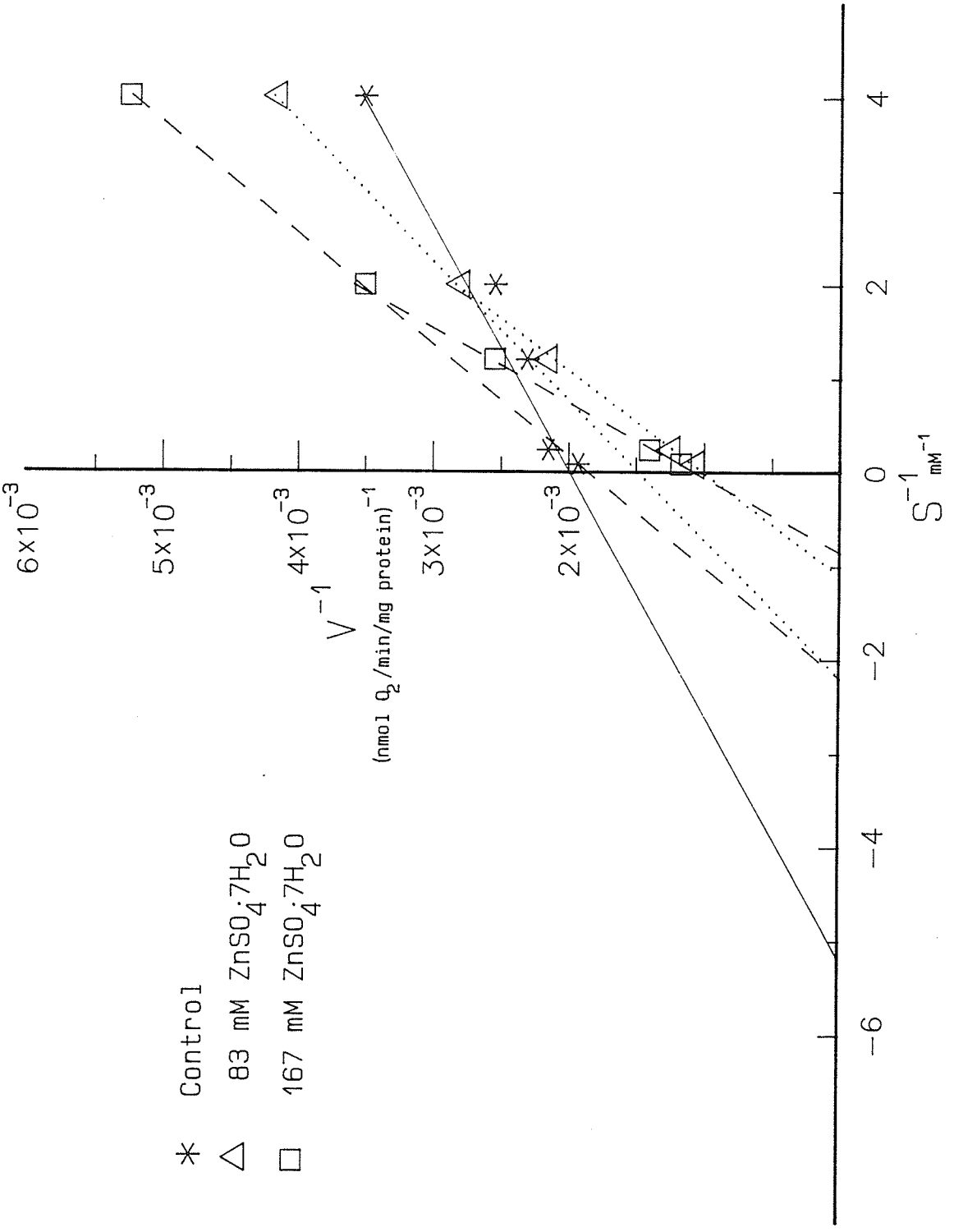

Control
83 mM $ZnSO_4 \cdot 7H_2O$
167 mM $ZnSO_4 \cdot 7H_2O$
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
6×10^{-3}
5×10^{-3}
4×10^{-3}
3×10^{-3}
2×10^{-3}
S^{-1} mM^{-1}
4
2
0
−2
−4
−6

Figure 8

Effect of Ferric Iron on Iron Oxidation Rates by SM-4

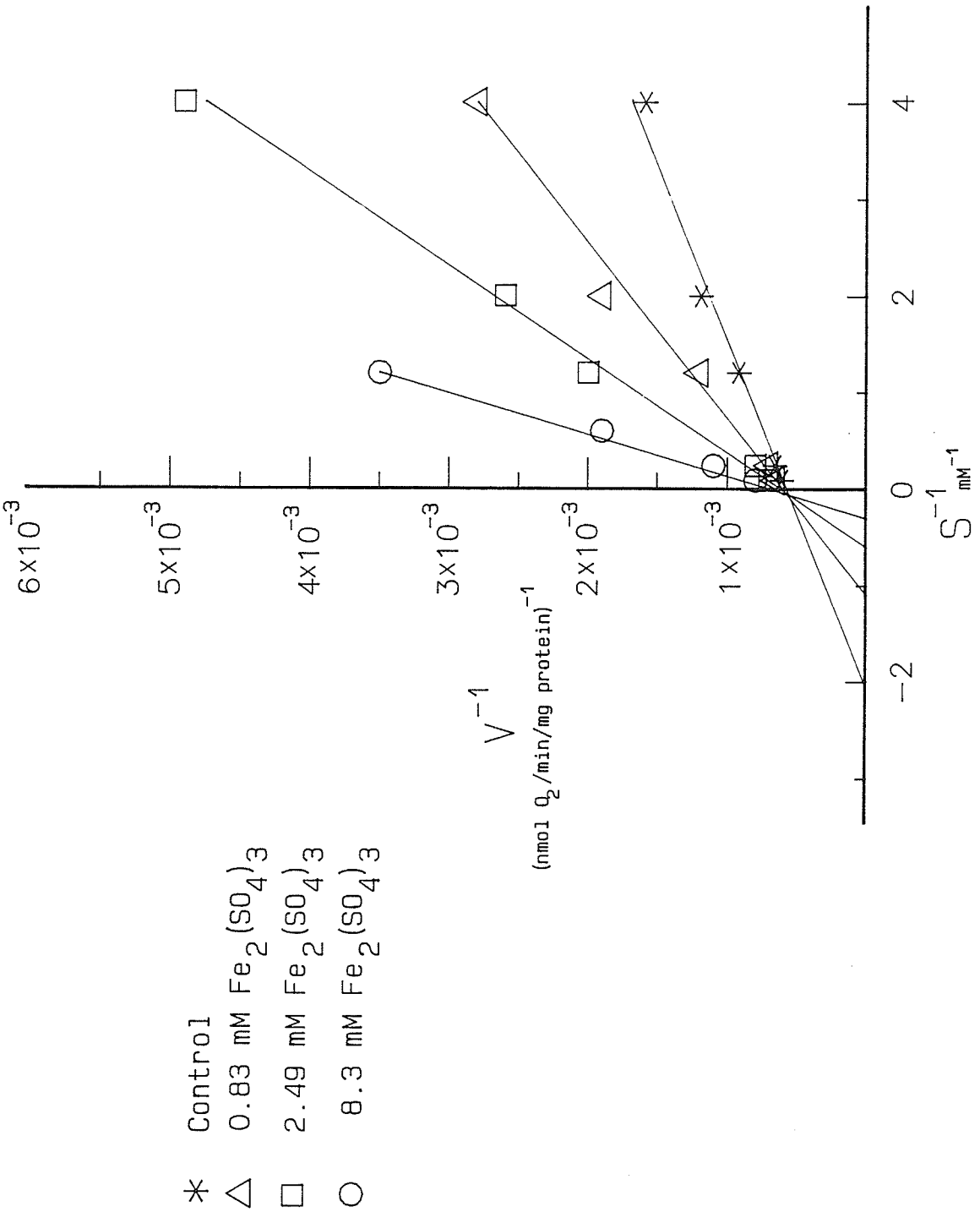

Control
0.83 mM $Fe_2(SO_4)_3$
2.49 mM $Fe_2(SO_4)_3$
8.3 mM $Fe_2(SO_4)_3$
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
$6x10^{-3}$
$5x10^{-3}$
$4x10^{-3}$
$3x10^{-3}$
$2x10^{-3}$
$1x10^{-3}$
−2
0
2
4
$S^{-1}_{mM^{-1}}$

Figure 9

Effect of Ferric Iron on Iron Oxidation Rates by Copper-Adapted SM-4

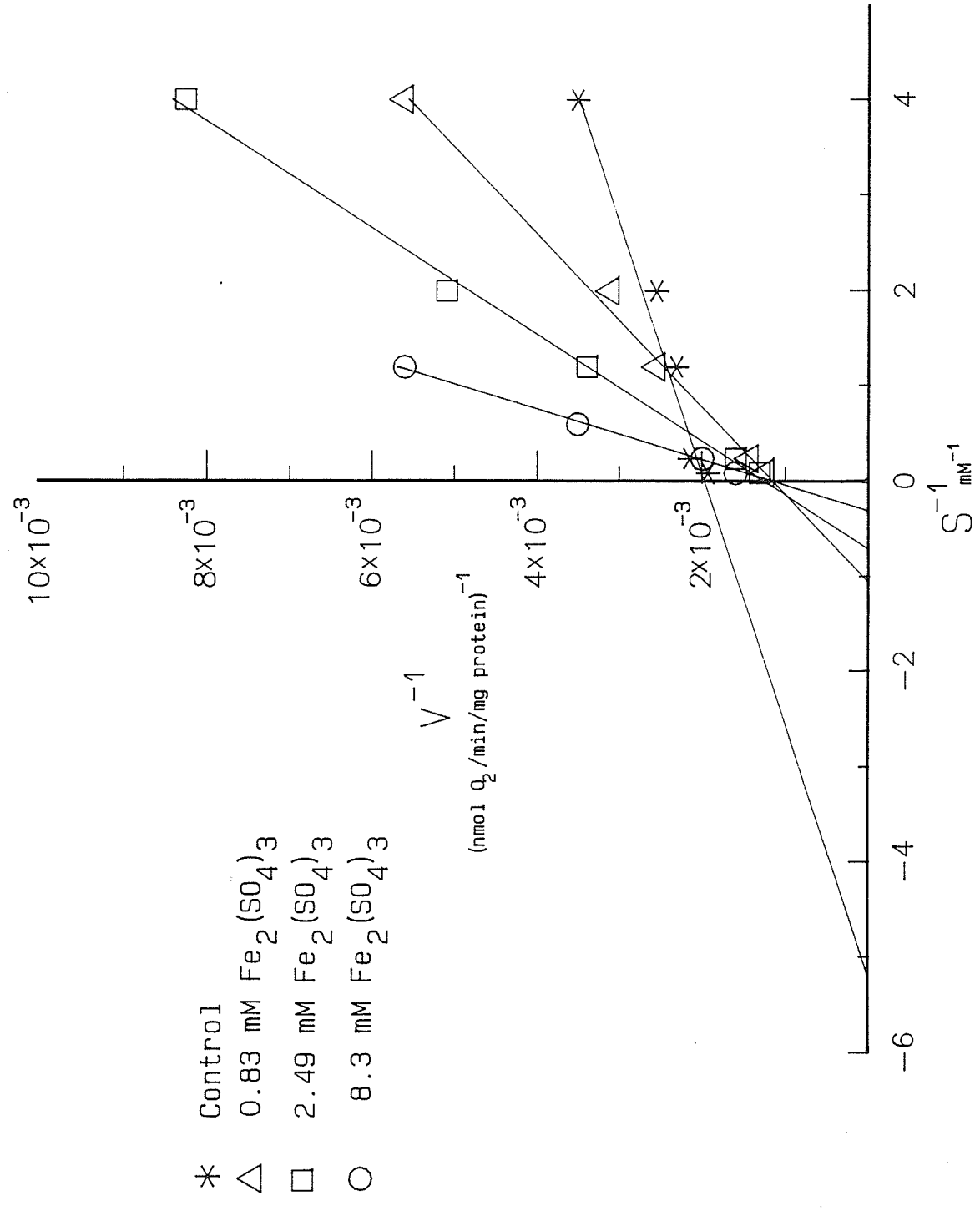

10X10^{-3}
8X10^{-3}
6X10^{-3}
4X10^{-3}
2X10^{-3}
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
-6
-4
-2
0
2
4
S^{-1} mM^{-1}
* Control
△ 0.83 mM $Fe_2(SO_4)_3$
□ 2.49 mM $Fe_2(SO_4)_3$
○ 8.3 mM $Fe_2(SO_4)_3$

Figure 10

Effect of Metal on Iron Oxidation by Copper-Adapted Tf-2

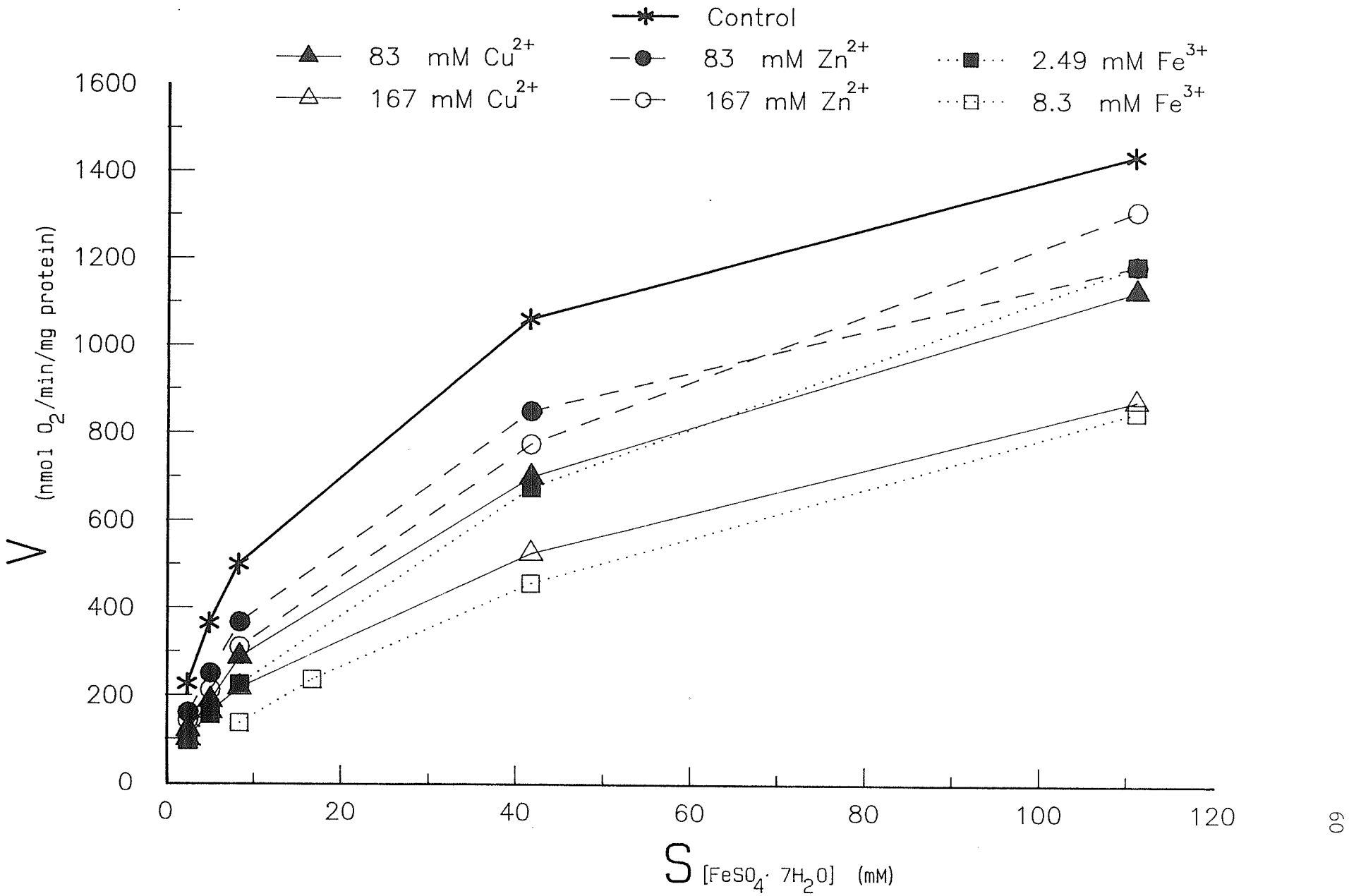
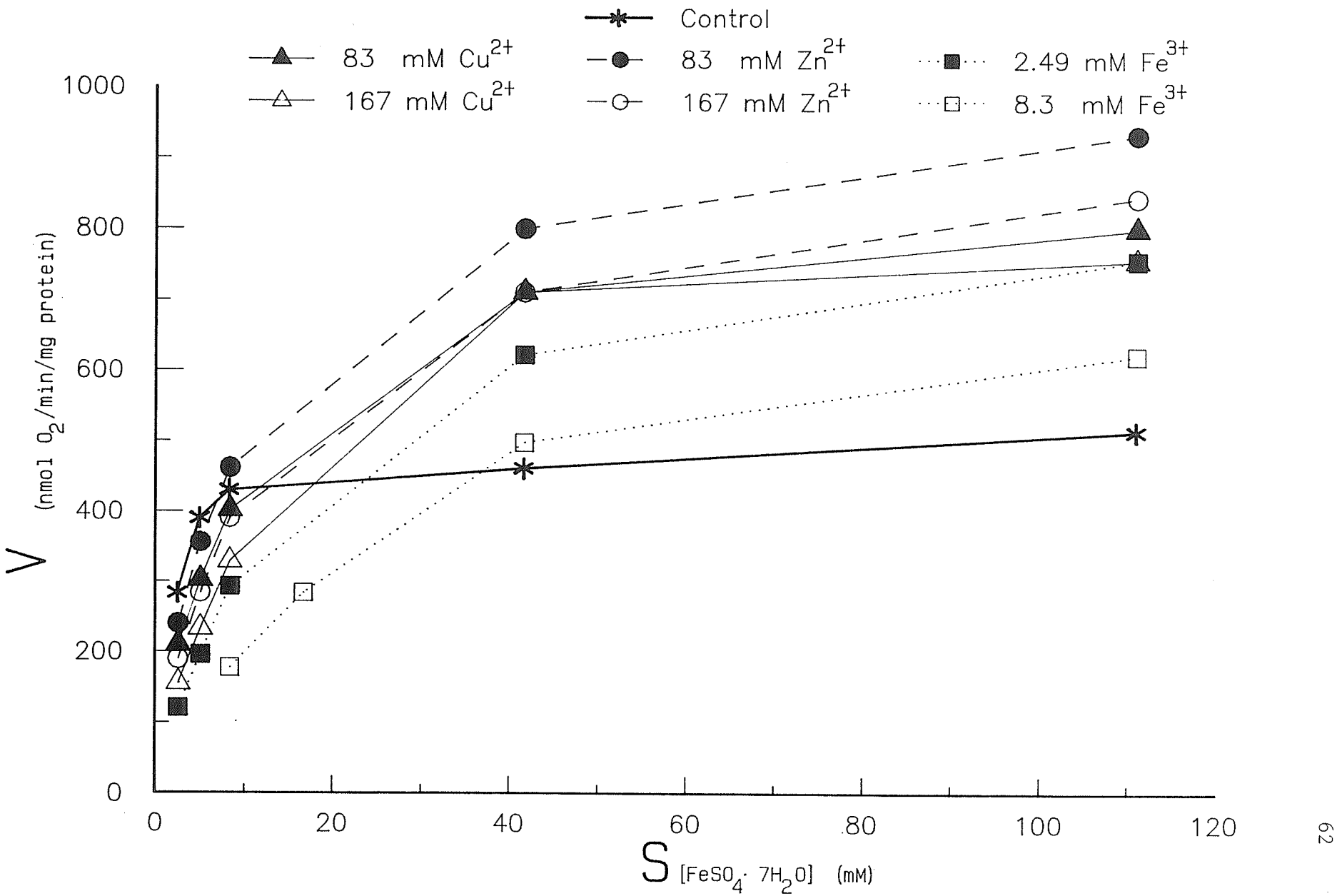

Control
83 mM Cu2+
83 mM Zn2+
2.49 mM Fe3+
167 mM Cu2+
167 mM Zn2+
8.3 mM Fe3+
V (nmol O2/min/mg protein)
S [FeSO4· 7H2O] (mM)
0
200
400
600
800
1000
1200
1400
1600
0
20
40
60
80
100
120

Figure 11

Effect of Metal on Iron Oxidation by Copper-Adapted SM-4


Control
83 mM Cu2+
83 mM Zn2+
2.49 mM Fe3+
167 mM Cu2+
167 mM Zn2+
8.3 mM Fe3+
V (nmol O2/min/mg protein)
1000
800
600
400
200
0
0
20
40
60
80
100
120
S [FeSO4· 7H2O] (mM)

Zinc

Both laboratory strains and mine strains generally adapted very well to growth in the medium with 150 mM $ZnSO_4$, except SM-1 which failed to grow in 150 mM $ZnSO_4$ after 2 transfers (Table 5).

The lab strains did show some differences in growth characteristics. Tf-1 was the only strain (of all 8 mine & laboratory strains) that showed a steady increase in the generation time over the 3 successive subcultures to double that of the control (7 hours to 14 hours). There was, however, no change in lag period over the same period. Tf-2, on the other hand, showed no real change in the generation time but did have an increased lag period initially compared to the control (0-5 hours to 32 hours), but returned to the control level upon adaptation. Similarities between the 2 laboratory strains upon adaptation included increased growth yields, relatively stable activity, and as mentioned above, lag periods the same as control cells.

The mine strains, with the exception of SM-1 and SM-2, adapted well to 150 mM $ZnSO_4$. The fully adapted cells (SM-3, SM-4, SM-5 and SM-8) had generation times as fast as the control cells and lag periods either similar (SM-3, SM-4) to or shorter (SM-5, SM-8) than control cells. Initially the lag periods increased for SM-5 and SM-8 as did the generation times for SM-3 and SM-8. The difficult to grow SM-2 showed some adaptation by decreasing the lag from 200

TABLE 5. Growth characteristics of Tf- and SM- cells in the presence/absence of zinc ($ZnSO_4.7H_2O$)

Strain	[$CuSO_4$] (mM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield mg protein	Growth yield mg wet cells	Activity (V_{max}) nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1	0	0-5	7	15	8.8	83	1250
Sensitive[a]	150	0-5	9	25			
Adapted[b]	150	0-5	14	40	20	170	1200
Tf-2	0	0-5	8	25	15.4	80	1250
Sensitive	150	32	9	55			
Adapted	150	0-5	9	30	16	125	1050
SM-1	0	325	20	350	2.6	20	680
Sensitive	150						
Adapted	150	NG[c]					
SM-2	0	200	20	265	3.3	23	360
Sensitive	150	280	30	360			
Adapted	150	110	30	210	7.1	50	600
SM-3	0	0-5	12	35	7.2	49	230
Sensitive	150	0-5	45	135			
Adapted	150	0-5	12	35	5.0	50	280
SM-4	0	0-5	12	25	3.4	40	1740
Sensitive	150	0-5	14	50			
Adapted	150	0-10	12	50	14	85	670
SM-5	0	40	10	70	49.1	332	800
Sensitive	150	80	10	105			
Adapted	150	20	10	45	24.2	220	750
SM-8	0	30	20	100	5.0	28	690
Sensitive	150	60	30	150			
Adapted	150	0-5	17	50	8.6	50	115

[a]Initial growth in metal containing media.
[b]Final growth in metal containing media.
[c]NG: no growth.

TABLE 6. Effect of Zn^{2+} on the ferrous iron oxidizing activity of Zn^{2+} -adapted/unadapted Tf and SM cells

Strain	$[CuSO_4]$ in growth medium (mM)	Km Fe2+ (mM)		Type of inhibition[a]		K_i Zn^{2+}(mM)	
		Low[S]	High[S]	Low[Fe^{2+}]	High[Fe^{2+}]	Low[S]	High[S]
Tf-1	0	1.2	1.2	Uc	C	80(i)	75(s)
Tf-1 adapted	150	0.6	4.6	Nc	C	400(i)	150(s)
Tf-2	0	0.95	0.95	C	C	140(s)	140(s)
Tf-2 adapted	150	0.90	0.90	Nc	C	100(i)	160(s)
SM-1	0	0.35	0.35	C	C	77(s)	77(s)
SM-2	0	1.0	1.0	C	C	300(s)	300(s)
SM-2 adapted	150	1.7	1.7	C	C	170(s)	170(s)
SM-3	0	0.20	0.60	Nc	C	180(i)	180(s)
SM-3 adapted	150	0.25	0.25	Nc	A	600(i)	A
SM-4	0	0.45	0.45	Nc	C	215(i)	46(s)
SM-4 adapted	150	0.30	0.30	C	A	130(s)	A
SM-5	0	0.43	0.43	Nc	C	160(i)	55(s)
SM-5 adapted	150	0.25	1.14	Nc	C	500(i)	325(s)
SM-8	0	0.33	0.33	Nc	C	120(i)	116(s)
SM-8 adapted	150	0.10	0.10	C	A	70(s)	A

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.
(i):y-intercept.
(s):slope.

TABLE 7. Effect of Cu^{2+} on the ferrous iron oxidizing activity of Zn^{2+} -adapted/unadapted Tf and SM cells

Strain	$[ZnSO_4]$ in growth medium (mM)	Type of inhibition[a]		K_i Cu^{2+} (mM)	
		Low[S]	High[S]	Low[S]	High[S]
Tf-1	0	Uc	C	50(i)	83(s)
Tf-1 adapted	150	Nc	C	185(i)	105(s)
Tf-2	0	Nc	C	90(i)	130(s)
Tf-2 adapted	150	Nc	C	65(i)	130(s)
SM-1	0	C	C	90(i)	90(i)
SM-2	0	Uc	C	100(i)	130(s)
SM-2 adapted	150	Uc	C	80(i)	70(s)
SM-3	0	Nc	C	150(i)	70(s)
SM-3 adapted	150	Nc	A	900(i)	A
SM-4	0	Nc	C	155(i)	30(s)
SM-4 adapted	150	C	A	80(i)	A
SM-5	0	Nc	C	75(i)	50(s)
SM-5 adapted	150	Nc	C	250(i)	165(i)
SM-8	0	Nc	C	60(i)	68(s)
SM-8 adapted	150	C	A	30(s)	A

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.

(i):y-intercept.

(s):slope.

TABLE 8. Effect of Fe^{3+} on the ferrous iron oxidizing activity of Zn^{2+} -adapted/unadapted Tf and SM cells

Strain	$[ZnSO_4]$ in growth medium (mM)	Type of inhibition[a]		K_i Fe^{3+} (mM)	
		Low[S]	High[S]	Low[S]	High[S]
Tf-1	0	C	C	1.5(s)	1.5(s)
Tf-1 adapted	150	Nc	C	1.6(i)	4.0(s)
Tf-2	0	C	C	1.8(s)	1.8(s)
Tf-2 adapted	150	Nc	C	4.0(i)	4.6(s)
SM-1	0	C	C	1.9(s)	1.9(s)
SM-2	0	C	C	2.0(s)	2.0(s)
SM-2 adapted	150	C	C	3.5(s)	3.5(s)
SM-3	0	C	C	1.2(s)	10.0(s)
SM-3 adapted	150	C	A	4.5(s)	A
SM-4	0	C	C	2.1(s)	2.1(s)
SM-4 adapted	150	C	A	2.0(s)	A
SM-5	0	C	C	1.4(s)	1.4(s)
SM-5 adapted	150	C	C	1.4(s)	1.4(s)
SM-8	0	C	C	3.2(s)	3.2(s)
SM-8 adapted	150	C	C	0.5(s)	0.5(s)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); A, activation; NI, no or little inhibition.

(i):y-intercept.

(s):slope.

hours to 110 hours but the generation time remained increased from 20 to 30 hours. Growth yields increased in SM-2, SM-4 and SM-8 while the specific activity of cells increased in SM-2 and decreased in SM-4 and SM-8 after adaptation to zinc.

Growth in zinc and the resulting iron oxidation properties (Table 6) provided further evidence for the distinctness of the 2 laboratory strains as well as the differentiation between them and the mine strains. A biphasic response (Uc to C) to zinc inhibition at low Fe^{2+} and high Fe^{2+} concentrations was present in the control cells of Tf-1 while Tf-2 showed only the competitive type inhibition. The Zn-adapted cells were biphasic in both cases switching from noncompetitive to competitive inhibition at high Fe^{2+} concentrations. The Zn-adapted cells of Tf-1 also showed a biphasic nature to iron oxidation in the absence of zinc having high affinity for Fe^{2+} at low [S] (K_m = 0.6mM) and low affinity for Fe^{2+} at high [S] (K_m = 4.6 mM). With adaptation, Tf-1 became considerably more tolerant of zinc (Table 6). Tf-2 did not show increased tolerance but instead maintained relatively constant K_m values and K_i values. Iron oxidation in the presence of copper makes the 2 laboratory strains appear more similar. Both had biphasic inhibition patterns at low [S] and high [S] (Table 7) either before or after adaptation to Zn. Ferric iron also inhibited both strains in a similar manner (Table 8). The control cells showed a simple competitive inhibition with one low Ki value, while the Zn adapted cells showed biphasic noncompetitive to competitive switching at high [S] with higher K_i values.

The mine strains, with the exception of SM-1 and SM-2, were fairly constant in their response to Zn, Cu or Fe^{3+} during iron oxidation (Tables 6-8). The Fe^{2+} oxidizing activities of control cells were affected by Zn similarly in 4 strains (SM-3, SM-4, SM-5 and SM-8) with biphasic double reciprocal plots (Nc to C), while SM-1 and SM-2 showed simple competitive inhibition by Zn (Table 6). The Zn-adapted cells of SM-3, SM-4 and SM-8 showed activation by zinc, particularly at high Fe^{2+} concentrations and SM-5 showed increased tolerance (increasing Ki) upon adaptation. SM-3 was activated by 83 mM $ZnSO_4$ at all Fe^{2+} concentrations but at 167 mM Zn it was activated only at high Fe^{2+} and slightly inhibited at low Fe^{2+} concentrations. They all retained the biphasic response at low and high Fe^{2+} concentrations. SM-2 retained competitive inhibition upon adaptation but did not increase the tolerance to zinc. Response to Cu of the Zn-adapted cells (Table 7) was similar to that to Zn. SM-2 retained the biphasic (Uc to C) response of control cells with no increased tolerance, while SM-3, SM-4 and SM-8 were activated by copper at high Fe^{2+}, and SM-5 showed increased tolerance. Response of the zinc-adapted cells to Fe^{3+} (Table 8) was the same with SM-3 and SM-4 (activation by Fe^{3+} at high Fe^{2+}), while no activation or increased tolerance was observed with SM-5 and SM-8. In fact, the K_i for Fe^{3+} decreased upon Zn-adaptation in SM-8 but the K_m for Fe^{2+} (Table 6) was also decreased (0.33 to 0.10 mM). SM-5 also had different K_m values for Fe^{2+} after adaptation to Zn (0.43 to 0.25 mM and 1.14 mM with biphasic activity - Fe^{2+} double reciprocal plot). SM-3 was opposite in that the biphasic plot of control cells changed

to a linear plot of Zn-adapted cells with a single K_m (0.2, 0.6 to 0.25 mM).

Molybdate

Growth characteristics of various strains in the presence of Na_2MoO_4 are shown in Table 9. Growth of Tf-2, SM-3, SM-4 and SM-5 in 1.0 mM Mo-containing medium initially resulted in longer lag periods, longer generation times and longer complete growth times. Subsequent growth in the same medium enabled these strains to decrease these three parameters but not to the extent of the control rates. SM-2 grew in 1 mM Mo twice but stopped growing in the third transfer. The other strains Tf-1, SM-1 and SM-8 grew only in 0.1 mM Mo with no effect or full adaptation. Adaptation to molybdate then, does occur, except in SM-2, in both the lab strains and mine strains when the growth parameters of the strain initially exposed to the metal are compared to those of a third subculture in molybdate. The Mo-grown "adapted" strains showed the iron-oxidizing system to be relatively unaffected in the absence of molybdate. Apparent K_m values (Table 10) were relatively unchanged upon adaptation as were the V_{max} values. Exceptions did occur, as in SM-1 and SM-4 where activity decreased considerably and SM-5 where the K_m value for Fe^{2+} decreased by 50% in the adapted cells. Molybdate was generally an uncompetitive inhibitor of Fe^{2+}-oxidation, although other types of inhibition also appeared in few cases (Table 10).

Tolerance of the iron-oxidizing activity to molybdate, according to the K_i values in Table 10 was quite a variable parameter. Among the

TABLE 9. Growth characteristics of Tf- and SM- cells in the presence/absence of molybdate ($Na_2MoO_4 \cdot 2H_2O$)

Strain	[Na_2MoO_4] (mM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield mg protein	Growth yield mg wet cells	Activity (V_{max}) nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1	0	0-5	7	22	8.0	87	500
Sensitive[a]	0.1	0-5	8	25			
Adapted[b]	0.1	0-5	8	26	10.0	33	490
Tf-2	0	0-5	7	21	6.0	42	670
Sensitive	1.0	45	14	82			
Adapted	1.0	21	9	52	8.5	50	595
SM-1 new iron	0	0-5	7	24	21.6	60	570
Sensitive new	0.1	0-5	7	25			
Adapted new	0.1	0-5	8	22	4.2	13	345
SM-2	0	50	8	72	13.8	42	333
Sensitive	1.0	50	8	72			
Adapted	1.0	NG[c]					
SM-3	0	0-5	10	32	10.7	93	690
Sensitive	1.0	270	12	295			
Adapted	1.0	0-5	17	50	16.7	167	740
SM-4	0	0-5	10	38	4.3	27	910
Sensitive	1.0	140	20	200			
Adapted	1.0	210	25	280	3.9	44	590
SM-5	0	0-5	8	30	10.8	100	833
Sensitive	1.0	0-5	8	30			
Adapted	1.0	210	25	280	3.9	44	590
SM-8	0	0-5	9	26	6.4	53	1667
Sensitive	0.1	23	9	47			
Adapted	0.1	0-5	8	24	4.5	53	1540

[a]Initial growth in metal containing media.
[b]Final growth in metal containing media.
[c]NG: no growth.

TABLE 10. Effect of Molybdate on the ferrous iron oxidizing activity of molybdate-adapted/unadapted Tf and SM cells

Strain	$[Na_2MoO_4]$ in growth medium (mM)	K_m Fe^{2+}(mM)	Type of inhibition[a]		K_i MoO_4^{2-}(mM)	
			Low[Mo]	High[Mo]	Low[Mo]	High[Mo]
Tf-1	0	0.40	NI	N	NI	0.08(i)[b]
Tf-1 adapted	0.1	0.40	Uc(C)	Uc(C)	0.28(i) (0.08(s))	0.15(i) (0.01(s)[b]
Tf-2	0	0.36	Nc(C)	Nc(C)	0.09(i) (0.01(s))	0.09(i) (0.01(s))
Tf-2 adapted	1.0	0.40	C	C	0.09(s)	0.09(s)
SM-1	0	0.19	Uc	Uc	0.60(i)	1.3(i)
SM-1 adapted	1.0	0.27	C	Uc	0.15(s)	0.03(i)[b]
SM-2	0	0.16	Uc	Uc	0.24(i)	0.08(i)[b]
SM-3	0	0.12	Uc	Uc	11.5(i)	11.5(i)
SM-3 adapted	1.0	0.08	Uc	Uc	10.5(i)	45(i)
SM-4	0	0.14	Uc	Nc	0.16(i)	0.05(i)[b]
SM-4 adapted	1.0	0.13	Uc	Uc	0.09(i)	0.06(i)
SM-5	0	0.30	C	Nc	0.002(s)	0.005(s)
SM-5 adapted	1.0	0.15	Uc	Uc	0.13(i)	0.02(i)[b]
SM-8	0	0.24	Uc	Nc	0.18(i)	0.06(i)[b]
SM-8 adapted	1.0	0.23	Uc	Uc	1.2(i)	0.05(i)[b]

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); NI, no or little inhibition. Type of inhibition was often affected by the concentration of MoO_4^{2-} and sometimes (Tf-1-Mo and Tf-2) by Fe^{2+} concentrations (high Fe^{2+} in brackets) leading to different Ki values.

[b]Unproportionately high degree of inhibition when MoO_4^{2-} concentration was raised.

(i):y-intercept.

(s):slope.

Figure 12

Effect of Molybdate on Iron Oxidation Rates by SM-8

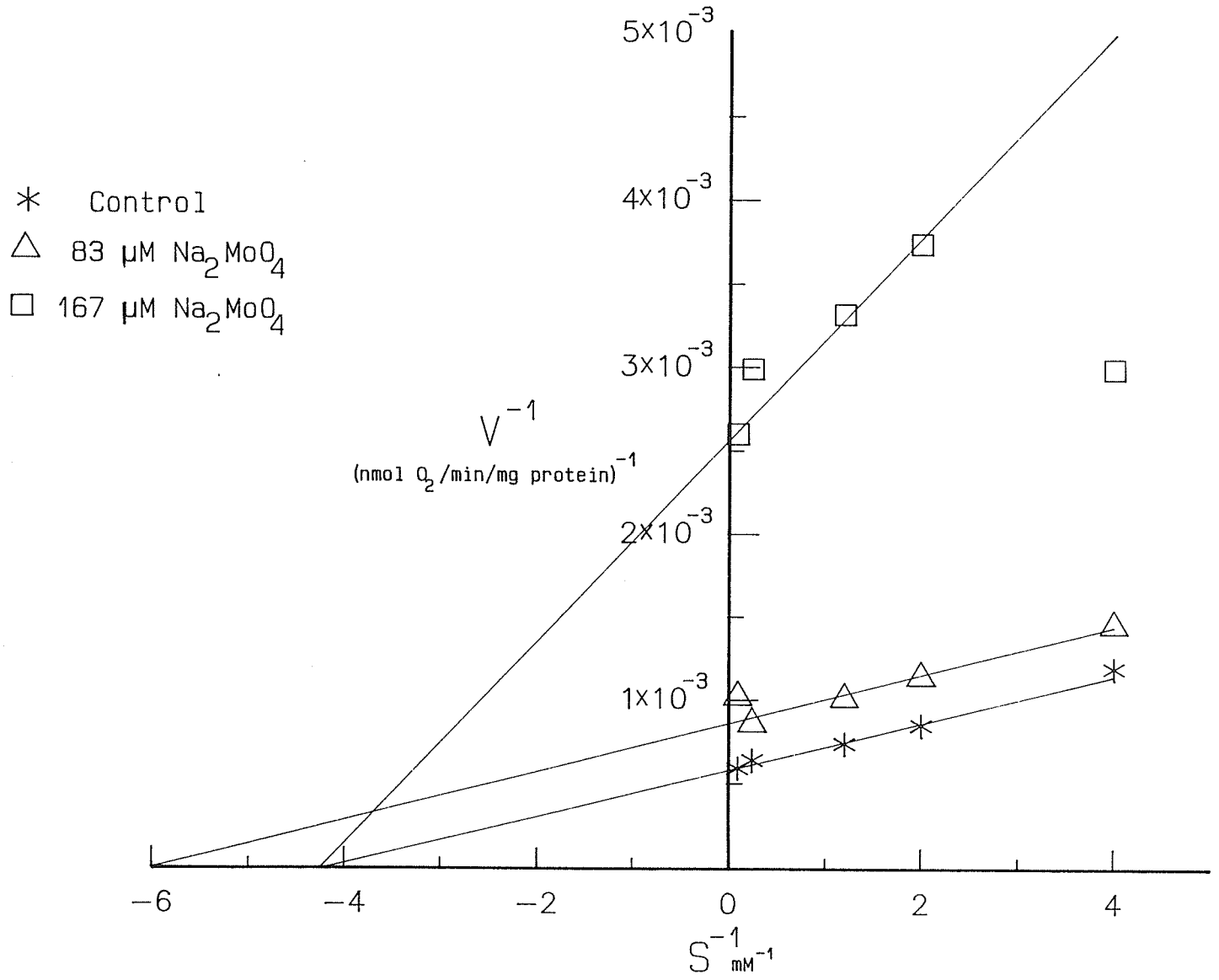
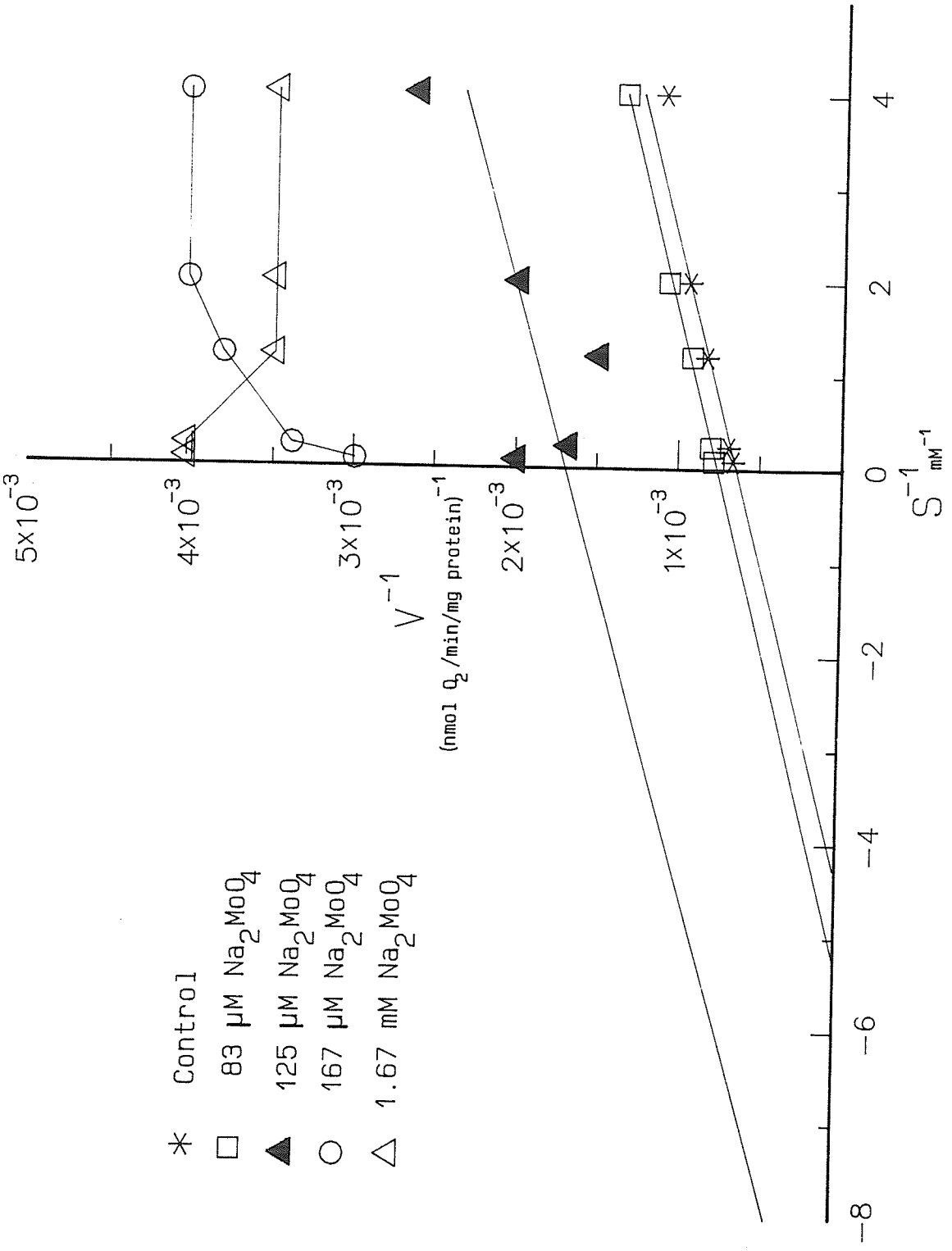

* Control
△ 83 μM Na_2MoO_4
□ 167 μM Na_2MoO_4
$5x10^{-3}$
$4x10^{-3}$
$3x10^{-3}$
$2x10^{-3}$
$1x10^{-3}$
V^{-1}
$(nmol\ O_2/min/mg\ protein)^{-1}$
−6
−4
−2
0
2
4
S^{-1}
mM^{-1}

Figure 13

Effect of Molybdate on Iron Oxidation Rates by Molybdate-Adapted SM-8


Control
83 μM Na_2MoO_4
125 μM Na_2MoO_4
167 μM Na_2MoO_4
1.67 mM Na_2MoO_4
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
$5X10^{-3}$
$4X10^{-3}$
$3X10^{-3}$
$2X10^{-3}$
$1X10^{-3}$
$S^{-1}_{mM^{-1}}$
-8
-6
-4
-2
0
2
4

control (unadapted) cells the K_i values ranged from 5 μM (SM-5) to 11.5 mM molybdate (SM-3). In addition there was an anomalous response to Mo concentrations with some strains. Thus the degree of inhibition was the same at 0.83 mM and 1.67 mM with SM-1 and at 1.67mM and 2.09 mM Mo with SM-3. With SM-5 16.7 μM Mo was less inhibitory than 8.3 μM. With SM-4 and SM-8 (Figure 12), on the other hand, 83 μM was only moderately inhibitory (20-25% inhibition), but the inhibition by 167 μM Mo was unexpectedly high (80-90% inhibition).

The Mo-adapted cells also showed a variable response to Mo in the Fe^{2+}-oxidizing activity. SM-3 was again the most tolerant strain with the highest K_i value of 45 mM and the inhibition was actually less when the Mo concentration was raised from 1.67 mM to 4.15 mM. SM-5, the most sensitive control cells, became more tolerant of Mo after adaption while SM-1, the second most tolerant control cells, became more sensitive to Mo inhibition after adaptation. In addition many strains (Tf-1, SM-1, SM-4, SM-5 and SM-8) showed disproportionately high degrees of inhibition when the Mo concentration was raised from 83 to 124 or 167 μM. With SM-8 the degree of inhibition decreased when the Mo concentration was further raised from 167 μM to 1.67 mM (Fig. 13). The reason for these complex responses to Mo is not understood but the accurate determination of K_i values was made difficult by the complexity.

Based on the estimated K_i values in Table 10 the order of tolerance to Mo of control (unadapted) cells is SM-3 > SM-1 > SM-4, SM-8 >

SM-2, Tf-1, Tf-2 > SM-5. Among Mo-adapted cells SM-3 is still by far the most tolerant to Mo inhibition, but the rest of the strains cannot be placed in an order of tolerance because the K_i values changed with Mo concentrations in a complex manner in each strain.

Arsenite

Arsenite precipitated at high concentrations in the Fe^{2+} iron medium and therefore could not be tested at concentrations higher than 1 mM. All the strains grew in 1.0 mM $NaAsO_2$ with only a slight initial increase in the growth time due to a slight increase in generation time or lag period (Table 11). SM-2 & SM-5 were the only strains to show a decrease in the lag and generation time upon adaptation. A significant increase in activity (V_{max}) was also observed with this SM-2 adapted strain. SM-8 was the only strain to have an increased lag period through the sensitive and adapted cells although the generation time have remained stable. No real trend can be described for a relation between the growth yield parameters and any other parameter.

Iron oxidation studies at high ferrous ($\geq$10 mM) and arsenite (16.7 mM) concentrations were hampered by probable removal of arsenite by ferrous iron, therefore results from low [Fe^{2+}] iron assays were used. With some strains the oxygen uptake levelled off before Fe^{2+} depletion in the presence of 16.7 mM arsenite making the initial velocity measurement difficult. This time dependent effect was observed only after incubation of all three cells, Fe^{2+} and arsenite.

The type of inhibition by arsenite was uncompetitive (Fig. 14) throughout the study. In higher concentrations of arsenite [16.7 mM], however, the inhibition turned competitive for some strains.

TABLE 11. Growth characteristics of Tf- and SM- cells in the presence/absence of arsenite($NaAsO_2$)

Strain	[$NaAsO_2$] (mM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield mg protein	Growth yield mg wet cells	Activity (V_{max}) nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1	0	0-5	7	21	8.0	87	530
Sensitive[a]	1.0	0-5	8	26			
Adapted[b]	1.0	0-5	9	26	19	60	640
Tf-2	0	0-5	7	21	6.0	42	670
Sensitive	1.0	0-5	8	28			
Adapted	1.0	0-5	8	28	32?	100	750
SM-1	0	0-5	7	24	21.6	60	570
Sensitive	1.0	0-5	9	32			
Adapted	1.0	0-5	10	31	12	42	445
SM-2	0	50	8	72	13.8	42	333
Sensitive	1.0	50	8	72			
Adapted	1.0	5-10	4	20	8.75	25	570
SM-3	0	0-5	11	33	10.7	93	690
Sensitive	1.0	0-5	11	39			
Adapted	1.0	0-5	11	34	17.5	120	625
SM-4	0	0-5	11	40	4.3	27	910
Sensitive	1.0	0-5	14	45			
Adapted	1.0	5	12	47	12.2	100	1190
SM-5	0	5-10	8	31	10.8	100	833
Sensitive	1.0	0-5	7	25			
Adapted	1.0	0-5	7	23	6.1	62.5	950
SM-8	0	0-5	9	27	6.4	53	1667
Sensitive	1.0	15	9	40			
Adapted	1.0	10	9	38	8.2	60	1430

[a]Initial growth in metal containing media.
[b]Final growth in metal containing media.

TABLE 12. Effect of arsenite on the ferrous iron oxidizing activity of arsenite -adapted/unadapted Tf and SM cells.

Strain	[$NaAsO_2$] in growth medium (mM)	K_m (mM)	Type of inhibition[a]		K_i AsO_2(mM)	
			Low[Fe^{2+}]	High[Fe^{2+}]	Low[As]	High[As]
Tf-1	0	0.16	Nc	C	6.0(i)	6.0(i)
Tf-1 adapted	1.0	0.20	C	NI(Nc)	2.3(s)	NI(17.5(i))
Tf-2	0	0.36	Uc(C)	NI(C)		NI(2.0(s))
Tf-2 adapted	1.0	0.36	Uc	NI(C)		NI(1.5(s))
SM-1	0	0.20	Uc	Uc	37(i)	90(i)
SM-1 adapted	1.0	0.20	NI	NI(C)	NI	NI(10.0(s))
SM-2	0	0.16	Uc	Uc	40(i)	40(i)
SM-2 adapted	1.0	0.13	Uc	Uc	37(i)	37(i)
SM-3	0	0.12	Uc	C	60(i)	2.5(s)
SM-3 adapted	1.0	0.28	Uc	C	40(i)	2.5(s)
SM-4	0	0.14	Uc	C	20(i)	2.5(s)
SM-4 adapted	1.0	0.14	Uc	C(Uc)	30(i)	2.0(s)(45(i))
SM-5	0	0.30	Nc	Nc	4.0(i)	4.0(i)(0.5(i))
SM-5 adapted	1.0	0.30	Uc	C	40(i)	2.5(s)
SM-8	0	0.24	Uc	NI(Uc)	23(i)	NI945(i))
SM-8 adapted	1.0	0.24	Uc	Uc	45(i)	45(i)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change); NI, no or little inhibition. Type of inhibition was often affected by the concentration of AsO_2^- and Fe^{2+} concentrations (high Fe^{2+} in brackets) leading to different Ki values. In some cases there was no inhibition (NI) at 8.3 or 16.7 mM AsO_2^- (high As) particularly at low Fe^{2+} concentrations.
(i):y-intercept.
(s):slope.

Figure 14

Effect of Arsenite on Iron Oxidation Rates by Arsenite-Adapted SM-8

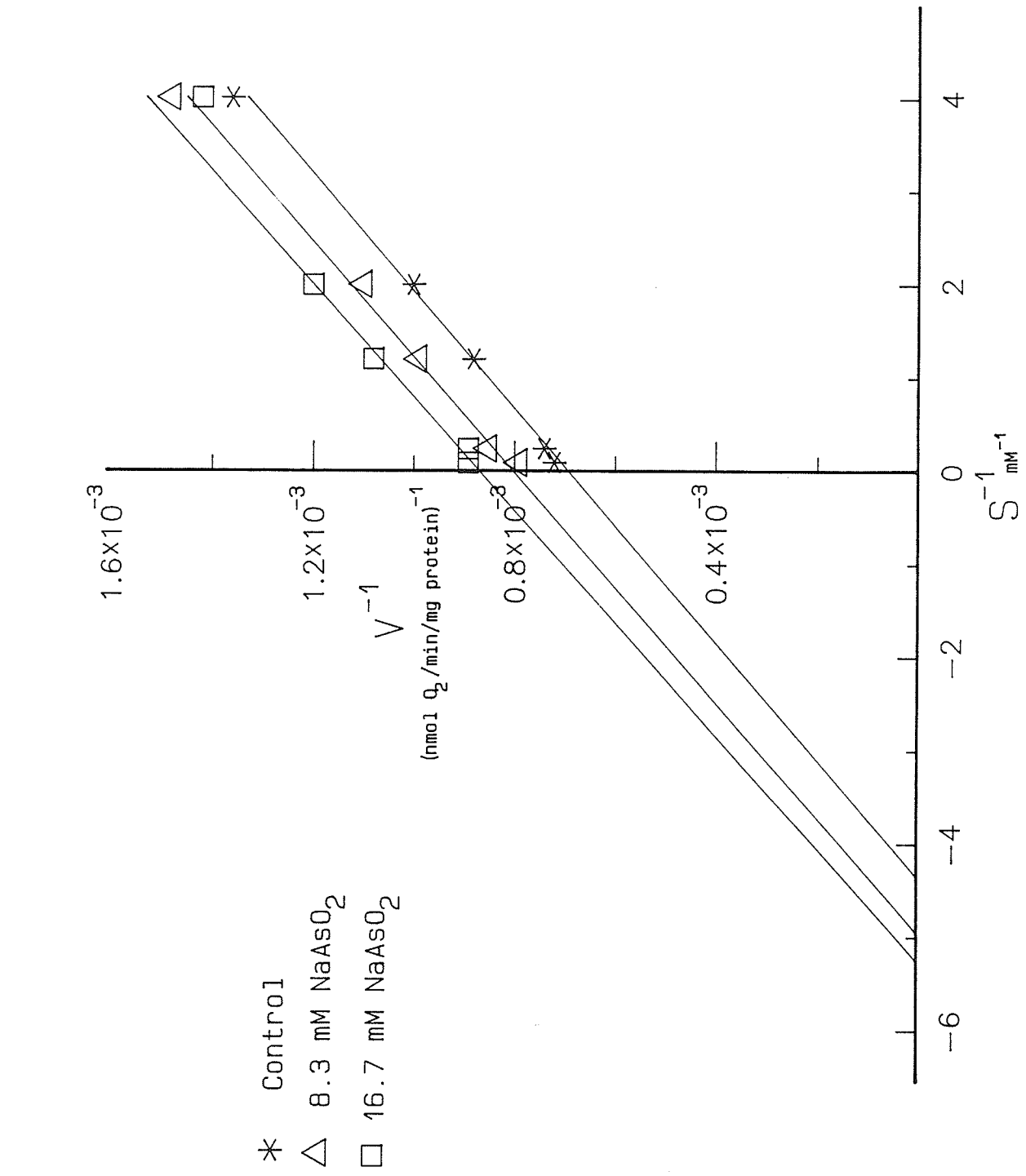
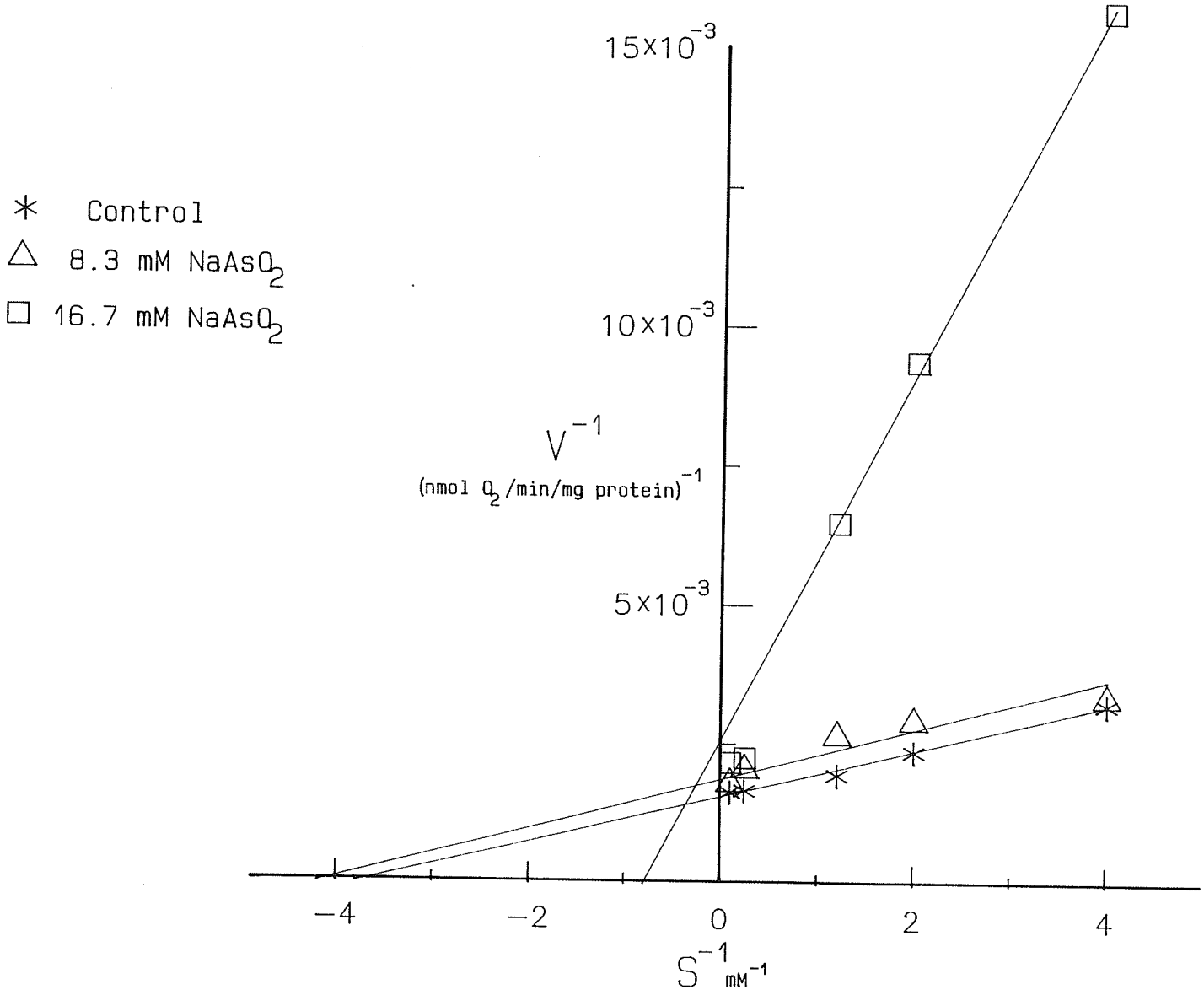

* Control
△ 8.3 mM $NaAsO_2$
□ 16.7 mM $NaAsO_2$
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
1.6X10^{-3}
1.2X10^{-3}
0.8X10^{-3}
0.4X10^{-3}
4
2
0
−2
−4
−6
$S^{-1}_{mM^{-1}}$

Figure 15

Effect of Arsenite on Iron Oxidation Rates by Arsenite-Adapted SM-3


* Control
△ 8.3 mM NaAsO2
□ 16.7 mM NaAsO2
15x10-3
10x10-3
5x10-3
V-1
(nmol O2/min/mg protein)-1
-4
-2
0
2
4
S-1
mM-1

SM-3 (Fig. 15) and SM-4 showed this uncompetitive to competitive switch for both adapted and control cells while SM-5 showed it for only the control cells. With the switch, a decreased K_i value resulted, changing by a full 10-25 times the uncompetitive value. The laboratory strains were the only strains to show a significant reduction in tolerance to arsenite upon adaptation. The mine strains demonstrated either no change or an increased tolerance to arsenite upon adaptation with SM-5, SM-1 and SM-8 showing the greatest change.

The laboratory strains and SM-1, both adapted and control, had less inhibition of iron oxidation at the higher [8.3 mM vs 16.7 mM] arsenite concentration while SM-2 showed this only for adapted cells and SM-8 for control cells. The other strains then, have typical increasing inhibition with increasing inhibitor concentration.

Adaptation to arsenite did not result in any change in the K_m values for iron, except for a doublings of the value for the adapted strain of SM-3.

The degree of tolerance to arsenite as measured by the K_i value, once again finds the laboratory strains to be the most sensitive in both adapted and control cells.

control : SM-3 > SM-2 > SM-1 > SM-8 > SM-4 > Tf-2 > Tf-1 > SM-5

adapted : SM-1 > SM-8 > SM-5 > SM-3 > SM-2 > SM-4 > Tf-2 > Tf-1

SM-1, SM-5 and SM-8 all showed the greatest degree of adaptation when comparisons are made of the K_i values for control (unadapted) versus adapted cells.

Mercury and silver

Mercuric chloride and silver nitrate were found to be more toxic to T. ferroxidans strains than other metals tested. Silver and mercury concentrations of both 1 μM (10^{-6} M) and 0.1 μM (10^{-7} M) were used for growth experiments (Table 13 and 14). The laboratory strains, Tf-1 and Tf-2, both grew in 0.1 μM mercury. The lag periods in Tf-1 did not change, but in Tf-2 it initially increased from almost zero to 80 hours and returned to the control level upon adaptation. The generation times of both Tf-1 and Tf-2 increased in the first exposure to Hg, but returned to the control level after adaptation. The effects of silver were similar. Tf-1 differed from Tf-2 here in that Tf-1 grew in 1 μM silver after a long lag of 210 hours with a long generation time (over 10 times the control), but on adapting decreased the lag to the control level and the generation time to only twice that of the control. Tf-2 did not grow in 1 μM Ag but did so in 0.1 μM silver after an extended lag of 300 hours. The generation time was only doubled. Both returned to the control level after adaptation. Oddly enough the activity increased about 2 times in both Hg and Ag adapted Tf-1, while both the activity and yield decreased considerably in Tf-2 after Ag adaptation.

Most of the mine strains had a great deal of difficulty in adapting to mercury and silver. SM-1 did not grow in 0.1 μM silver while SM-2 did grow once in 1 μM silver but was not able to grow after further transfer. Both grew in 0.1 μM mercury. With both strains, the lag

TABLE 13. Growth characteristics of Tf- and SM- cells in the presence/absence of mercury ($HgCl_2$)

Strain	[$HgCl_2$] (mM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield		Activity (V_{max})
					mg protein	mg wet cells	nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1 control	0.0	0-5	8	20	6.0	45	770
Sensitive[a]	0.1	0-5	29	90			
Adapted[b]	0.1	0-5	10	35	6.1	42	1400
Tf-2	0.0	0-5	9	30	8.5	50	1428
Sensitive	0.1	80	18	135			
Adapted	0.1	0-5	8	28	5.7	48	1700
SM-1	0	60	8	80	2.1	22	600
Sensitive	1.0	120	24	200			
Adapted	1.0	40	13	75	6.8	64	1200
SM-2	0.0	85	23	140	*		
Sensitive	0.1	150	35	240			
Adapted	0.1	75	35	160	0.28	6	385
SM-3	0.0	0-5	12	35	3.0	25	667
Sensitive	0.1	40	62	150			
Adapted	0.1	115	28	180	1.0	50	2200
SM-4	0	0-5	10	40	5.2	45	770
Sensitive	1.0	285	25	350			
Adapted	1.0	500	25	550	4.0	30	510
SM-5	0.0	0-5	8	35	4.3	42	667
Sensitive	0.1	170	90	325			
Adapted	0.1	140	12	170	1.8	20	570
SM-8	0	0-5	30	90	6.4	53	1667
Sensitive	1.0	260	20	310			
Adapted	1.0	0-5	23	80	0.4	13	1500

[a]Initial growth in metal containing media

[b]Final growth in metal containing media

*No growth in large flasks; therefore no cell harvest possible (see Ag results).

TABLE 14. Growth characteristics of Tf- and SM- cells in the presence/absence of silver ($AgNO_3$)

Strain	[$AgNO_3$] (μM)	Lag period (hrs)	Generation time (hrs)	Growth time (hrs)	Growth yield mg protein	Growth yield mg wet cells	Activity (V_{max}) nmol O_2 min^{-1} mg $protein^{-1}$
Tf-1 control	0	0-5	10	45	6.0	45	770
Sensitive [a]	1.0	210	110	600			
Adapted [b]	1.0	0-5	20	80	6.1	42	1428
Tf-2	0.0	0-5	8	30	8.5	50	1428
Sensitive	0.1	300	20	380			
Adapted	0.1	0-5	8	30	1.25	12.5	715
SM-1	0.0	80,60	70,22	200	2.1	22	600
Sensitive	0.1	NG[c]					
Adapted	0.1	NG[c]					
SM-2 new iron	0	50	8	72	13.8	42	333
Sensitive	1.0	50	8	72			
Adapted	1.0	NG[c]					
SM-3	0	0-5	12	35	3.0	25	667
Sensitive	1.0	15-20	15	60			
Adapted	1.0	15-20	70	190	0.8	23	111
SM-4	0	0-5	10	40	5.2	45	770
Sensitive	1.0	160	20	235			
Adapted	1.0	120	25	190	3.2	25	588
SM-5	0	0-5	8	35	4.3	42	667
Sensitive	0.01	285	25	410			
Adapted	1.0	20	10	55	9.0[d]	50[d]	385
SM-8	0	0-5	9	27	6.4	53	1667
Sensitive	0.1	0-5	10,22	100			
Adapted	0.1	0-5	9	28	16.8	42	667

[a] Initial growth in metal containing media.

[b] Final growth in metal containing media.

[c] NG: no growth.

[d] Cells freed from ferric precipitate by Fe^{2+} wash (7g $FeSO_4.7H_2O$/200 mL M9K).

Figure 16

Adaptation of SM-5 to $AgNO_3$

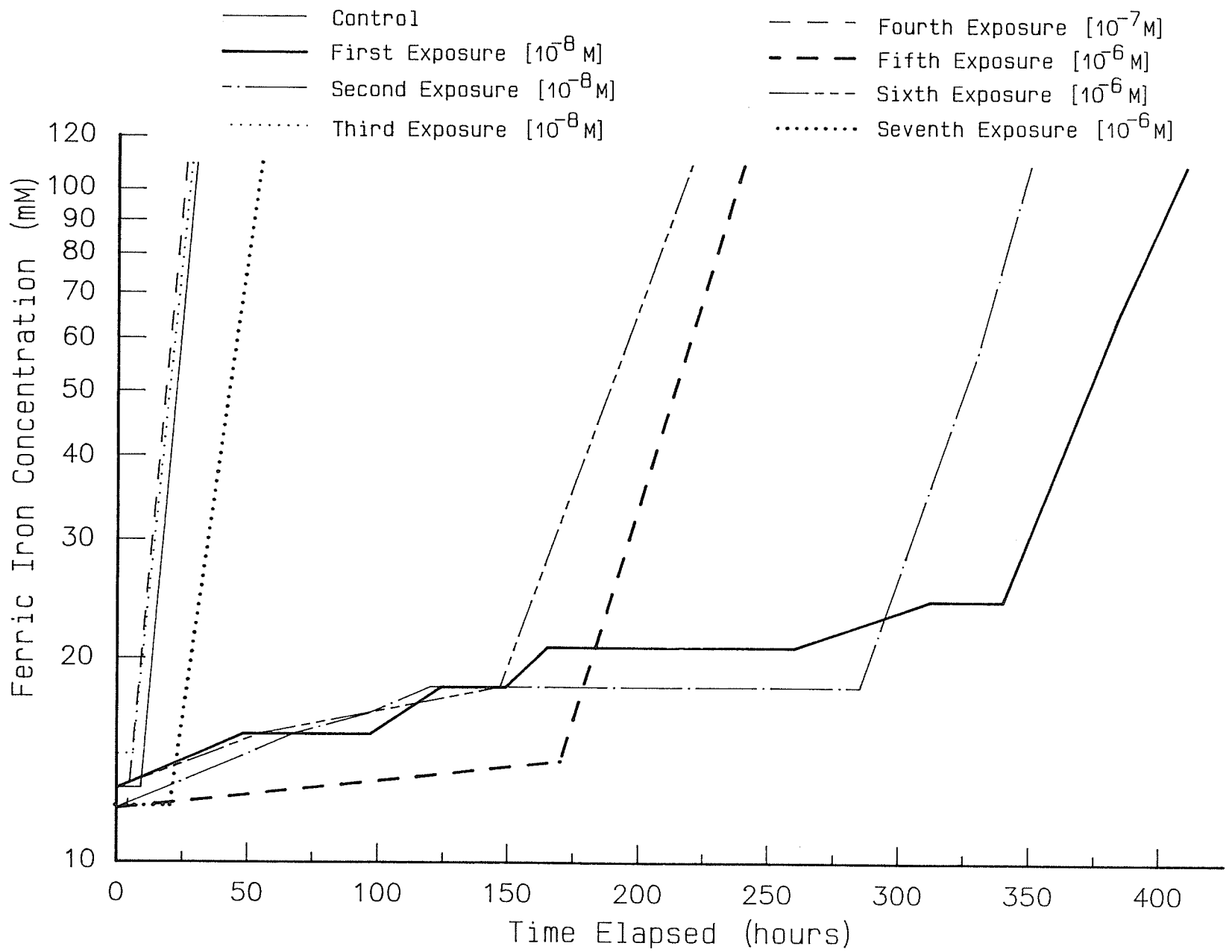

Control
First Exposure [10⁻⁸ M]
Second Exposure [10⁻⁸M]
Third Exposure [10⁻⁸M]
Fourth Exposure [10⁻⁷M]
Fifth Exposure [10⁻⁶M]
Sixth Exposure [10⁻⁶M]
Seventh Exposure [10⁻⁶M]
Ferric Iron Concentration (mM)
120
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
Time Elapsed (hours)

Figure 17

Effect of Mercury on Iron Oxidation Rates by Mercury-Adapted SM-4

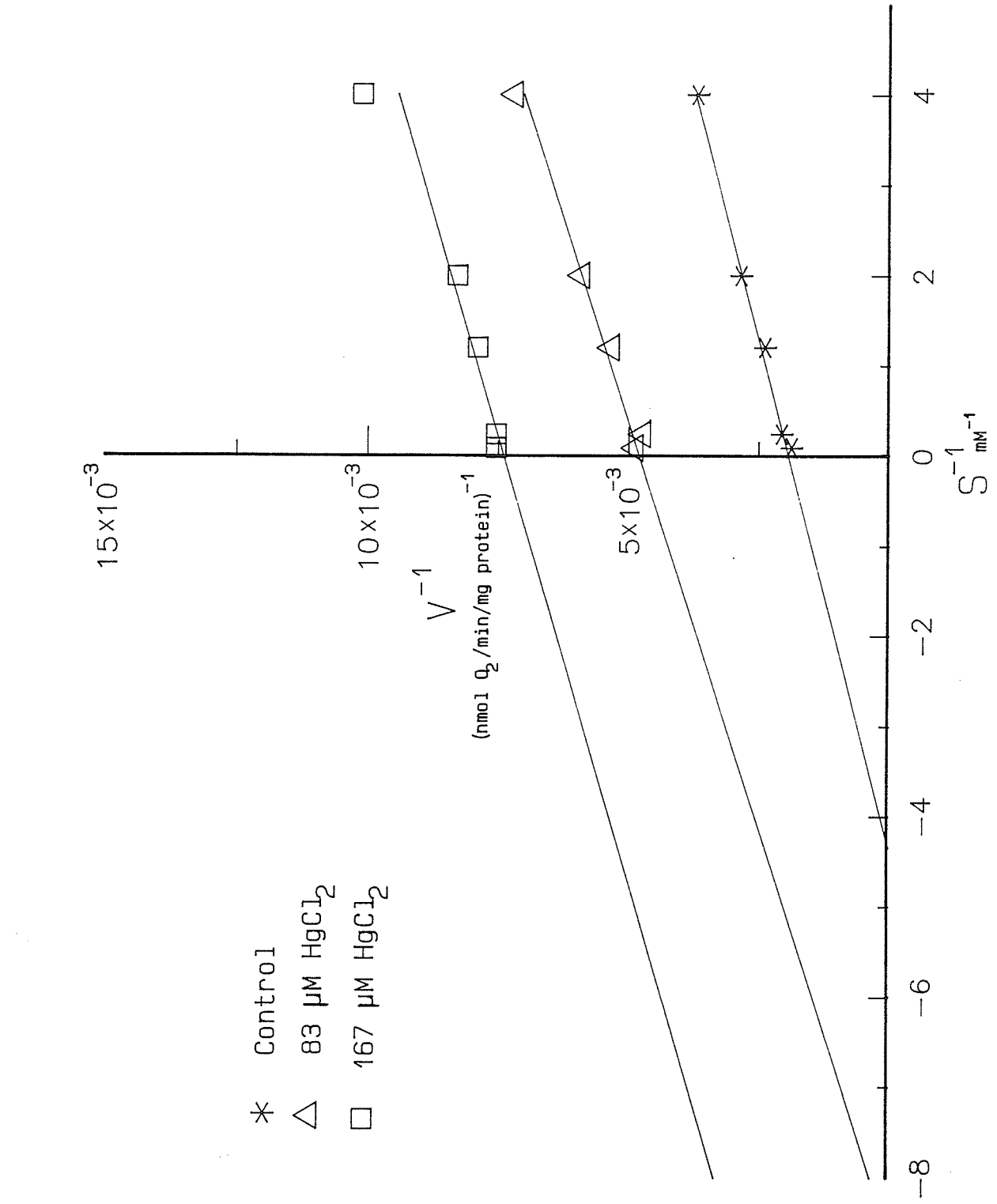

15x10^-3
10x10^-3
5x10^-3
V^-1
(nmol O2/min/mg protein)^-1
-8
-6
-4
-2
0
2
4
S^-1 mM^-1
Control
83 µM HgCl2
167 µM HgCl2

Figure 18

Effect of Mercury on Iron Oxidation Rates by SM-5

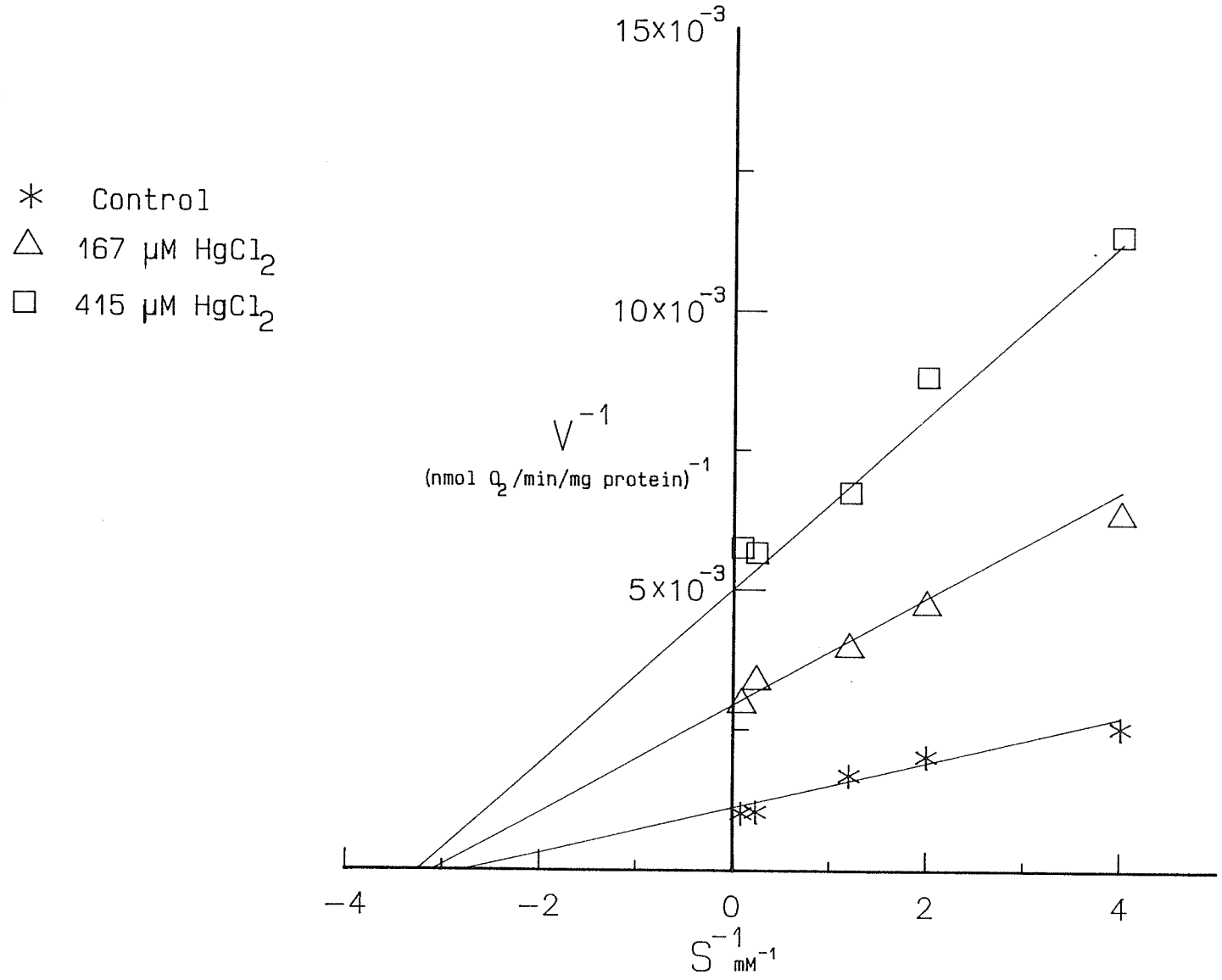
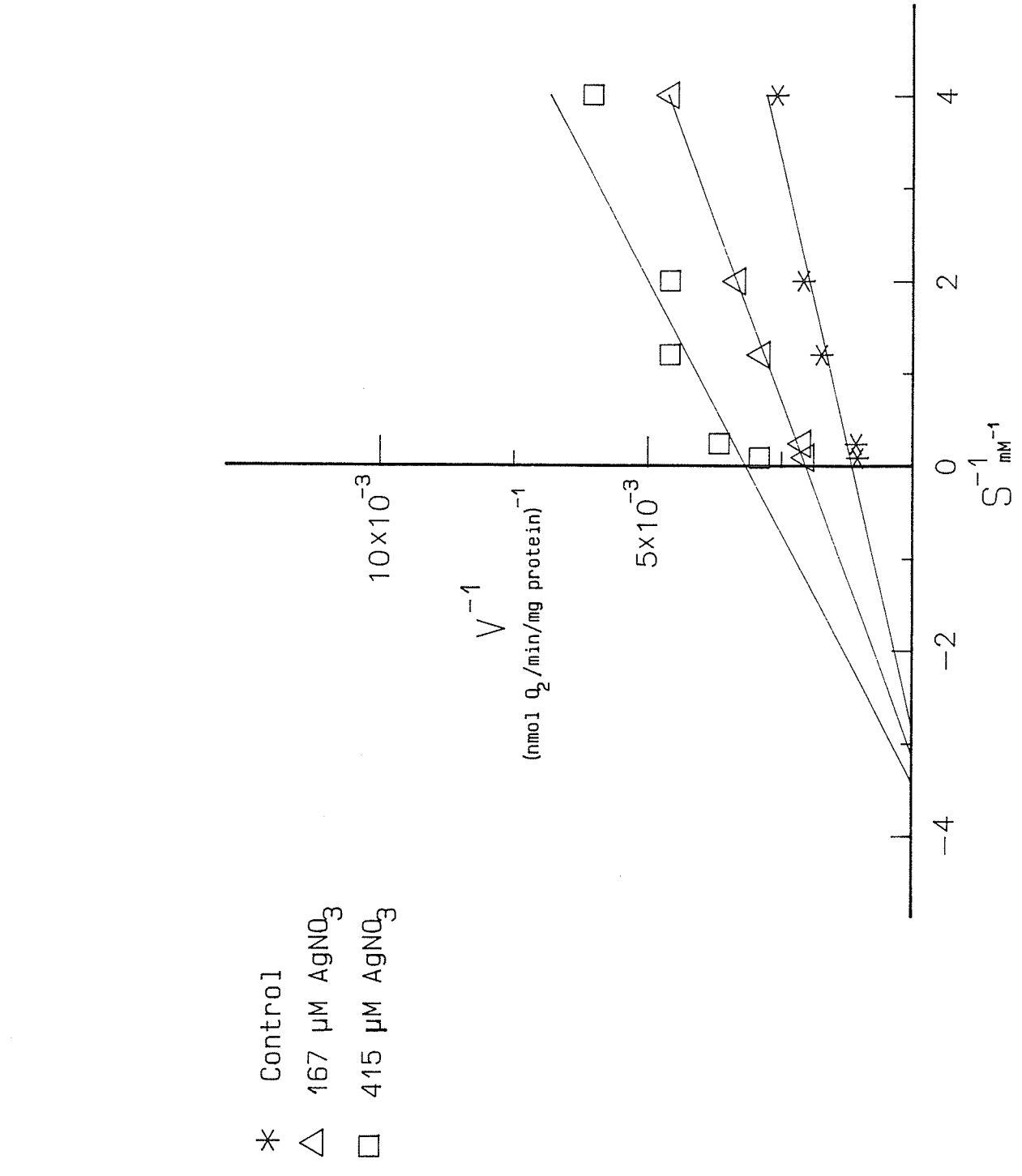

* Control
△ 167 μM $HgCl_2$
□ 415 μM $HgCl_2$
$15x10^{-3}$
$10x10^{-3}$
$5x10^{-3}$
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
−4
−2
0
2
4
S^{-1} mM^{-1}

Figure 19

Effect of Silver on Iron Oxidation Rates by SM-5


* Control
△ 167 μM $AgNO_3$
□ 415 μM $AgNO_3$
10×10^{-3}
5×10^{-3}
V^{-1}
(nmol O_2/min/mg protein)$^{-1}$
−4
−2
0
2
4
S^{-1} mM^{-1}

period increased in mercury then decreased to about the control level in the adapted cells while the generation times remained increased compared to the control rate. The strains which had shown the best adaptive potential in copper and zinc, SM-3, SM-4, SM-5 & SM-8 had mixed results in mercury and silver. SM-3 (0.1 μM) and SM-4 (1 μM) showed progressively increasing lag periods during adaptation in mercury media. Although not shown in Table 13 SM-4 adapted fully in 0.1 μM $HgCl_2$ with the lag period and generation time similar to control values. Adapted cells of SM-3 and SM-4 grew with reasonable generation times of only 2 to 3 times the control values. Both SM-3 and SM-4 adapted to 1 μM $AgNO_3$ with increased lag periods and generation times. The growth of SM-3 was adversely affected by Ag with a greatly increased generation time (12 to 70 hours) while with SM-4 the lag period was much extended (0-5 to 120 hours). SM-5 grew in 0.1 μM Hg but not in 1 μM Ag unless it was gradually adapted by successive transfers from 0.01 μM Ag to 0.1 μM Ag and finally to 1 μM Ag (Figure 16). SM-5 growth in 0.1 μM Hg resulted in a much extended lag period (140 hours) even after adaptation, but the generation time was reduced to the control level by adaptation. In the case of silver, SM-5 adapted well, decreasing both the lag period and generation time just slightly above the control levels. SM-8 was the only strain to show control like lag periods and generation times upon adaptation in both 1 μM mercury and 0.1 μM silver.

Iron oxidation was uncompetitively inhibited (Fig. 17) by silver and mercury in most strains (both adapted and control cells). In exceptional cases noncompetitive inhibition (Figs. 18 and 19) was observed. Both laboratory strains with and without mercury adaptation were fairly uniform in response to mercury during iron oxidation (Table 15). The inhibition was uncompetitive and the K_m and K_i values did not significantly change. Silver was also an uncompetitive inhibitor (Table 16), but silver adaptation resulted in increased sensitivity to silver (lower K_i) and decreased affinity for iron (higher K_m) in Tf-1 while in Tf-2 the opposite was observed (lower K_m, higher K_i). Although SM-1 and SM-2 did not grow in silver media, it is interesting to note that in the control cells, noncompetitive inhibition by silver was observed. Uncompetitive inhibition by mercury was observed in control cells and Hg adapted cells of both SM-1 and SM-2 with very low K_i values. SM-2 cells adapted to mercury were difficult to obtain, requiring special wash treatments with Fe^{2+} and Tween 80 in order to free cells from ferric iron precipitate. Because of the low yield and the special treatments of cells, the results are considered not as reliable as others. SM-3, SM-4 and SM-5 developed noncompetitive inhibition as well as increased sensitivity (lower K_i) to silver upon adaptation to 1 μM $AgNO_3$. SM-4 stood out from the others in that the K_m for Fe^{2+} increased to about 5 times that of the control (0.38 to 2.00 mM). Characteristics of SM-8 did not change upon adapting to silver and mercury. SM-5, SM-4 and SM-3 all showed increased sensitivity to mercury upon adaptation. SM-5 was the only one to be noncompetitively inhibited in the unadapted cells by both silver and

mercury. Both SM-4 and SM-5 developed slightly higher affinities for iron upon adapting to mercury while SM-3 developed a lower affinity.

The order of tolerance to Hg or Ag by various strains based on growth experiments is:

Hg: SM-8, SM-4 > Tf-2, SM-1, Tf-1, SM-2 > SM-5 > SM-3

Ag: SM-5, Tf-1 > SM-4, SM-3 > SM-2 > Tf-2, SM-8 > SM-1

Based on Ki values during Fe^{2+} oxidation:

Hg: control cells: SM-3 > Tf-2, SM-4, SM-5 > SM-8, SM-1 > Tf-1, SM-2

Hg: adapted cells: very little difference (K_i 50-80 μM)

Ag: control cells: SM-4 > SM-3 > SM-1 > Tf-1, SM-5, SM-8, SM-2 > Tf-2

Ag: adapted cells: very little difference among TF-2, SM-3, SM-4, SM-5, SM-8 ; K_i (175-260 μM) except Tf-1 (K_i = 30 μM)

TABLE 15. Effect of Hg on the ferrous iron oxidizing activity of Hg-adapted/unadapted Tf and SM cells

Strain	[$HgCl_2$] in growth medium (μM)	K_m Fe^{2+}(mM)	Type of inhibition[a]	K_i Hg^{2+}(μM)
Tf-1		0.23	Uc	50(i)
Tf-1 adapted	0.1	0.33	Uc	50(i)
Tf-2		0.34	Uc	130(i)
Tf-2 adapted	0.1	0.30	Uc	70(i)
SM-1		0.33	Uc	60(i)
SM-1 adapted	0.1	0.44	Uc	50(i)
SM-2		0.16	Uc	50(i)
SM-2 adapted[b]	1.0	0.09	Uc	50(i)
SM-3		0.17	Uc	210(i)
SM-3 adapted	0.1	0.56	Uc	60(i)
SM-4		0.38	Uc	125(i)
SM-4 adapted	1.0	0.23	Uc	50(i)
SM-5		0.36	Nc	125(i)
SM-5 adapted	0.1	0.24	Uc	60(i)
SM-8		0.24	Uc	65(i)
SM-8 adapted	1.0	0.16	Uc	80(i)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change).

[b]Cells freed from flasks and Fe^{3+} precipitate by washing with Fe^{2+} (118 mM) and 0.05% Tween 80 in M9K.

(i):y-intercept.

(s):slope.

TABLE 16. Effect of Ag on the ferrous iron oxidizing activity of Ag-adapted/unadapted Tf and SM cells

Strain	[$AgNO_2$] in growth medium (μM)	K_m Fe^{2+}(mM)	Type of inhibition[a]	K_i Ag^+(μM)
Tf-1	0	0.23	Uc	250(i)
Tf-1 adapted	1.0	0.36	Uc	30(i)
Tf-2	0	0.34	Uc	150(i)
Tf-2 adapted	0.1	0.20	Uc	260(i)
SM-1	0	0.33	Nc	300(i)
SM-2	0	0.16	Nc	200(i)
SM-3	0	0.17	Uc	340(i)
SM-3 adapted	1.0	0.17	Nc	175(i)
SM-4	0	0.38	Uc	525(i)
SM-4 adapted	1.0	2.0	Nc	250(i)
SM-5	0	0.36	Nc	230(i)
SM-5 adapted	1.0	0.24	Nc	180(i)
SM-8	0	0.24	Uc	230(i)
SM-8 adapted	0.1	0.29	Uc	250(i)

[a]Type of inhibition : C, competitive (slope change, no Y-intercept change); Nc, non-competitive (slope change, Y-intercept change); Uc, uncompetitive (no slope change, Y-intercept change).

(i):y-intercept.

(s):slope.

DISCUSSION

DISCUSSION

Copper

Most strains, laboratory and mine, showed the ability to partially or fully adapt to the 50 mM copper containing M9K medium. Since the laboratory strains adapted as well as the mine strains (except SM-2) the ability was not a characteristic peculiar to the mine isolates. It should be remembered, however, that the growth medium used contained 120 mM $FeSO_4$ (more than 100 times the K_m values for most strains) and the results might have been different at lower Fe^{2+} concentrations.

The most striking effect of Cu adaptation was that many mine isolates developed dependency on the presence of metals (Cu^{2+}, Zn^{2+}, or Fe^{3+}) for the activation of Fe^{2+} oxidation either at high Fe^{2+} concentrations only or at both low and high Fe^{2+} concentrations. Thus the activation was observed in Cu adapted cells of SM-1 (Cu, Zn), SM-3 (Cu, Zn), SM-4 (Cu, Zn, Fe^{3+} at high Fe^{2+}) and SM-5 (Cu, Zn, Fe^{3+} at high Fe^{2+}). With laboratory strains only a slight activation was noted with Cu adapted Tf-1 by Zn. The reason for this activation is not known, but the fact that 83 mM Cu^{2+} or Zn^{2+} or 2.5 mM Fe^{3+} can activate SM-4 and SM-5 suggests a mechanism where these metals specifically bind to the Fe^{2+} oxidase or cell surface iron lattice structure for increased activity. In some

experiments at high Fe^{2+} concentrations, the activity was reduced by increasing Fe^{2+} in the absence of metals, but increased in their presence. A possibility exists that the substrate inhibition site for Fe^{2+} can be occupied by Fe^{3+}, Cu^{2+} or Zn^{2+} eliminating the substrate inhibition, which results in activation in comparison. This ability to use metals to increase the ferrous iron oxidation rate may be an important feature of mine isolates in successful leaching of sulfide ores.

An interesting characteristic of the Cu and Zn inhibition of Fe^{2+} oxidation by most of the control cells (laboratory and mine) was the switch from noncompetitive to competitive inhibition at high ferrous iron concentrations creating a biphasic double reciprocal plot. This biphasic inhibition pattern was retained with Cu adapted cells of laboratory strains. With the Cu-adapted mine strains the plots were often biphasic but the switch was from competitive inhibition at low Fe^{2+} concentration to activation at high Fe^{2+} concentrations, increasing the V_{max} beyond the value in the absence of Cu or Zn. This activation was always more with 83 mM Cu or Zn and the activity was less when the metal concentration was increased to 167 mM, i.e. the inhibition was still operating. Noncompetitive or uncompetitive inhibition by Cu or Zn at low Fe^{2+} concentration indicates that Cu or Zn can bind the iron oxidase system at a site different from the Fe^{2+} (as substrate) binding site, thus causing inhibition indirectly. When the ferrous concentration increases, Fe^{2+} now can effectively compete with Cu^{2+} or Zn^{2+} at the inhibitor binding site. This site must have a lower affinity for

Fe^{2+} than the substrate-binding site operating at low Fe^{2+} concentrations since the apparent K_m (reciprocal of intercept at X-axis) increases.

A trend common to the mine strains is the generally lower K_m values compared to the laboratory strains. Also unique to three of the mine strains SM-4, SM-5 and SM-8 are the decreased K_m values (or increased affinity for Fe^{2+} substrate) upon adaptation in the 50 mM Cu - medium. The lower K_m values may indeed represent a selective advantage that these mine isolates may have over laboratory strains with higher K_m values, especially under the natural environmental conditions of mines where Fe^{2+} concentrations will be much lower than 120 mM Fe^{2+} of laboratory growth medium.

Ferric iron is considerably more inhibitory than copper or zinc to these cells, with much lower K_i values. The competitive inhibition and the proximity of the K_i values to the K_m values for Fe^{2+} suggests the same binding site for ferric iron as for ferrous iron substrate. This would indeed present a problem in bacterical leaching even when it is removed from solution as ferric hydroxide or jarosite. The strain such as SM-4 which is activated by Fe^{3+} would have a definite advantage.

The biphasic (activity - Fe^{2+} concentration) double reciprocal plots with two K_m values observed with some strains is not unusual in T. ferrooxidans. Kelly and Jones (1978) reported K_m values of 0.7 and

20-40 mM. They also reported substrate inhibition at high Fe^{2+} concentrations and competitive inhibition by Fe^{3+}. They also noted stimulation by Fe^{3+} (20 mM) when fresh cells were used, but not when cells were stored at 20°C. The K_i values of 2.5 to 28 mM Fe^{3+} are higher than those reported here, but the pH used for Fe^{2+} oxidation by these workers was pH 1.6, considerably lower than pH 2.3 used here.

Zinc

In agreement with reports by other workers (Imai et al., 1975, Tuovinen et al., 1971, 1974) zinc was found to be not highly toxic to the growth of either the laboratory strains or the mine strains. All strains except SM-1 adapted to 150 mM $ZnSO_4$ (10% Zn). Although either lag periods or generation times or both initially increased in sensitive cells, the zinc-adapted cells all showed shortening of the extended lag periods or generation times (with only 2 exceptions).

The effect of Zn adaption to the Fe^{2+} oxidations was similar to that of Cu adaption. Many mine isolates, but not laboratory strains, developed dependancy on the presence of metals for the activation of ferrous iron oxidation. Thus the activation was observed in Zn-adapted cells of SM-3 (Zn at low or high Fe^{2+}, Cu or Fe^{3+} at high Fe^{2+}), SM-4 (Zn, Cu, Fe^{3+} at high Fe^{2+}) and SM-8 (Zn, Cu at high Fe^{2+}). The discussion presented earlier relating to such an activation applies here also.

The biphasic inhibition pattern of control cells by Cu and Zn, the switch from noncompetitve or uncompetitive to competitive inhibition at high Fe^{2+}, was retained by laboratory strains after adaptation to Zn, but only by SM-5 among mine isolates. The other mine isolates after adaptation, although retaining the biphasic nature of the double reciprocal plots, were inhibited mostly competitively at low Fe^{2+} and activated at high Fe^{2+} concentrations.

Therefore the results were similar to the Cu-adapted cells and the discussion presented earlier is also pertinent here.

It is interesting that SM-8 which showed lower K_m for Fe^{2+} and lower K_i for Fe^{3+} after Zn-adaptation is the same strain that showed these properties after Cu adaptation.

From these results it is evident that Cu and Zn have a similar effect on T. ferrooxidans. Their inhibition patterns are similar and adaptation to Cu or Zn results in similar changes in the properties of cells. Cu, however, is a stronger inhibitor than Zn in terms of K_i values and growth tolerance (50 mM or 3% Cu and 150 mM or 10% Zn used in our experiments).

Molybdate

Molybdate (Na_2MoO_4) has been shown to inhibit most processes with respect to iron oxidation, including growth parameters. Indeed, half of the six mine isolates could not grow in 1.0 mM Mo-containing medium. Of these, only SM-3 showed a high tolerance to molybdate with a corresponding higher affinity for iron upon adaptation. This strain is certainly the exception as all the others, including the laboratory strains, showed extreme sensitivity to the metal anion, much like that demonstrated by some other investigators. Near complete inhibition of growth by molybdate at 0.2 mM was noted by Roy and Mishra (1981), and at 0.1 mM by Tuovinen et al., (1974). Imai et al., (1975), on the other hand reported that 1 mM Na_2MoO_4 delayed the growth by only one day and 10 mM Mo was required to inhibit the growth by 90%. Roy and Mishra (1981) also postulated that molybdate was more toxic to growth than to iron oxidation. In this study many strains showed K_i values lower than the Mo concentrations used for growth. This higher tolerance for Mo in growth experiments was probably related to the formation of phosphomolybdate and "molybdenum blue" in the growth medium which contained phosphate and a very high concentration of Fe^{2+} iron (120 mM $FeSO_4$). In fact 1 mM Na_2MoO_4 in the M9K medium produced a faint green color and 10 mM Na_2MoO_4 a darker green color. Presumably phosphomolybdate (yellow) was formed and was partially reduced by Fe^{2+} to "molybdenum blue" to produce a greenish color. The formation of "molybdenum blue" would have

reduced the effective concentration of Mo as inhibitor in the growth experiments and might have also led to an anomalous response to increasing Mo concentration in some inhibition experiments of Fe^{2+} oxidation.

For the most part, molybdate displayed an uncompetitive nature in the inhibition pattern. Since only the y-intercept changes, it may indicate that molybdate is combining somewhere in the scheme of iron oxidation that ferrous iron does not. It has been suggested by Tuovinen and Kelly (1974) that molybdate may be interfering with sulfate dependant initial steps in iron oxidation due to its similarity to the sulfate anion. An alternative explanation is that molybdate binds the iron oxidase system at the cytochrome oxidase and oxidizes a component essential for the action, similar to Ag (Sugio et al., 1981) and Hg (Imai et al., 1975). Variability does exist among the laboratory strains and mine strains indicating perhaps a very complex nature of molybdate inhibition.

Arsenite

The observation that the laboratory strains are the most sensitive to arsenite during iron oxidation seems to testify that the SM-isolates are representative of the "natural" growth conditions of mine waters, while laboratory strains were maintained for many years in an unnatural protective environment. Not only did they display greater sensitivity to arsenite but they became less tolerant of arsenite (during iron oxidation) upon adaption, as witnessed by decreased K_i values.

The uncompetitive nature of arsenite inhibition indicates that the iron oxidation system is indirectly affected. To determine exactly what occurs during iron oxidation is beyond this study but some possible explanations are presented for the results. Arsenite is an inhibitor known to bind to adjacent dithiol groups. In this manner, arsenite may bind the iron oxidase protein (or one of the proteins). Because uncompetitive inhibition is noted (i.e. increase in y-intercept) it appears that arsenite combines with an enzyme form different from that of the ferrous ion binding enzyme form. Perhaps ferrous ion binds to the disulfide form and reduces it to the dithiol form which binds arsenite. The fact that the inhibition sometimes increases with time after ferrous ion addition lends support to this idea.

There may also be a complication due to interaction between arsenite and Fe^{2+}. The visible precipitation at high concentration of

ferrous iron and arsenite could also be a factor affecting the degree of inhibition at lower concentrations of either ferrous or arsenite without causing visible precipitation. Depending on the isolates' properties this ferrous/arsenite interaction may result in either the observed increased or decreased inhibition at lower ferrous iron concentrations. The characteristic of three of the mine isolates (SM-3,4,5) to change from uncompetitive inhibition to competitive inhibition with an increase in arsenite concentration may be a result of the above interaction, where arsenite removal of Fe^{2+} may cause inhibition which is eliminated at higher Fe^{2+} concentrations. It is also possible that the ferrous arsenite interaction complex as inhibitor competes with Fe^{2+} as substrate. A more detailed study is required to elucidate this complex inhibition.

The fact that the other mine strains did not respond in the same way as the three named above suggest the general variability of characteristics found in naturally occurring thiobacilli.

Variability was also noted in the adaptive nature to arsenite with iron oxidation. While SM-2, 3 and 4 remained relatively unchanged with respect to the tolerance to arsenite (at low ferrous concentrations), SM-1, 5 and 8 became more tolerant after adaptation (increased K_i).

Mercury & silver

Mercury and silver were found to be strong inhibitors of growth in T. ferrooxidans strains, stopping the growth of most strains at 1 μM and allowing the growth and adaptation at 0.1 μM. Even when they grew, the generation times and lag periods were usually extended, the severity dependent on the strain. SM-4 was the only strain that grew repeatedly in the presence of 1 μM Hg or Ag, although the lag period remained extended. Other strains adapted to only one metal at 1 μM (SM-8, Ag; Tf-1, SM-3 and SM-5, Hg) and to the other metal at 0.1 μM or to both metals only at 0.1 μM (Tf-2). Both SM-1 and SM-2 adapted to 0.1 μM Hg, but failed to grow successively in 0.1 μM Ag (SM-1) or 1 μM Ag (SM-2). So for both Ag and Hg the upper limit of concentration which allows the growth is 0.1 to 1 μM. Other investigators have reported 0.1 ppm (1 μM) silver (Hoffman and Hendrix, 1976), (Sugio et al., 1981) and 1 ppm silver as well as 10 μM mercury (Imai et al., 1975), 2 μM Hg^{2+} (Norris and Kelly., 1978) and 0.1 μM Hg^{2+} (Olson et al., 1981) as inhibitory concentrations for the growth of various strains of T. ferrooxidans. A possible explanation for the extended lag period and generation time that mercury produces has been proposed (Olson et al., 1981) as a requirement by the cells to reduce the concentration of the mercury in the medium by volatilization to nearly zero before iron oxidation (and therefore growth) may occur. This mercury volatilization mechanism proposed is probably quite different from the silver

accumulation (Norris and Kelly, 1978) (Sugio et al., 1981) described as a possible resistance mechanism in silver resistant cells.

Extended lag periods or generation times in the presence of Ag or Hg observed in this study are not unusual. A silver-resistant culture grown by Norris and Kelly (1978) had a 75 to 100 hour lag period in 0.2 μM $AgNO_3$ 9K medium compared to 25-50 hours in the control cells without silver in the medium. Olson (1981) observed a similar lengthening of growth parameters in the presence of Hg. Although a considerable degree of variability was seen in the growth parameters of all the strains, those strains that adapted to 1 μM metal should be considered as more tolerant to that metal than those which only grew in 0.1 μM.

The Fe^{2+} oxidizing activity was more tolerant of higher concentrations of both metals than the growth and slightly more tolerant of Ag^{+} than Hg^{2+}. Silver had K_i values of control cells between 150 and 525 μM and of adapted cells between 30 and 250 μM. Mercury by comparison had K_i values of between 50 and 210 μM for control cells and 50 and 80 μM for adapted cells. Interestingly the K_i values for mercury in many strains decreased upon adaptation and the values in all adapted cells converged to a narrow range of 50 to 80 μM. The K_i values for silver also changed upon adaptation (mostly decreased) and again the values for all adapted cells converged to a range of around 200 to 250 μM except in Tf-1 where it was reduced from 250 μM to 30 μM.

Because the inhibition was uncompetitive or noncompetitive (with no competitive inhibition displayed) by silver or mercury, it suggested binding of the toxic metal to someplace other than the site where the Fe^{2+} substrate binds. Imai et al. (1975) and Sugio et al. (1981) reported that both mercury and silver prevented cytochromes c and a type from being reoxidized after reduction by Fe^{2+} as substrate. They proposed, therefore, that cytochrome oxidase was the binding site of the mercury or silver ions. In the current study, the inhibition of Fe^{2+} oxidation by mercury or silver in Oxygraph experiments showed the initial linear inhibited activity followed sometimes by a time-dependant further slowing down of the activity often resulting in near complete inhibition of Fe^{2+} oxidation even in the presence of high substrate concentrations. This time dependant inhibition required the presence of Fe^{2+} and was observed with most strains and more at higher concentrations of Hg or Ag used. These results suggest that Hg or Ag binds irreversibly with the enzyme form reduced by Fe^{2+} and agree with the theory of cytochrome oxidase as the site of binding. The K_m for Fe^{2+} does not significantly change between the control cells and the adapted cells of most of the strains which also lends support to this idea.

Since the tolerance level of metals for Fe^{2+} oxidation was 100 to 1000 fold higher, the metal tolerance during growth is more important in the selection of strains for sulfide ore leaching. SM-4 is considered the most tolerant to both Hg and Ag.

CONCLUSION

CONCLUSION

The six strains of Thiobacillus ferrooxidans which have been isolated from different sulfidic mine waters of the same Cu-Zn mine display considerable differences in growth and iron oxidation. Two of the strains would either grow poorly or not at all in the presence of most of the metals while the other four strains showed varying degrees of adaptability.

Silver and mercury were the most inhibitory of the metals studied. Although each strain is affected detrimentally, differences in the ability to tolerate higher concentrations of either metal are evident. Most of the strains never fully adapt to silver or mercury, indicating the difficulty they probably have in detoxifying these metals in their environment.

Molybdate is a stronger inhibitor than arsenite although arsenite shows a time-dependant inhibition of iron oxidation, similar to silver or mercury inhibition, a possible result of inhibition at cytochrome oxidase. Molybdate inhibits the iron oxidation more strongly than the growth of most strains in a complex manner.

Zinc and copper are tolerated at the highest concentrations for both growth and iron oxidation while ferric iron reflects more or less the K_m values for ferrous iron. Copper, zinc and ferric iron all seem to interfere directly with iron oxidation at the point where ferrous iron binds. The competitive nature of the inhibition patterns

by all three cations, especially at higher substrate concentrations, indicate this. The activation by metals of iron oxidation in some mine strains at higher ferrous substrate concentrations may be important in successful metal leaching.

Generally, the mine strains (SM-) exhibit an advantage over the laboratory (Tf-) strains in both adaptability to and iron oxidation in the presence of these six metals. The advantage, however, is not universal and there is an extreme variability among various strains. Even the two laboratory strains are not consistently similar although more so than the mine strains. However, the fact that some strains of mine isolates do show a consistently high tolerance to metals in these growth and iron oxidation experiments, supports such a strain selection methodolgy in the implementation of a bioleaching operation. Because there seems to be a wide variety of natural strains associated with different locations in a mine, applying the parameters characterized in this report to each strain should aid in allowing the selection of an optimal strain(s) of Thiobacillus ferrooxidans.

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