A physiological study of *Thiobacillus thiooxidans* on sulfur adhesion and oxidation and sulfite oxidation

By
Travis L. Takeuchi

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A PHYSIOLOGICAL STUDY OF *Thiobacillus thiooxidans* ON SULFUR ADHESION AND OXIDATION AND SULFITE OXIDATION

BY

TRAVIS L. TAKEUCHI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Thiobacillus thiooxidans cells oxidize elemental sulfur to sulfate. Two important steps in the oxidation are the initial attack of particulate sulfur for oxidation and the oxidation of sulfite.

Sulfite oxidation was studied in the first part of this thesis using an oxygen probe to determine the kinetic parameters, K_m, K_i, and V_{max}, for *T. thiooxidans* cells. The results showed Michaelis-Menten kinetics with substrate inhibition at high concentrations. The optimal pH for oxidation shifted to a higher pH when the concentration of potassium sulfite was raised suggesting that sulfurous acid is the species which enters the cell and is oxidized. These kinetic patterns were shown for all strains of *T. thiooxidans* cells tested. Therefore, the concentration of sulfurous acid, which is pH dependent, and not that of potassium sulfite, determines the rate of entry into the cell cytoplasm and subsequent oxidation. Substrate inhibition was also determined by the concentration of sulfurous acid, thus a fixed concentration of potassium sulfite was more inhibitory at lower pH because of increased sulfurous acid.

A second important step is the initial attack of sulfur and oxidation by *T. thiooxidans* cells. Cell adhesion to sulfur particles, Bacterial Adhesion to Hydrocarbon (BATH), and sulfur oxidation assays were used to study *T. thiooxidans* cells. Decreased numbers of cells adhered to sulfur when potassium phosphate concentrations were increased from 50 mM (wash buffer concentration) to 500 mM

(assay buffer concentration). The adhesion was not affected by metabolic inhibitors (2,4-dinitrophenol, iodoacetate, *p*-chloromercuribenzoate, or *N*-ethyl maleimide) suggesting adhesion is not energy related. The hydrophobicity of the cell surface was studied using *n*-hexadecane in the BATH test. Increased cell surface hydrophobicity was shown with increased potassium phosphate concentration. This was confirmed by direct microscopic observation. Sulfur oxidation was inhibited by high concentrations of potassium phosphate. Cells washed in 50 mM potassium phosphate were strongly inhibited when assayed in 500 mM potassium phosphate. Similar results were shown for sulfur dissolved in dimethylsulfoxide. Soluble substrates, potassium sulfite and sodium sulfide were affected by concentration of potassium phosphate used in washing procedures. In both cases the oxidation rates observed may be a combination of various effects. The results show that at increasing concentrations of potassium phosphate buffer, a decrease in cell adhesion and an increase in cell surface hydrophobicity occurs. This inhibits the ability of *T. thiooxidans* cells to oxidize sulfur.

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PART I

SULFITE OXIDATION

INTRODUCTION

Thiobacillus thiooxidans oxidizes reduced sulfur compounds to derive the energy and reducing power necessary for growth. The organism was isolated by Waksman and Joffe (1922) and further studied and described by Starkey (1925). Several papers have been published describing the physiology (Vishniac and Santer, 1957), metabolism (Suzuki, 1974) and industrial importance (Lizama and Suzuki, 1988) of *T. thiooxidans*.

Although much work has been done on this organism, the overall mechanism of sulfur oxidation remains to be proven for intact cells. The overall mechanism of sulfur oxidation formulated by Suzuki (1974) considers the formation of sulfite as an intermediate (Suzuki et al., 1992) before oxidation to sulfate.

This investigation involved the use of a Clarke oxygen electrode and a Gilson oxygraph to assay oxidation of sulfite. The apparatus allows for rapid and simple assay under varied conditions. In performing these variations, sulfite oxidation was measured by intact cells, with or without inhibitor at varied pH. Also, the kinetic parameters, K_i and V_{max} for sulfite oxidation by intact cells were determined.

The conclusions derived from the experiments strongly suggest that intact cells oxidize externally added sulfite solutions as sulfurous acid. Sulfurous acid at high concentrations also inhibits its own oxidation. The K_m for sulfite was affected by the washing conditions and assay conditions of the cells. The importance of these conditions must be considered in future studies on metabolism of *Thiobacillus thiooxidans*.

HISTORICAL

General physiology of the organism

Thiobacillus thiooxidans is a gram negative, rod shaped, motile, chemolithotroph. Originally isolated from a compost of soil, sulfur and rock phosphate (Waksman and Joffe, 1922), the organism is a strict aerobe, distinguished by the ability to withstand extreme acid conditions (growth optimum pH 1.0-3.5) and the ability to oxidize reduced inorganic sulfur compounds to generate energy. The organism grows optimally at 28°-30° C. Standard taxonomical methods such as DNA analysis (Jackson et al., 1968; Harrison, 1982) and fatty acid profile (Levin et al., 1971) have been reported for the organism.

Sulfur metabolism of the organism

Numerous reports have been made on the metabolism of the organism (for review Vishniac and Santer, 1957; Suzuki, 1974). The overall oxidation step as originally reported by Waksman and Starkey (1923) is unquestioned currently. Waksman and Starkey established that *T. thiooxidans* can completely oxidize elemental sulfur to sulfate according to the equation:

$$S^0 + 11/2\ O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$$

It was further suggested that no intermediate oxidation products are formed for either sulfur or thiosulfate in the complete oxidation to sulfate. Since this work, much debate has occurred as to which intermediates may be formed during oxidation.

Sulfide oxidation:

Hydrogen sulfide was shown to be oxidized to sulfate with small amounts of elemental sulfur accumulating (Parker and Prisk, 1953). Other authors such as Kelly have suggested that other reduced sulfur intermediates form during the oxidation steps (Steudel et al., 1989). Similarly, bacterial extracts (Suzuki and Werkman, 1959; Charles and Suzuki, 1966) showed sulfur precipitation when sulfide is oxidized. This would suggest that in fact sulfide is converted to sulfur before further oxidation occurs. Moriarity and Nicholas (1969, 1970) confirmed this using cell free extracts of *T. thiooxidans* to show enzymic sulfide oxidation to form membrane polysulfide chains.

Sulfur oxidation

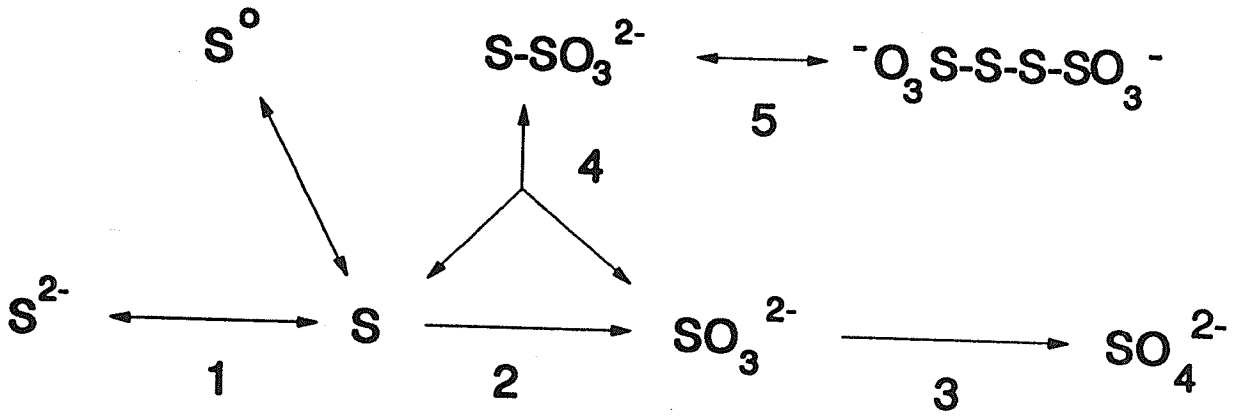
Historically, studies were carried out on intact *T. thiooxidans* cells to determine the nature of the sulfur oxidation mechanism.

Thiosulfate intermediates. Various authors (e.g., Kelly, 1982), proposed that thiosulfate was the initial intermediate in the oxidation of sulfur. However, cell-free systems were necessary to prove the steps involved in the mechanism of oxidation. Suzuki and Werkman (1959) reported that cell-free extracts could oxidize elemental sulfur only in the presence of reduced glutathione to produce thiosulfate. This would agree with previous results that thiosulfate could be oxidized to form tetrathionate (Vishniac, 1952) and sulfate in growth media (Parker and Prisk, 1953).

Tetrathionate intermediate. Later, Vishniac (1953) reported that tetrathionate could be oxidized to sulfate agreeing with Parker and Prisk that tetrathionate was an intermediate in thiosulfate oxidation. Iwatsuka and Mori (1960) attempted to repeat these results for a *T. thiooxidans* isolate and found that thiosulfate could not be oxidized and tetrathionate could be oxidized slightly. As a result, these points remain in question to some current researchers (e.g., Kelly, 1982) who continue to try to unify the sulfur oxidation pathway for all *Thiobacillus* species.

Sulfite intermediate. Suzuki refined earlier work (Suzuki, 1965a; 1965b; Suzuki and Silver, 1966) to report that sulfite is the initial oxidation product of sulfur in the cell-free sulfur oxidation by *T. thiooxidans*. This agrees with reports by Peck (1960, 1962) which suggested that sulfite oxidation is the terminal step in oxidation by other thiobacilli. It was proposed that elemental sulfur (S° or S_8) reacted with catalytic amounts of reduced glutathione (GSH) to generate (GSS_8H) sulfane sulfur which was oxidized to sulfite one atom at a time. Thiosulfate would be expected to accumulate by condensation of sulfite with sulfur under the experimental conditions because of the neutral pH. Although enzymes have been isolated and characterized for each step of the sulfur oxidation pathway, few studies have been able to prove the overall stoichiometry of the complete oxidation to sulfate. In fact, few studies have been able to indicate that sulfite is an intermediate using intact cells (Pronk et al., 1990). This has been a significant objective for many researchers in attempting to prove the overall scheme, because thiosulfate breaks down under acidic conditions to sulfite and sulfur (Iwatsuka and Mori, 1960). Roy and Trudinger (1970) concur

Figure 1. Sulfur oxidation pathway (Suzuki, 1974; Suzuki et al., 1993) unifying the various proposed and possible intermediates of the thiobacilli. Known enzymes include (1) sulfide oxidase, (2) sulfur oxidizing enzyme, and (3) sulfite: cytochrome c oxidoreductase, (4) thiosulfate cleavage enzyme, and (5) thiosulfate oxidizing enzyme.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that "in weakly acidic media thiosulfate is converted almost entirely to sulfur and bisulfite."

The stoichiometry for sulfur oxidation and the steps involved were unified by Suzuki (1974)(Fig. 1). Suzuki suggested that sulfide, elemental sulfur and thiosulfate form reactive sulfur which is then oxidized sequentially first to sulfite and then sulfate. Tetrathionate or polythionate can be similarly considered as reactive sulfur plus sulfite. The scheme has satisfied experimental results for different species of thiobacilli published by most groups of researchers. As well, recent suggestions (Pronk et al., 1990) that thiosulfate or polythionates form as intermediates or products can be explained because these compounds result from known chemical reactions between sulfur and intermediates such as sulfite (Suzuki, 1965b).

Enzymes involved

The initial oxidation step by *T. thiooxidans* involves a step mediated by sulfur-oxidizing enzyme reported and partially purified by Suzuki (1965b). A second step, sulfite oxidation, as suggested by Suzuki (1965b) and reported by Adair (1966) and measured by Kodama and Mori (1968b), has been shown to be carried out by sulfite oxidase enzymes in cell free systems (Kodama et al., 1970).

Sulfite oxidation

The oxidation of sulfite appears to be the regulatory step of the sulfur oxidation pathway (Suzuki et al., 1993).

Although sulfite oxidation by thiobacilli is easily shown at neutral pH such as in *Thiobacillus novellus* cells (Charles and Suzuki, 1966), it is difficult to show in acidophilic thiobacilli such as *T. thiooxidans* and *Thiobacillus acidophilus*, without raising the pH above the acid growth pH. In fact, growth on sulfite has not been shown to date and intact *T. thiooxidans* cells show only low oxygen consumption rates at acid pH with a pH optimum of 6.5 for 50 mM sulfite (Kodama and Mori, 1968b) suggesting that the enzyme is located cytoplasmically. An exception to this is the sulfite:ferric ion oxidoreductase enzyme of *Thiobacillus ferrooxidans* which couples sulfite oxidation to iron reduction (Sugio, 1988, 1992) and has a pH optimum near the optimum growth pH.

Enzymes involved

At the enzyme level, two different pathways, APS reductase (adenosine phosphosulfate reductase) and sulfite oxidase (coupled to cytochrome *c* reduction or ferric ion reduction in *T. ferrooxidans*) have been proposed for the oxidation of sulfite. The APS reductase pathway was first proposed by Peck (1959) in *Desulfovibrio desulfuricans*, a sulfate reducing organism. The mechanism involves the reduction of sulfate to sulfite via a high energy intermediate. Studies with *Thiobacillus thioparus*, Peck (1960) proposed the intermediary formation of APS (adenosine phosphosulfate) from sulfite and AMP by APS reductase. During substrate level phosphorylation, APS is converted to ADP and sulfate by ADP sulfurylase. ATP and AMP are produced by means of adenylate kinase as follows:

$2\ SO_3^{2-} + 2\ AMP \rightarrow 2\ APS + 4e^-$ (APS reductase)

$2\ APS + 2\ Pi \rightarrow 2\ ADP + 2\ SO_4^{2-}$ (ADP sulfurylase)

$2\ ADP \rightarrow AMP + ATP$ (adenylate kinase)

APS reductases have been purified from *Thiobacillus denitrificans* (Bowen et al., 1966; Aminuddin and Nicholas, 1974), *T. thioparus* (Lyric and Suzuki, 1970b; Adachi and Suzuki, 1977), sulfate reducing bacteria (Stille and Trüper, 1984; Speich and Trüper, 1988) and some photosynthetic bacteria (Trüper and Rogers,1971; Kirchoff and Trüper, 1974). A report by Peck (1961) who reported that *T. thiooxidans* has an APS reductase system conflicts with Adair (1966) who had indicated otherwise.

The finding of sulfite:cytochrome *c* oxidoreductase in *T. novellus* by Charles and Suzuki (1965) led to the proposal that an AMP independent system, which neither needs AMP nor produces APS, functions as an additional mechanism of sulfite oxidation. The purified enzyme oxidizes sulfite to sulfate with a reduction of cytochrome *c*:

$$SO_3^{2-} + 2\ \text{cyt } c\ Fe^{3+} + H_2O \rightarrow SO_4^{2-} + 2\ \text{cyt } c\ Fe^{2+} + 2\ H^+$$

The reduced cytochrome is then oxidized with molecular oxygen by cytochrome oxidase (cytochrome *c*:O_2 oxidoreductase)

$$2 \text{ cyt } c \ Fe^{2+} + 1/2O_2 + 2H^+ \rightarrow 2 \text{ cyt } c \ Fe^{3+} + H_2O$$

Sulfite oxidase (sulfite:cytochrome *c* oxidoreductase) has been isolated from *T. novellus* (Charles and Suzuki, 1965; Yamanaka et al., 1981; Southerland and Toghrol, 1983), *Thiobacillus versutus* (Lu and Kelly, 1984), *T. thioparus* (Lyric and Suzuki, 1970a) and *T. ferrooxidans* (Vestal and Lundgren, 1971). Sulfite oxidase, cytochrome *c*-550 and cytochrome oxidase have been purified from *T. novellus* and then reconstituted to show electron flow from sulfite to molecular oxygen via cytochromes (Yamanaka et al., 1981). An unusual sulfite oxidizing enzyme from *Thiobacillus neopolitanus* has been isolated and characterized (Sklodowska, 1990). The enzyme was stimulated by AMP and reacted directly with either ferricyanide or oxygen, but did not reduce native horse heart cytochrome *c*. The enzyme was probably an intermediate to APS reductase and sulfite oxidase as it was stimulated by AMP but did not form APS.

Recently, the sulfite oxidation mechanism in *T. thiooxidans* has been reported to occur by the AMP-independent pathway (Nakamura et al., 1992) confirming earlier work by Kodama et al. (1970), Takakuwa (1976) and Tano et al. (1982). A recent addition to Suzuki's scheme (1974) is the addition of enzymes which couple the reduction of ferric ion to the oxidation of sulfur and/or sulfite (Sugio et al., 1987; 1992). Sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase have only been shown for *T. ferrooxidans* which is very similar in metabolism but is complicated due to the role of iron in metabolism and in the physiology of the organism

(Ingledew, 1982).

Sulfite oxidation by intact cells of *T. thiooxidans* remains at the preliminary stage of investigation. However, sulfite oxidase appears to be membrane associated and localized in the cytoplasmic fraction (Takakuwa, 1992) with a pH optimum of around 6.5. This complicates the suggestion by Hooper and DiSpirito (1985) that the enzymes involved in the oxidation of reduced sulfur should operate extracytoplasmically with optimal pH values around 2.0.

The enzyme has been detected in the particulate membrane fractions of crude cell-free extracts (Takakuwa, 1976; Kodama et al., 1970) which also contain cytochrome *a*, *b*, *c* and *d*-types. The oxidation of sulfite is postulated as being coupled to either cytochrome *b* (Tano et al., 1980) or cytochrome *c* (Nakamura et al., 1992; Kodama et al., 1970) as shown with inhibitors and spectrophotometric evidence.

Given that sulfite can be oxidized in the cell free system of *T. thiooxidans*, oxidation of sulfite by intact cells was studied.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest grade commercially available. Soluble salts were obtained from Fisher Scientific (Fairlawn, New Jersey, U.S.A.) or Mallinkrodt Canada Inc. (Pointe Claire, Quebec, Canada). Precipitated sulfur powder was obtained from British Drugs Houses (BDH) Limited (Toronto, Canada). Potassium sulfite (K_2SO_3) was obtained from Matheson, Coleman and Bell (Norwood, Ohio and East Rutherford, New Jersey, U.S.A.).

Growth conditions. *Thiobacillus thiooxidans* (ATCC 8085), SM-6, SM-7 (Lizama and Suzuki, 1988), Iron Mountain isolate, *Thiobacillus ferrooxidans* [Tf-2](ATCC 19859) and SM-4 (Lizama and Suzuki, 1988) were grown stationary for 4 days at 28°C in Starkey's medium No. 1 [0.3 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.018 g $FeSO_4 \cdot 7H_2O$, 3.5 g KH_2PO_4 and 0.25 g $CaCl_2$ per liter distilled water] adjusted to pH 2.3 with H_2SO_4 with elemental sulfur (1 g/l) spread on the surface as previously described (Suzuki, 1965; Suzuki et al., 1990). Iron Mountain isolate [301-S Iron Mtn] was isolated from a water sample donated by Dr. Charles Alpers, U.S. Geological Survey, California. Varied growth conditions included the addition of 300 μg/l $NaMoO_4$ to Starkey's No. 1 or High Phosphate [HP] medium [0.4 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4 and 0.4 g $MgSO_4 \cdot 7H_2O$ per liter distilled water] acidified to pH 2.3 with H_2SO_4 or varied length of incubation of 4 or 5 days as stated.

Cell harvesting. Cells were filtered through Whatman No. 1 paper under suction to

remove sulfur and collected by centrifugation at 10,000 x *g* for 10 minutes. Cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 2.3) unless otherwise indicated at a final concentration of 50 mg wet cells per milliter for storage at 4°C. The washing processes took around one hour at 4°C. The cells were used normally on the same day for experiments.

Cell-free extracts. Cell-free extracts were prepared by breaking the cells with passage through an Aminco French Pressure cell (three times) at a pressure of 20,000 psi (138 MPa) of a cell suspension (200 mg wet cells per ml) in 50 mM, pH 7.5 phosphate buffer. The cells were previously washed in the same buffer and were treated with trypsin for 20 minutes with gentle stirring then with trypsin inhibitor for 5 minutes (1.5 μg/mg wet cells for either bovine pancreatic trypsin or soybean trypsin inhibitor, Sigma). The trypsin treated cells (Bhella, 1981) were ruptured more easily but untreated cells also produced extracts with similar sulfite oxidation properties. The cell-free extracts were obtained as supernatants by centrifugation of the broken cell suspension at 10,000 x *g* for 10 minutes.

Sulfite oxidation. Sulfite oxidation was followed in a Gilson oxygraph with a Clarke oxygen electrode at 25°C and the initial linear rate of O_2 consumption (nmoles per minute) was used as the oxidation rate. The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH as specified), 1 mg of wet cells (20 microliters of a 50 mg per milliter suspension) or 50 microliters of cell-free extracts (23 mg protein per milliliter, determined with bovine serum albumin as standard following the method of Charles and Suzuki (1966)) and varied amounts of potassium sulfite in a

total volume of 1.2 ml. The reactions were started by the addition of microliter volumes of 10 mM, 0.1 M and 1.0 M K_2SO_3 solutions prepared in 50 mM disodium EDTA. Non-biological oxidation rates were performed as for cell assay procedures except cells were omitted.

RESULTS

Optimal pH for sulfite oxidation. *T. thiooxidans* cells oxidize sulfite at various pH values, but the optimum was a function of potassium sulfite concentration used as substrate as shown in Figure 2. The optimal pH was low at low sulfite concentrations, but increased with increasing sulfite concentrations (nearly two pH units when the sulfite concentration was raised forty-fold). At a fixed pH it was possible to observe either an increasing rate of oxidation (pH 6.5) or a decreasing rate of oxidation (pH 4.0) with increasing substrate concentrations (Fig. 2). Non-biological oxidation of sulfite was insignificant under the conditions used in this study (Table 1). Therefore in the following experiments the effect of increasing substrate concentrations on the increase in activity (normal situation) and decrease in activity (inhibition) of sulfite oxidation by *T. thiooxidans* cells was studied.

Effect of sulfite concentrations on the increase in activity. Sulfite was oxidized by *T. thiooxidans* cells with a stoichiometry of $SO_3^{2-} + 1/2O_2 \rightarrow SO_4^{2-}$ in O_2 consumption, but the rate of oxidation was a function of both substrate concentration and pH as shown in Figure 3. At a fixed pH, the rate (v) increased with increasing substrate concentration [S] following a normal Michaelis-Menten kinetics giving a linear Lineweaver-Burke plot (Lineweaver and Burke, 1935), except at high inhibitory concentrations of substrate (data not shown in Fig. 3). The K_m values for potassium sulfite added as substrate increased sharply when the pH of the reaction mixture was increased as shown in Figure 3 (as much as sixty-fold when the pH was raised from

Figure 2. Effect of potassium sulfite concentration on the optimal pH for sulfite oxidation by *Thiobacillus thiooxidans* cells (1 mg wet cells washed in 50 mM potassium phosphate buffer, pH 4.7). The rate of oxygen consumption was determined as described in Materials and Methods.

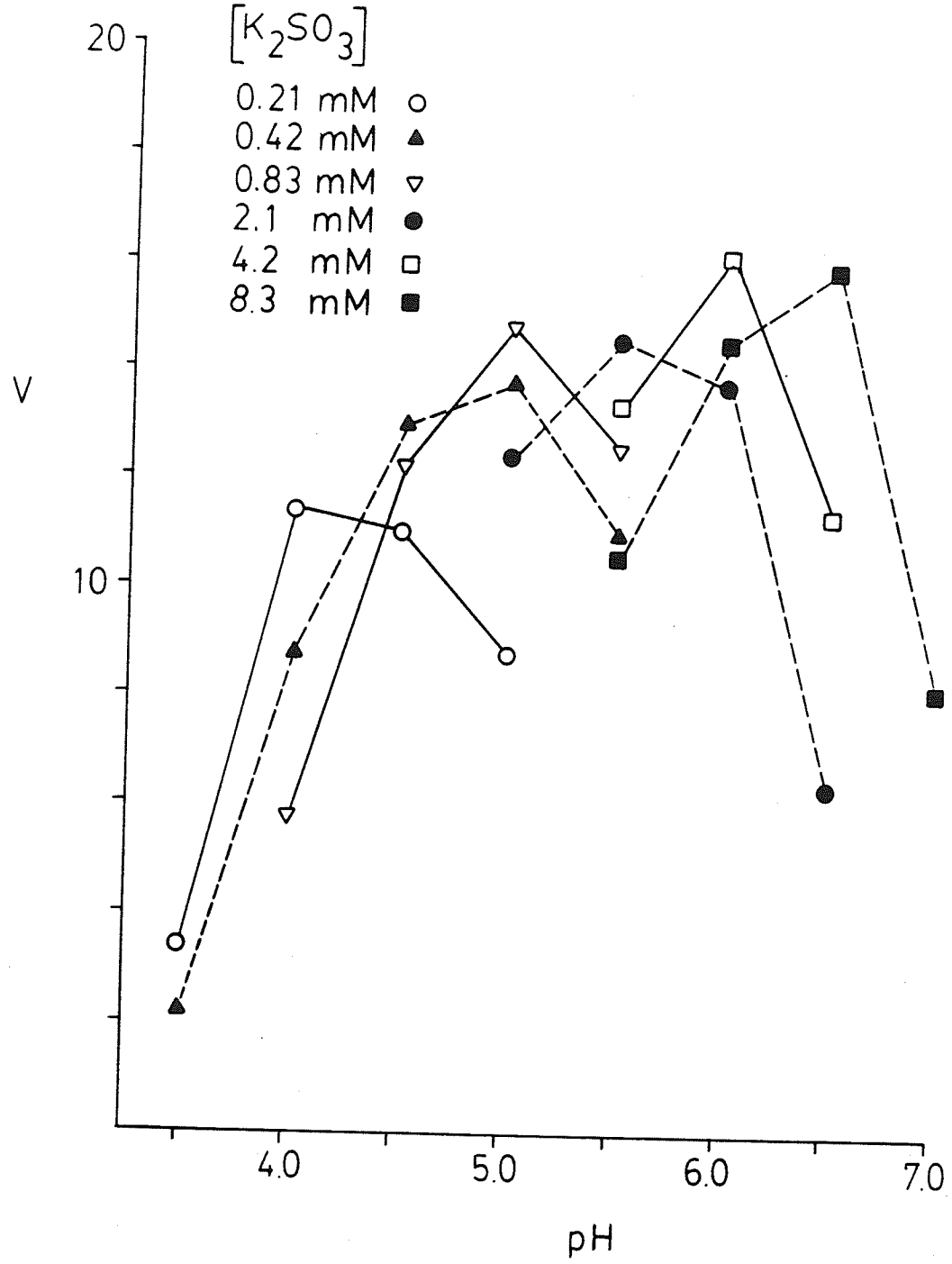

[K2SO3]
0.21 mM
0.42 mM
0.83 mM
2.1 mM
4.2 mM
8.3 mM
20
10
V
4.0
5.0
6.0
7.0
pH

Table 1. Chemical oxidation rates for sulfite in potassium phosphate buffer at varied pH

pH	K_2SO_3 (μmoles)	Chemical Rate[a] (nmoles O_2/min)	pH	K_2SO_3 (nmoles)	Chemical Rate[a] (nmoles O_2/min)
2.3	2.5	0.2	5.5	2.5	0.3
	5.0	0.3		5.0	0.5
	10.0	1.0		10.0	1.0
	50.0	1.2		50.0	4.3
3.5	5.0	0.3	6.0	5.0	0.6
	10.0	0.7		10.0	1.0
	50.0	2.1		50.0	3.9
4.0	5.0	0.5	6.5	2.5	0.4
	10.0	1.0		5.0	0.7
	25.0	4.0	7.0	1.0	0.1
4.5	2.5	0.3		2.5	0.3
	5.0	0.4		5.0	0.5
	10.0	1.0		10.0	1.0
	25.0	2.3		50.0	3.7
5.0	2.5	0.2			
	5.0	0.6			
	10.0	1.5			
	50.0	5.5			

[a]rates measured using assay procedures described in Materials and Methods.

Figure 3. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations. *Thiobacillus thiooxidans* cells (1 mg wet cells) were washed and assayed at 50 mM potassium phosphate buffer as described in Materials and Methods. K_m values are listed in Table 2.

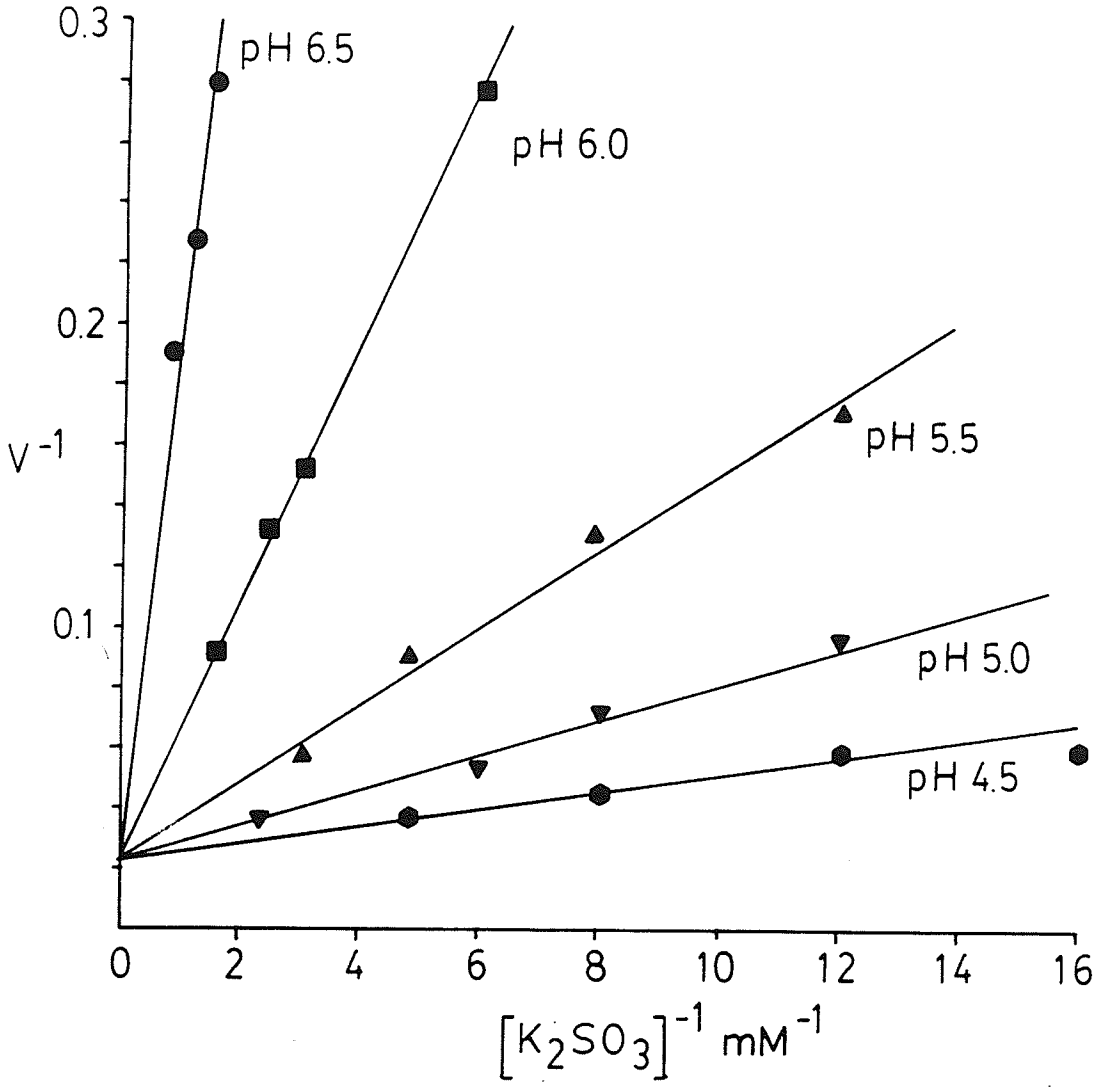
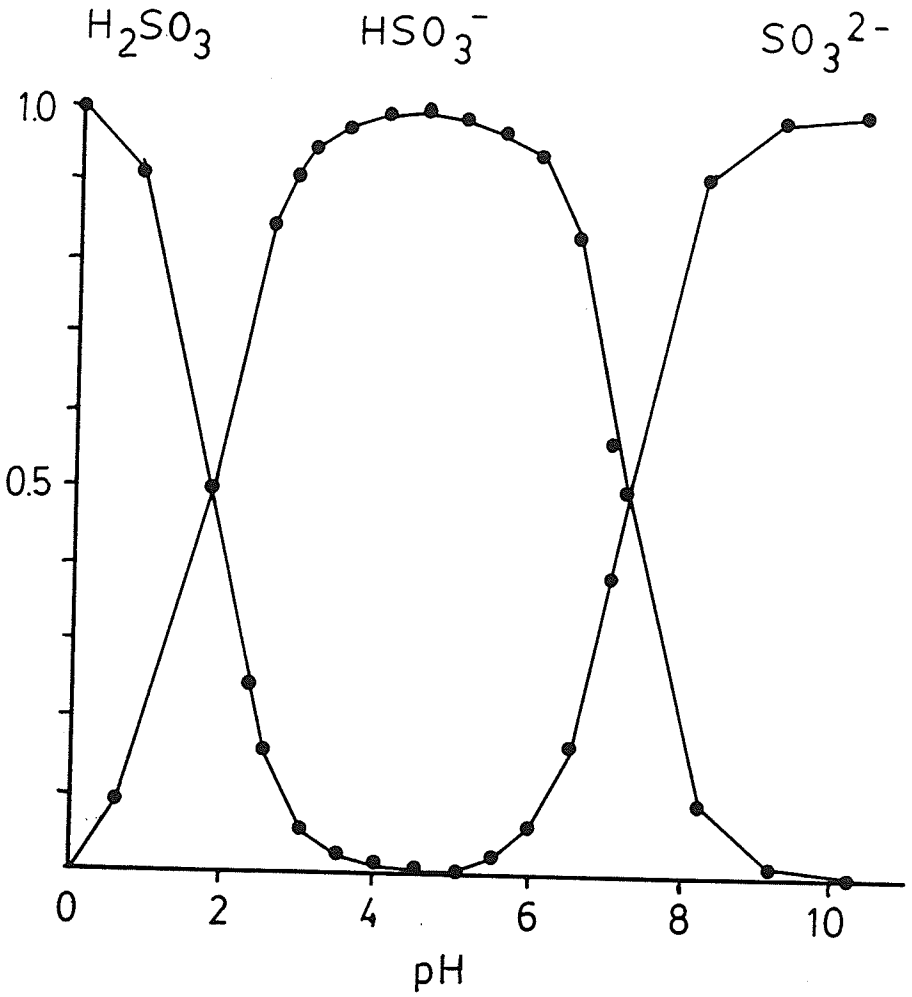

0.3
0.2
0.1
V -1
pH 6.5
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 4. Effect of pH on sulfurous acid, bisulfite and sulfite ion concentrations. pK_a values of 1.81 and 7.0 were used to calculate the fractions of ionic species.


H_2SO_3
HSO_3^-
SO_3^{2-}
1.0
0.5
0
2
4
6
8
10
pH

4.5 to 6.5). When the total sulfite concentration (potassium sulfite added) was converted to the concentration of sulfurous acid (H_2SO_3) based on the pKa_1 value of 1.81 and the pKa_2 value of 7.0 at various pH of the reaction mixture (Fig. 4), however, the K_m values converged to a narrow range of very low values (0.11 to 0.24 μM) as shown in Table 2. Remarkably all the lines in Figure 3 intersected the Y-axis with the same V_{max} value indicating that the maximal rate of sulfite oxidation by the cells is not influenced by the assay pH.

Inhibition by high sulfite concentrations. Sulfite inhibited its own oxidation by *T. thiooxidans* under acidic conditions and it was not possible to lower the pH below 4.5 for the K_m determination, because increasing potassium sulfite concentration resulted in decreased rate of oxidation (Fig. 5). Inhibition of enzyme reaction by high substrate concentrations can be treated (Dixon and Webb, 1965) as the inhibition by the binding of a second substrate molecule with a dissociation constant K_i (inhibition constant). The plot of 1/v versus [S] should give a straight line with an intercept on the X-axis corresponding to the concentration of substrate equal to K_i (Dixon and Webb, 1965). The experimental results (Fig. 6) showed a family of straight lines converging on the Y-axis with increasing slopes at decreasing assay pH (pH 4.5 → 2.3), indicating a stronger inhibition by potassium sulfite at lower pH. The K_i values obtained (Table 3) for total sulfite concentration decreased 190-fold from 380 μM at pH 4.5 to 2 μM at pH 2.3. Thus at pH 2.3 sulfite oxidation is inhibited by 50% at a low total sulfite concentration of 2 μM or by 90% at 20 μM. If the substrate concentration was converted to the concentration of sulfurous acid (H_2SO_3), however,

Table 2. K_m values for potassium sulfite and sulfurous acid for *Thiobacillus thiooxidans* cells washed in 50 mM potassium phosphate buffer, pH 2.3, obtained from Figures 3 and 4

pH	K_m for potassium sulfite (mM)	K_m for sulfurous acid (μM)
4.5	0.12	0.24
5.0	0.22	0.14
5.5	0.56	0.11
6.0	2.0	0.12
6.5	7.1	0.11

Figure 5. Effect of high potassium sulfite concentration on the double reciprocal plots for sulfite oxidation rates and sulfite concentrations. The rate of oxygen consumption by *Thiobacillus thiooxidans* cells (1 mg) was determined as described in Materials and Methods. The values for K_m and V_{max} are listed in Table 4.

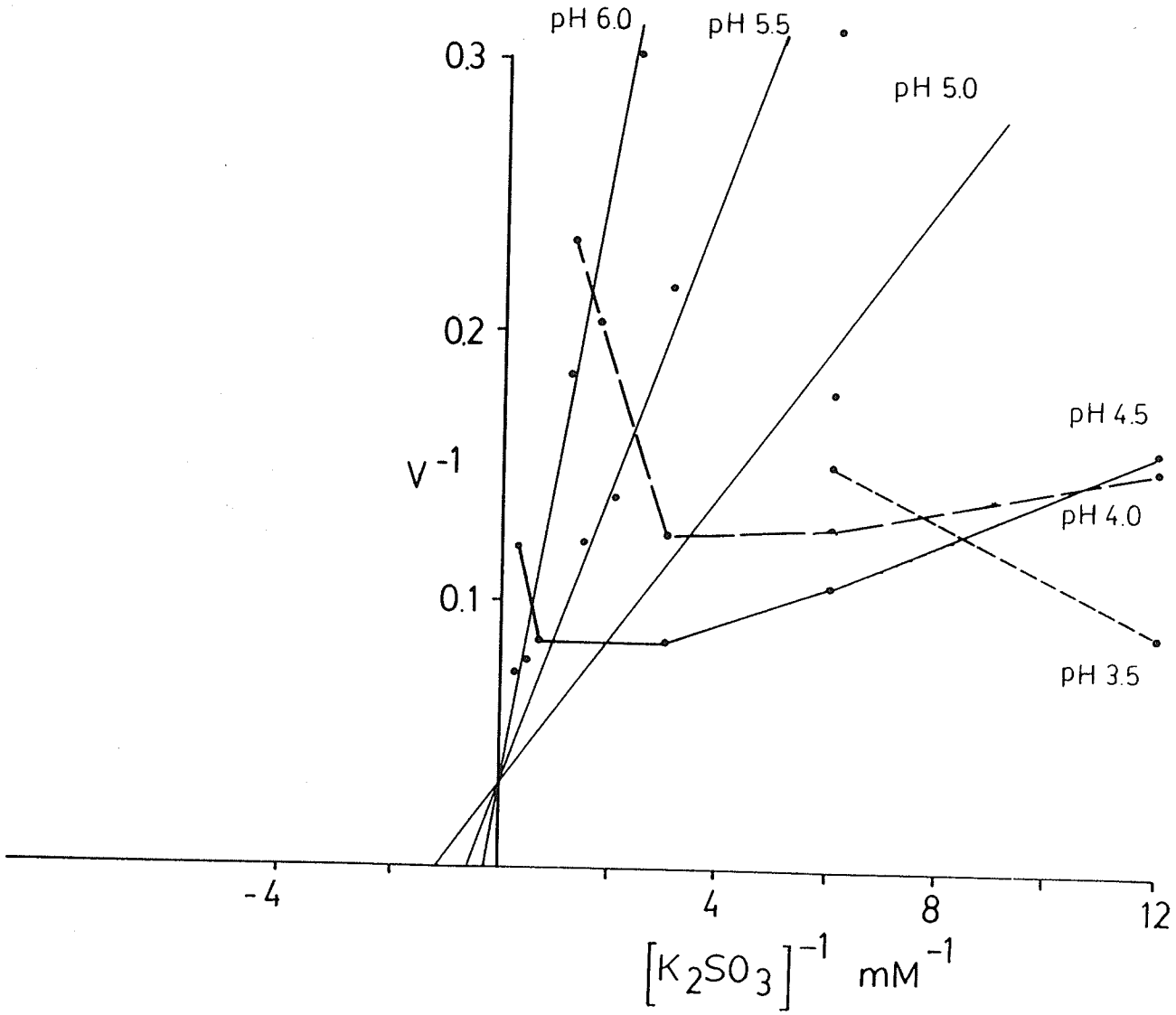

pH 6.0
pH 5.5
pH 5.0
pH 4.5
pH 4.0
pH 3.5
0.3
0.2
0.1
V -1
-4
4
8
12
[K2SO3]-1 mM-1

Figure 6. Inhibition by high concentrations of sulfite at various assay pH

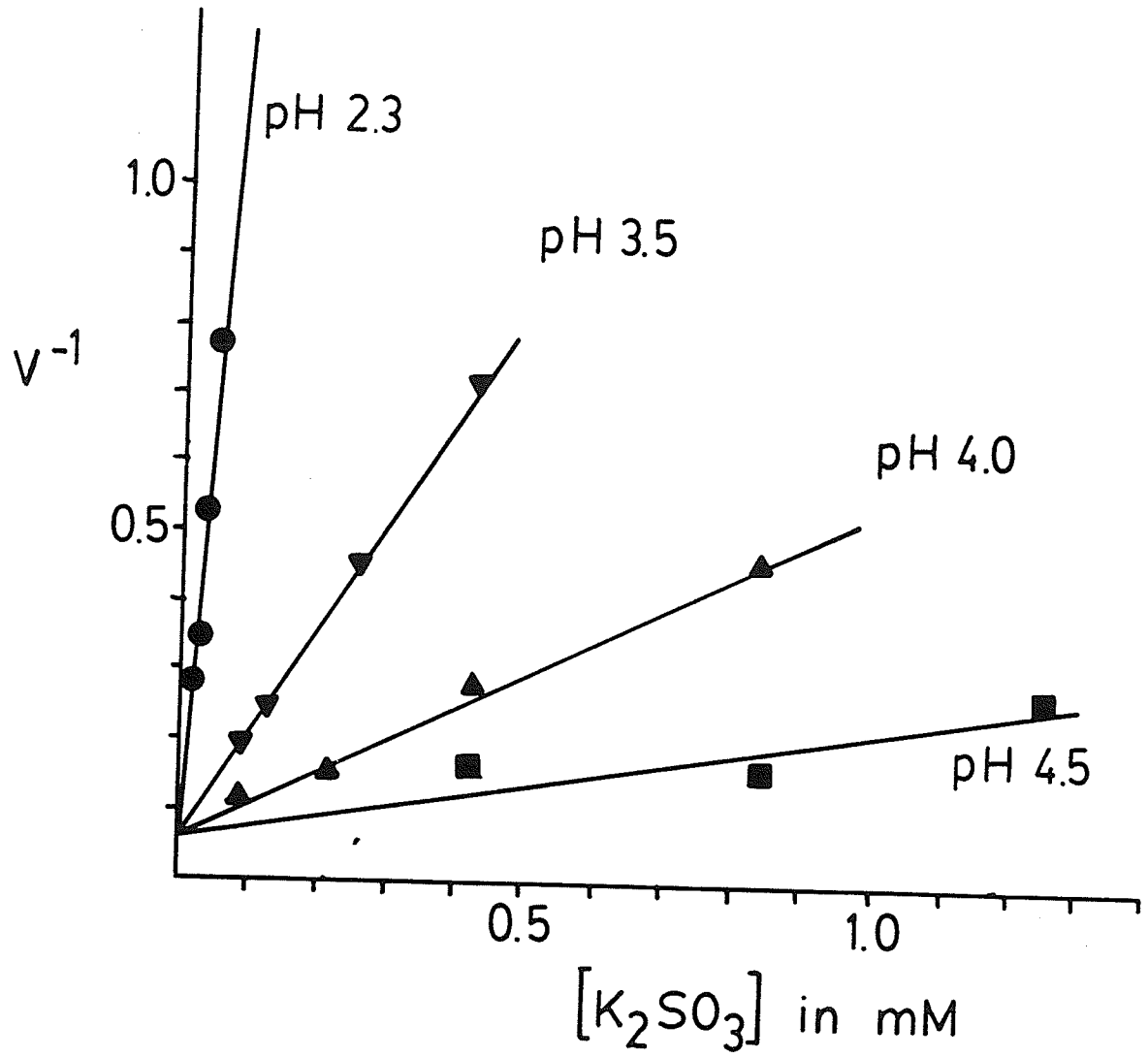

pH 2.3
pH 3.5
pH 4.0
pH 4.5
1.0
0.5
V -1
0.5
1.0
[K2SO3] in mM

Table 3. K_i values for potassium sulfite and sulfurous acid for *Thiobacillus thiooxidans* cells washed in 50 mM potassium phosphate buffer, pH 2.3. Constants are derived from Figure 3. V_{max} was 42 nanomoles O_2 consumed per minute

pH	K_i for potassium sulfite (mM)	K_i for sulfurous acid (μM)
2.3	0.002	0.5
3.5	0.035	0.7
4.0	0.13	0.8
4.5	0.38	0.8

Figure 7. Effect of potassium sulfite concentration on the optimal pH for sulfite oxidation by cell-free extracts of *Thiobacillus thiooxidans*. The rate of oxygen consumption by extracts (1.2 mg of protein per assay) was determined as described in Materials and Methods.

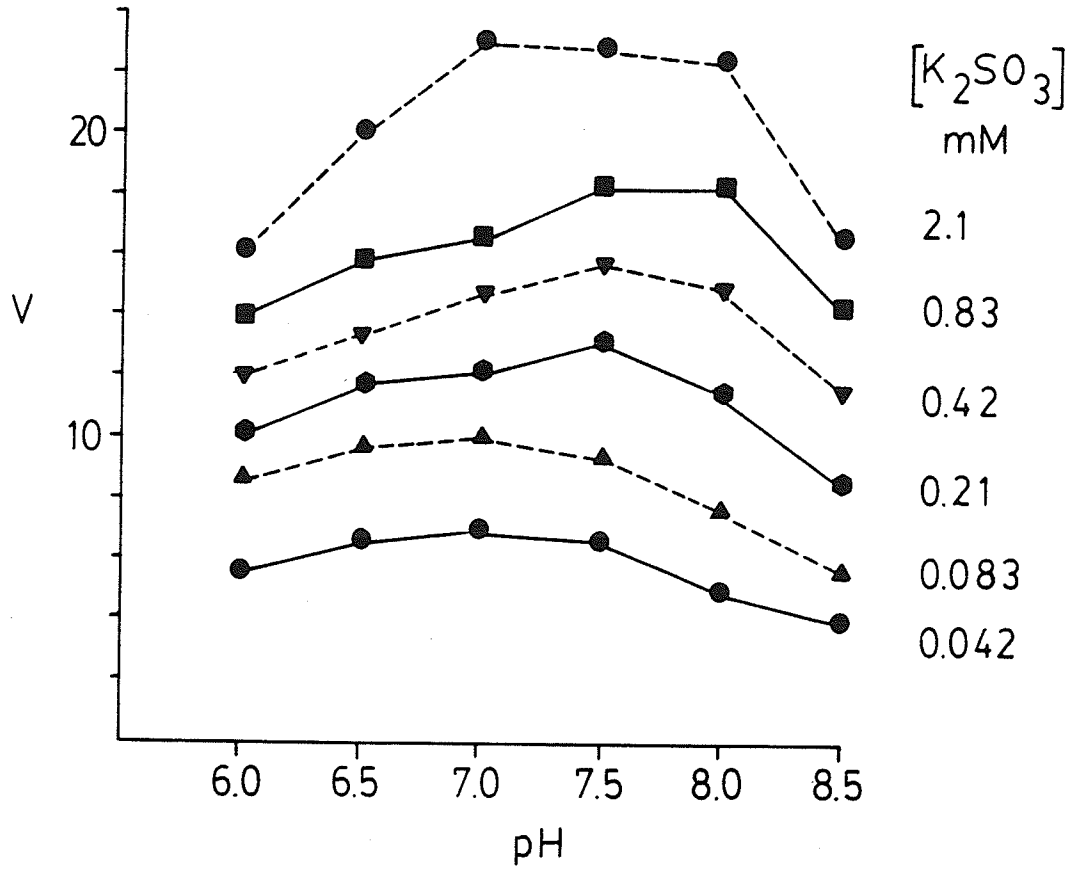
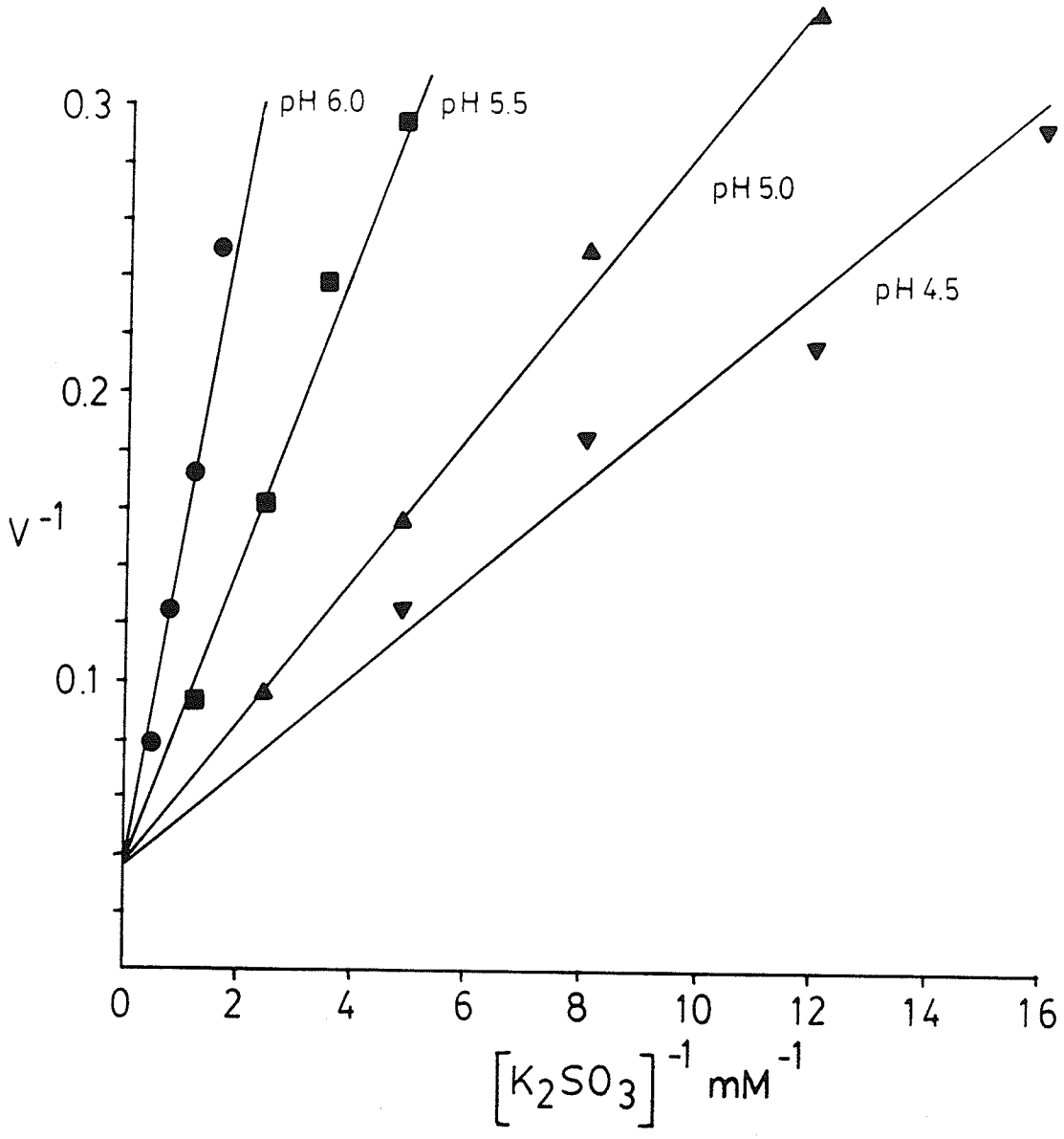

20
10
V
6.0
6.5
7.0
7.5
8.0
8.5
pH
[K2SO3]
mM
2.1
0.83
0.42
0.21
0.083
0.042

Figure 8. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (pH 7.5 washed cells). *Thiobacillus thiooxidans* cells (1 mg wet cells) were washed and resuspended in 50 mM potassium phosphate buffer, pH 7.5 as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.


0.3
0.2
0.1
V -1
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3] -1 mM -1

the K_i values converged again to a very narrow range of 0.5 to 0.8 μM (Table 3), as in the case of K_m values.

These results of pH dependency of sulfite oxidation by *T. thiooxidans* cells can be satisfactorily explained if fully protonated form of sulfite, sulfurous acid is the active species either as the substrate for oxidation or as the inhibitor of the oxidation. In fact if the concentration of sulfurous acid [H_2SO_3] is used instead of [K_2SO_3] in these plots the pH effect largely disappears.

Sulfite oxidation by cell-free extracts. The pH dependency study of sulfite oxidation by the cell-free extracts of *T. thiooxidans*, in contrast to those of intact cells (Fig. 2), showed little effect of substrate concentrations on the optimal pH (Fig. 7), where the rate of oxidation remained highest around pH 7-7.5 when the concentration of K_2SO_3 was varied fifty-fold. The double reciprocal plots (not shown) of cell-free extract activity versus K_2SO_3 concentrations did not show the characteristic pattern of Figure 2 (intact cells) with changing pH. In fact the data points were close together between pH 6.5 and 7.5 with similar K_m and V_{max} values. At pH 6.0 and 8.5 the rate was lower, but the K_m for the total sulfite concentration did not change more than two times within the pH range, i.e. 0.25 to 0.5 mM at high substrate concentrations and 50 to 100 μM at low concentrations. These results exclude the possibility of any one of the three ionic species of sulfite being the only substrate for oxidation by the cell-free system. The cell-free extracts became progressively more turbid when the pH was lowered below 6.0 and the activity also decreased.

Effect of washing cells at different pH's on sulfite oxidation. In previous

experiments of Figure 3 *T. thiooxidans* cells were washed and suspended in pH 2.3, 50 mM potassium phosphate. When the cells were washed at a higher pH (pH 7.5) their response to sulfite oxidation at various pH values changed considerably with increasing V_{max} and K_m values (Table 4, Fig. 8). The K_m values for K_2SO_3 still increased with increasing assay pH. The K_m values calculated for sulfurous acid, remained relatively constant.

Sulfite oxidation by different cells. Sulfite oxidation was assayed for *T. thiooxidans* cells grown in different growth media and washed in various buffers (Table 4). The double reciprocal plots followed similar kinetics patterns of a pH dependency (Experiments 5, 6, 8, 11, 12, 13 in Table 4; Figures 9 to 14).

In all experiments increasing assay pH had an effect of increasing K_m values for sulfite without affecting the V_{max} values. Both K_m and V_{max} values increased with increasing pH (2.3→4.7→7.5) of washing medium (Experiments 1,2,3 in Table 4). The high K_m and V_{max} values were observed when cells were washed at pH 7.5 either in potassium phosphate (Experiment 3 in Table 4) or Tris-HCl (Experiment 7 in Table 4). Assay in 0.5 M potassium phosphate buffer (Experiment 4 in Table 4) showed somewhat higher K_m and V_{max} values, similar to ß-alanine-H_2SO_4 washed cells and Starkey No.1 washed cells assayed in 0.1 M buffer (Experiments 5 and 6 in Table 4). KCl washed cells (Experiment 9 in Table 4) had a higher K_m and V_{max} than the cells washed under standard conditions (Experiment 1 in Table 4). Molybdate which is known to be involved in sulfite oxidation in some thiobacilli (Toghrol and Southerland, 1983; Kessler and Rajagopolan, 1972) increased the activity of cells

grown in its presence (Experiment 10 in Table 4) nearly three times. Cells grown in HP medium, whether washed in HP, P_i or H_2SO_4, had similar K_m and V_{max} values to those grown in Starkey No. 1 medium (Experiments 11-13 in Table 4).

T. ferrooxidans cells grown on sulfur showed a similar pH depency of K_m values (Experiment 1 in Table 5 and Fig. 15) to *T. thiooxidans*. Washing at pH 7.5 (Experiment 2 in Table 5 and Fig. 16) had the same effect of increasing K_m and V_{max} values.

Mine isolates, SM-6, SM-7 and 301-S Iron Mtn all showed similar pH response of increasing K_m values with increasing pH (Experiment 3,4 and 5 respectively in Table 5; Figs. 17,18 and 19 respectively). Thus all *T. thiooxidans* ATCC 8085, SM-6, SM-7 and 301-S Iron Mtn strains as well as *T. ferrooxidans* responded in an identical manner to the assay pH in sulfite oxidation.

Table 4. Summary of K_m and V_{max} values for sulfite oxidation by *Thiobacillus thiooxidans* (ATCC 8085)

Expt.	Growth medium[a]	Washing[b]		K_m (mM) at assay pH[c]				V_{max}
		medium	pH	6.5	6.0	5.5	5.0	4.5	
1	Starkey	P_i	2.3	7.1	1.8	0.6	0.8	0.12	41.7
2			4.7	10.0	4.0	1.7		0.25	57.0
3			7.5		10.0	2.9	0.7	1.0	83.0
4			2.3	10.0	3.3	1.3	0.6	0.3	66.7
5		ß-alanine	2.3	10.0	3.3	1.2	0.7	0.3	62.5
6		Starkey	2.3	12.5	4.6	2.0	1.0	0.5	66.7
7		Tris	7.5		10.0	3.3	1.5	0.1	71.4
8		KCl	3.0		3.3	0.7	0.4	0.3	31.3
9		K_2SO_4	3.0	20.0	6.7	1.8	0.9	0.4	83.3
10	Starkey + MoO_4^{2-}	P_i	2.3	10.0	6.7	2.5	1.4	0.5	125.0
11	HP	HP	2.3	6.7	2.0	0.6	0.3		40.0
12		P_i	2.3	10.0	2.5	0.7	0.3		40.0
13		H_2SO_4	3.0	6.0	4.0	1.4	0.6	0.2	30.3

[a] Growth medium was Starkey's medium No. 1 with or without 0.3 ppm Na_2MoO_4, or HP medium, each adjusted to pH 2.3.
[b] Washing medium was 50 mM potassium phosphate, 0.1 M ß-alanine-H_2SO_4, 50 mM Tris (HCl), 50 mM KCl (HCl) or 50 mM K_2SO_4 (H_2SO_4) of pH indicated; or Starkey's medium No. 1 or HP medium of pH 2.3; or H_2SO_4 at pH 3.0.
[c] Assay was carried out in 0.1 M potassium phosphate buffer of indicated pH, except in Experiment 4 where 0.5 M potassium phosphate buffer was used.

Figure 9. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (pH 3.0 ß-alanine washed cells). *Thiobacillus thiooxidans* cells (1 mg wet cells) were washed and resuspended in 0.1 M ß-alanine (pH 3.0 with sulfuric acid) as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

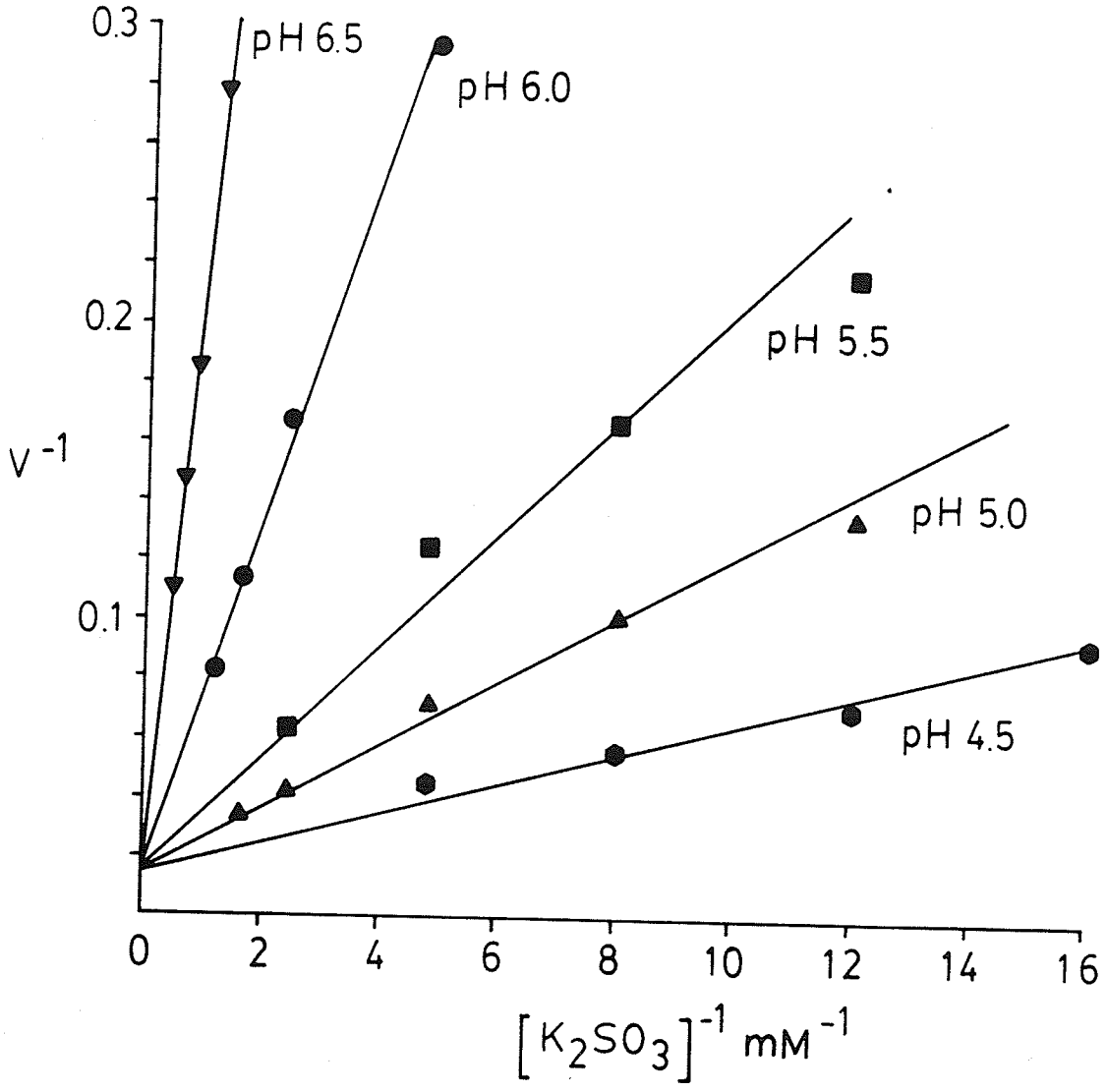

0.3
0.2
0.1
V -1
pH 6.5
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 10. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (Starkey's No.1 washed cells). *Thiobacillus thiooxidans* cells (1 mg wet cells) were washed and resuspended in Starkey's No. 1 medium, pH 2.3 as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

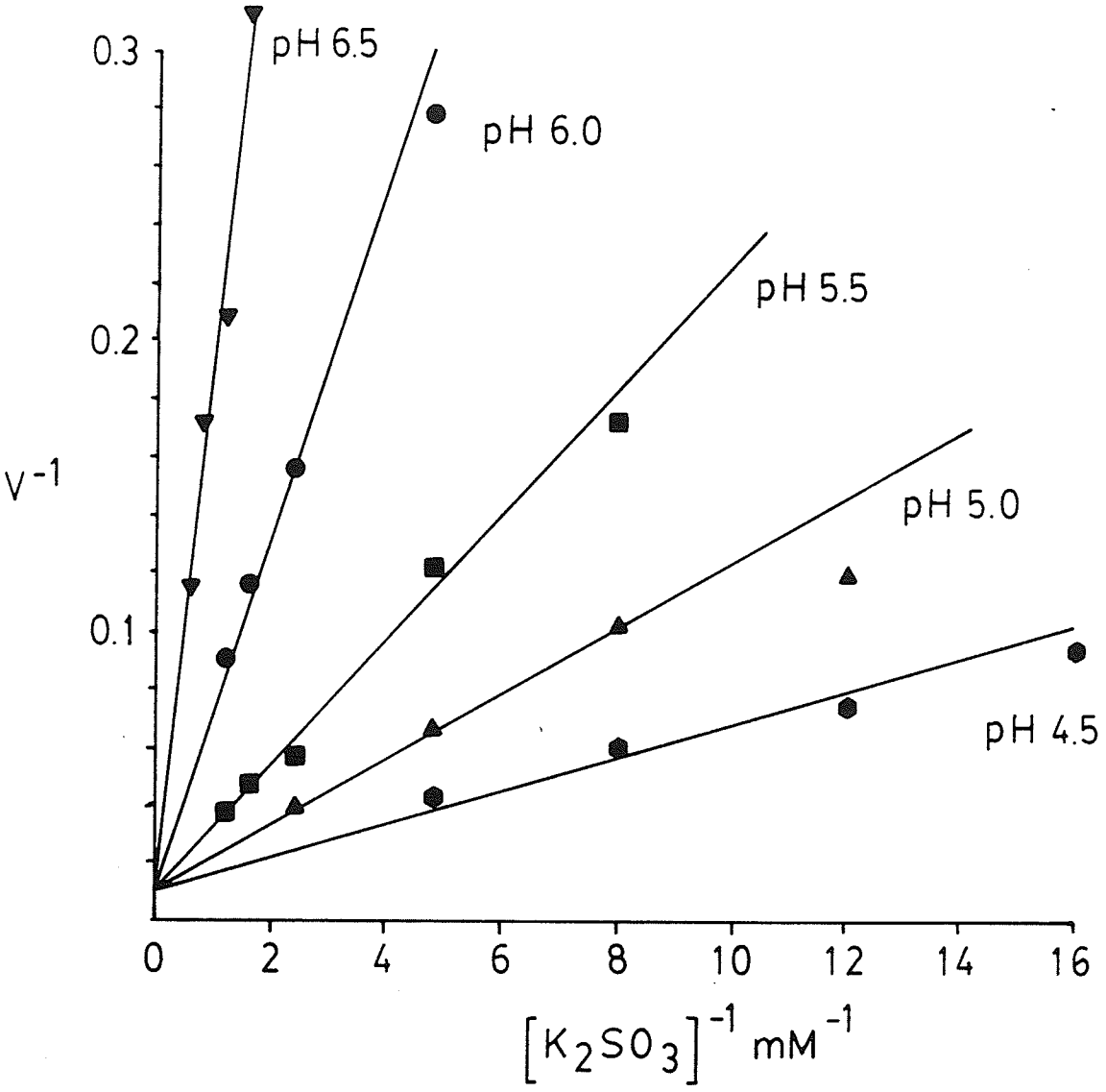

pH 6.5
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0.3
0.2
0.1
V-1
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 11. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (pH 3.0 KCl washed cells). *Thiobacillus thiooxidans* cells (1 mg wet cells) were washed and resuspended in 50 mM potassium chloride, pH 3.0 (with hydrochloric acid) as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

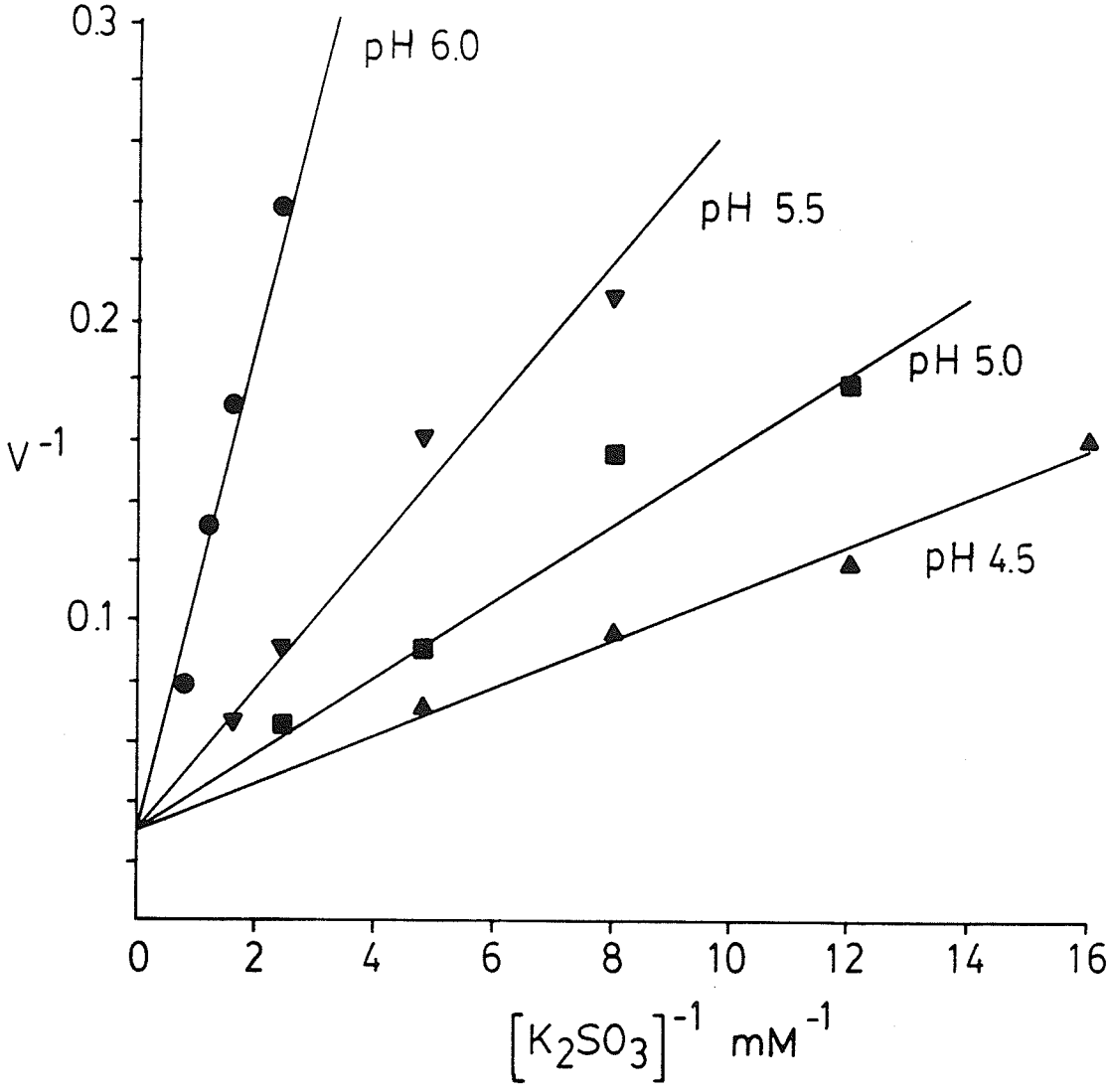

0.3
0.2
0.1
V -1
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 12. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (HP medium grown and washed cells). *Thiobacillus thiooxidans* cells (1 mg wet cells) were grown in HP medium (Lizama and Suzuki, 1988) for 4 days. Cells were washed and resuspended in same, pH 2.3 as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

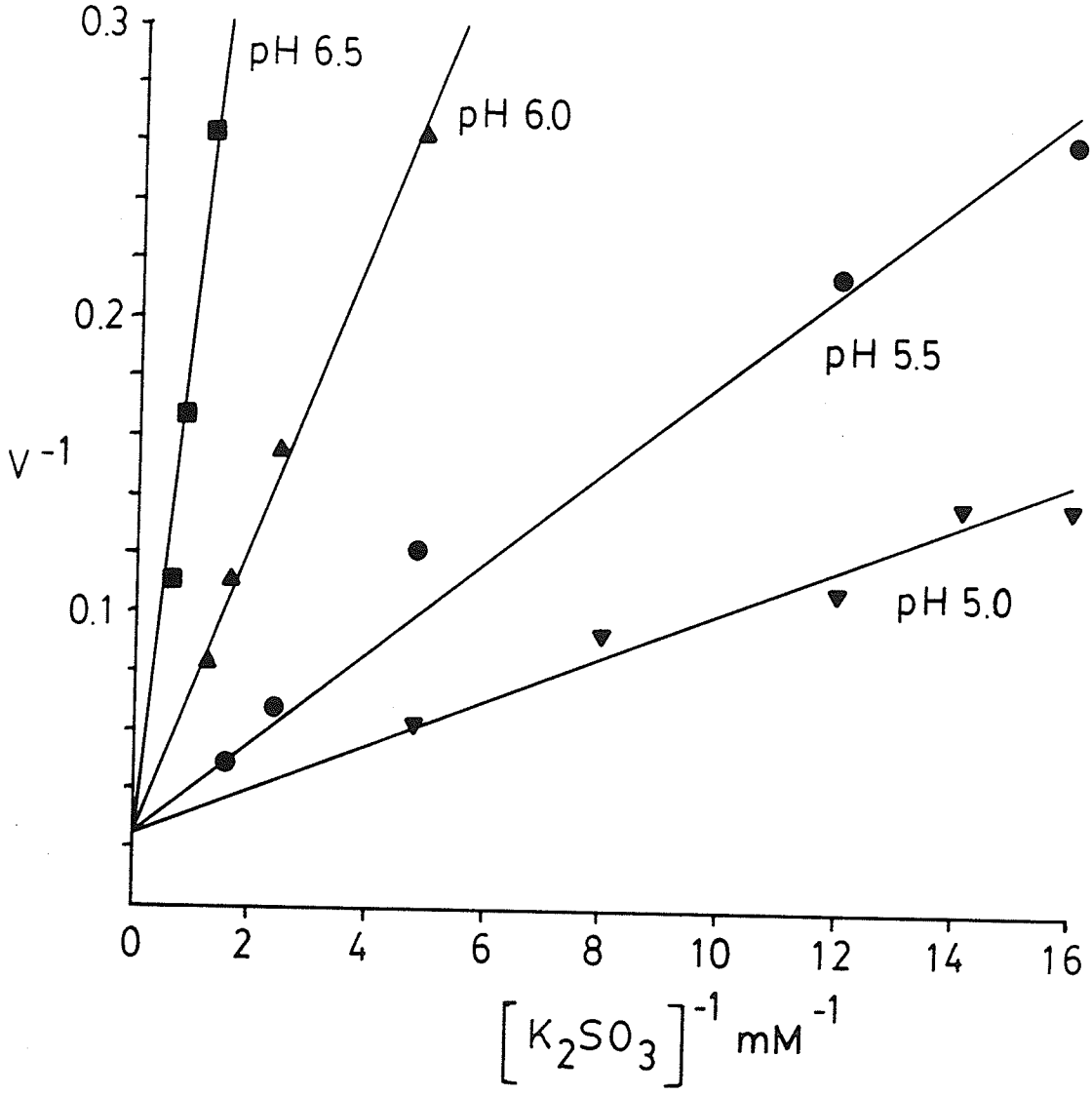

0.3
0.2
0.1
V -1
pH 6.5
pH 6.0
pH 5.5
pH 5.0
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 13. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (HP medium grown cells washed in potassium phosphate at pH 2.3). *Thiobacillus thiooxidans* cells (1 mg wet cells) were grown in HP medium (Lizama and Suzuki, 1988) for 4 days. Cells were washed and resuspended in 50 mM potassium phosphate buffer, pH 2.3 as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

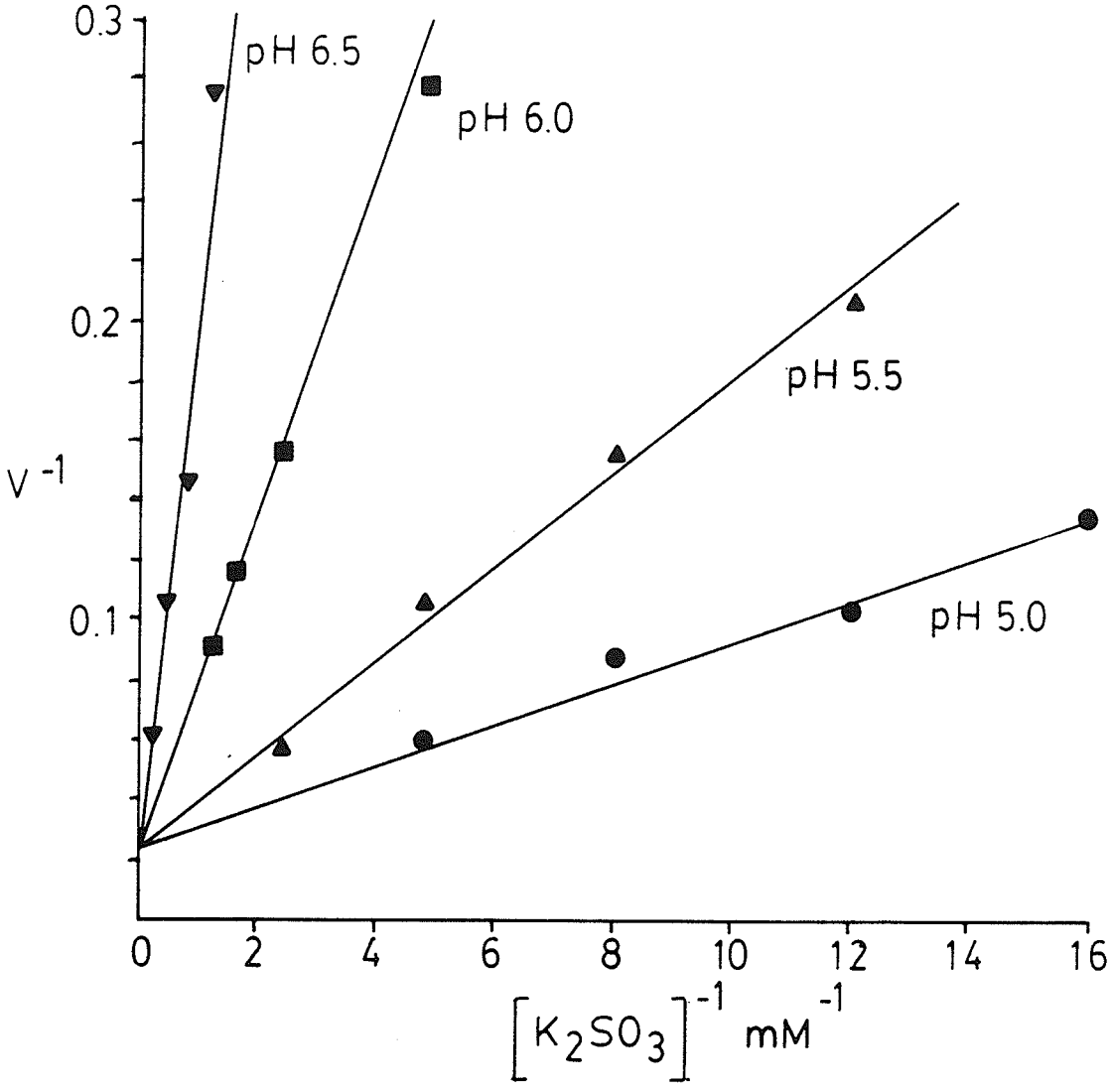

0.3
0.2
0.1
V -1
pH 6.5
pH 6.0
pH 5.5
pH 5.0
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 14. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations (HP medium grown cells washed in pH 3.0 H_2SO_4). *Thiobacillus thiooxidans* cells (1 mg wet cells) were grown in HP medium for 4 days. Cells were washed and resuspended in sulfuric acid, pH 3.0 as described in Materials and Methods. K_m and V_{max} values are listed in Table 4.

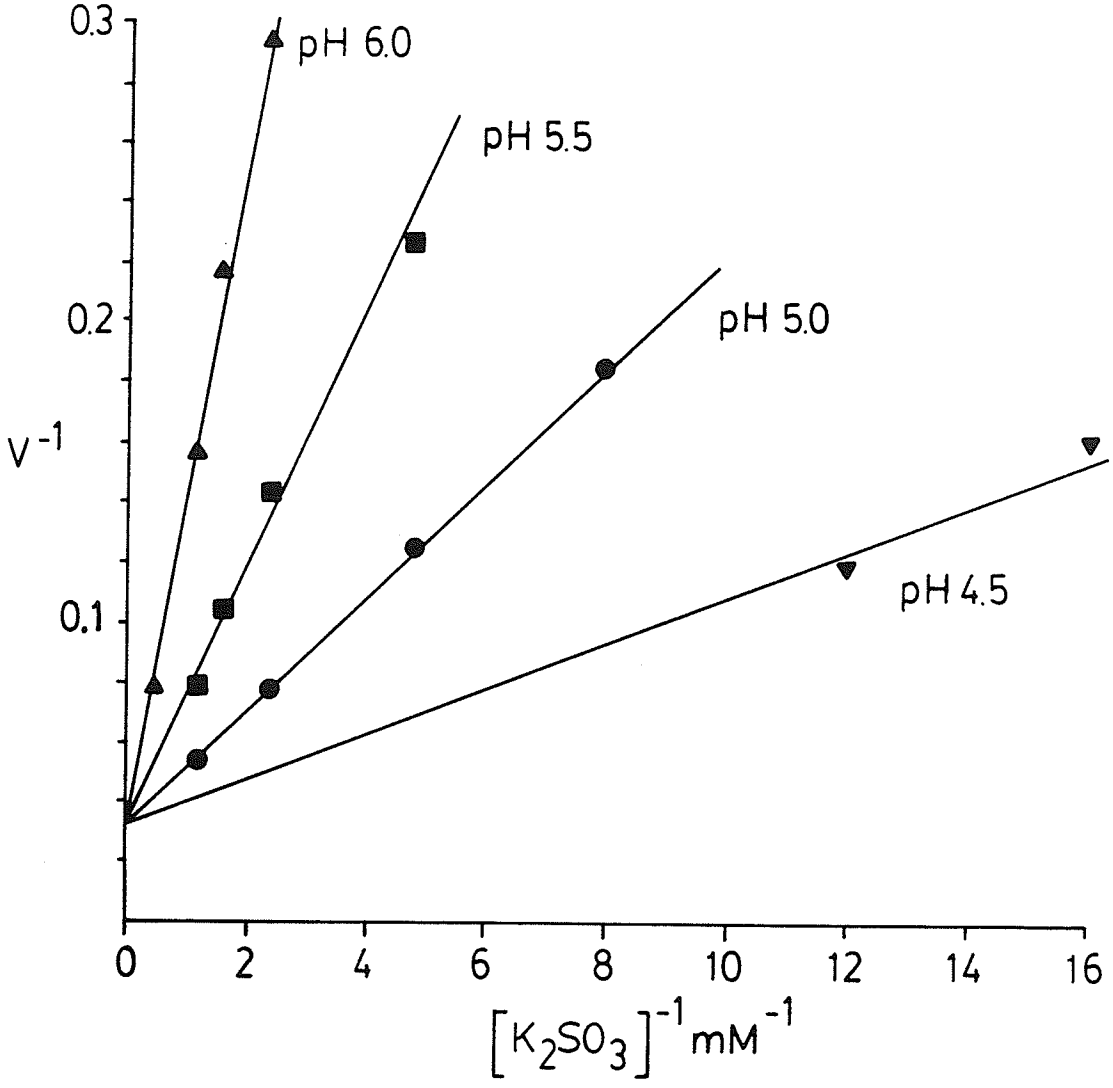

0.3
0.2
0.1
V^{-1}
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
$[K_2SO_3]^{-1}$ mM^{-1}

Table 5. Summary of K_m and V_{max} values for sulfite oxidation by *Thiobacillus ferroxidans* Tf-2 (ATCC 19859) and mine isolated *T. thiooxidans* strains

Expt	Strain[a]	Washing[b] pH	K_m at pH[c]					V_{max} (nmoles O_2/min)
			6.5	6.0	5.5	5.0	4.5	
1	Tf-2	2.3		1.1	0.7	0.3	0.2	22.7
2	Tf-2	7.5		5.0	2.5	1.4	1.1	43.5
3	SM-6	2.3		8.3	4.0	1.7	0.5	66.7
4	SM-7	2.3		6.3	2.4	0.7	0.2	34.5
5	301-S Iron Mtn	2.3	16.7	3.3	1.2	0.5		62.5

[a] Each strain was grown in Starkey's medium No.1 at pH 2.3 for 4 days except in Experiments 1 and 2 it was grown for 5 days.

[b] Washing medium was 50 mM potassium phosphate

[c] Assay was carried out in 50 mM potassium phosphate buffer of indicated pH.

Figure 15. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations by *Thiobacillus ferrooxidans* washed at pH 2.3. *Thiobacillus ferrooxidans* ATCC 19859 (Tf-2) cells were grown for 5 days. Cells (1 mg wet cells) were washed in 50 mM potassium phosphate buffer, pH 2.3 as described in Materials and Methods. K_m and V_{max} values are listed in Table 5.

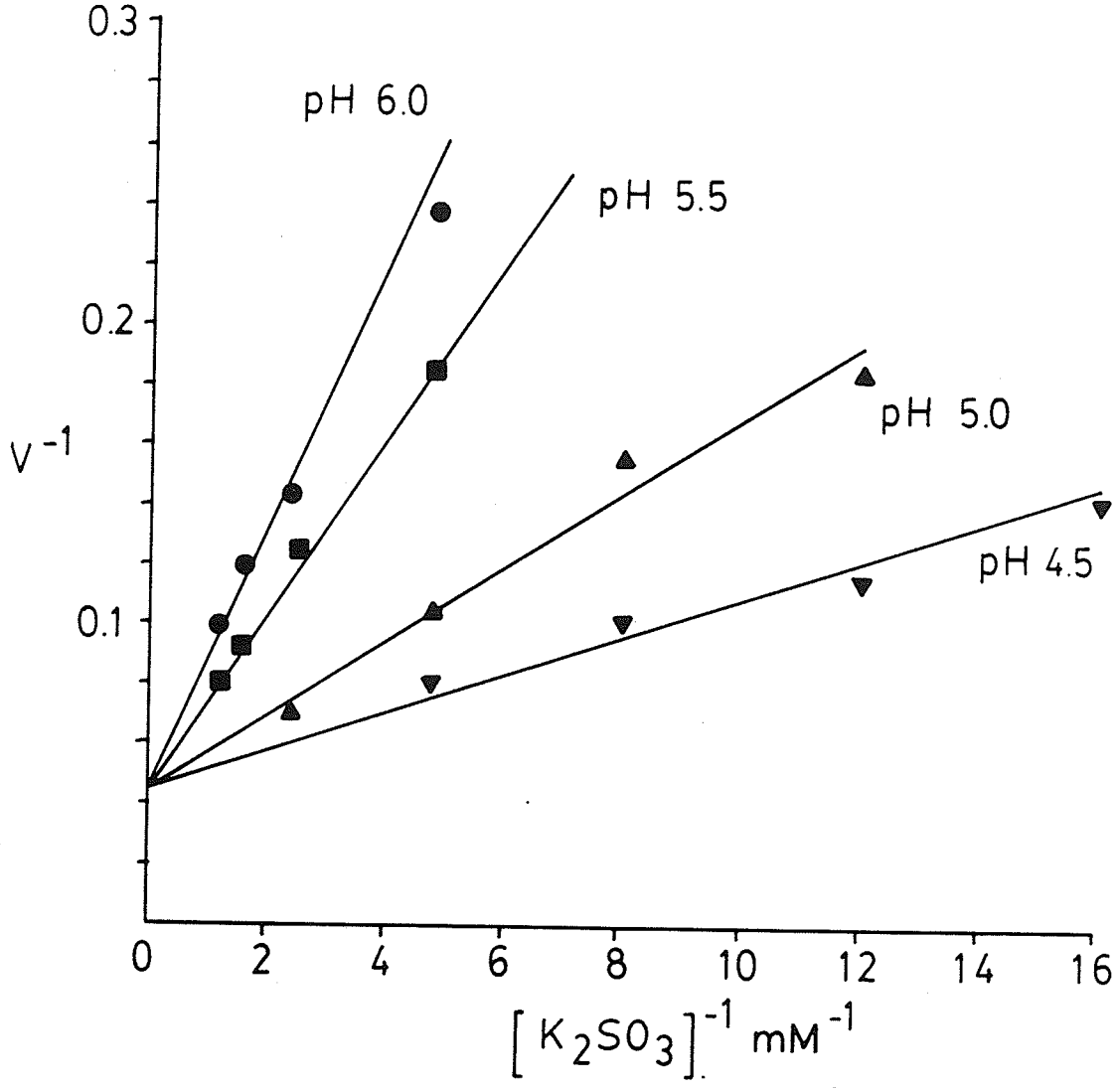

0.3
0.2
0.1
V -1
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 16. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations by *Thiobacillus ferrooxidans* washed at pH 7.5. *Thiobacillus ferrooxidans* ATCC 19859 (Tf-2) cells were grown for 5 days. Cells (1 mg wet cells) were washed in 50 mM potassium phosphate buffer, pH 7.5 as described in Materials and Methods. K_m and V_{max} values are listed in Table 5.

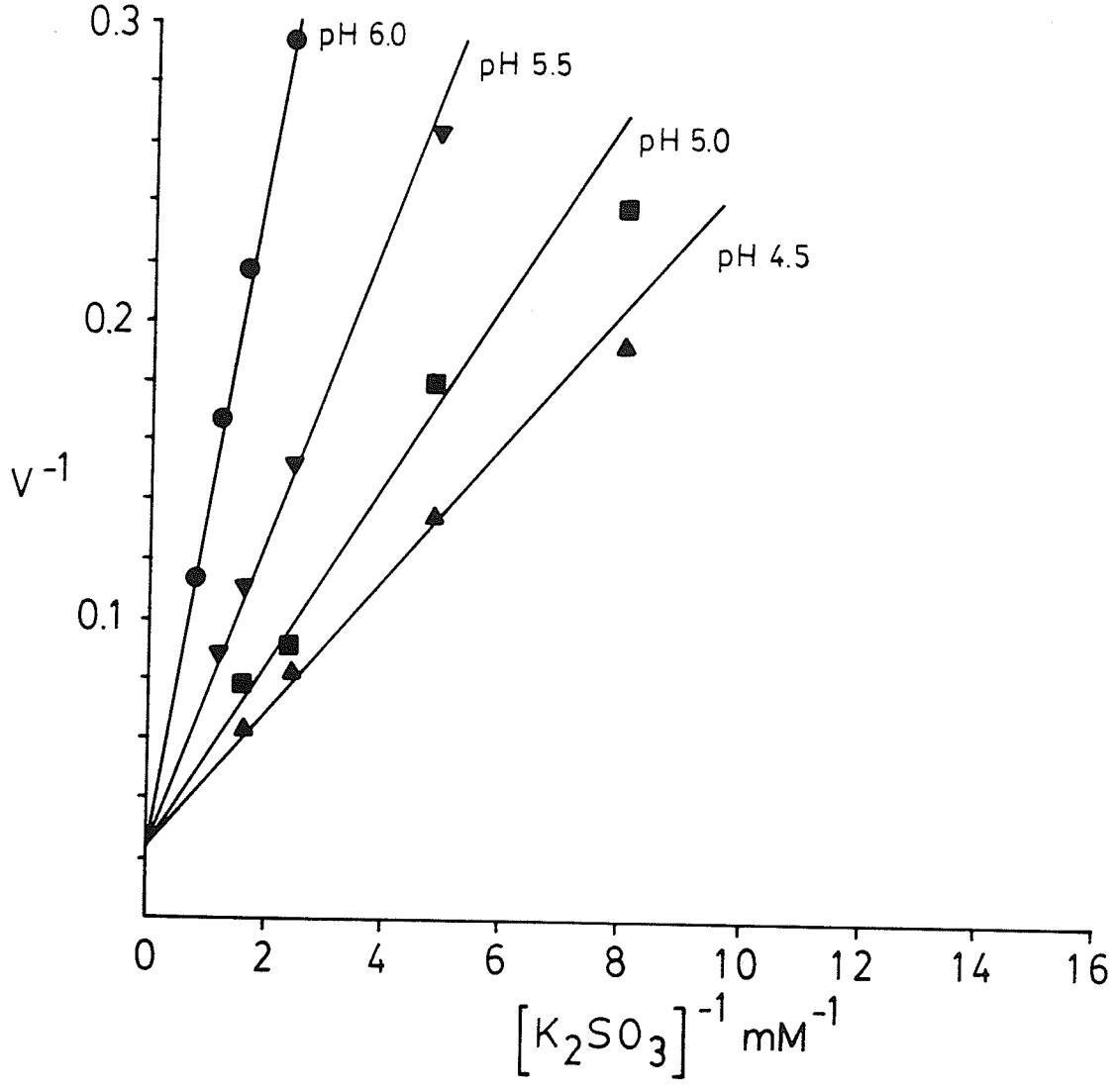

0.3
0.2
0.1
V -1
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 17. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations by SM-6. SM-6 mine isolate (Lizama and Suzuki, 1987) cells (1 mg wet cells) were washed and assayed at 50 mM potassium phosphate buffer as described in Materials and Methods. K_m and V_{max} values are listed in Table 5.

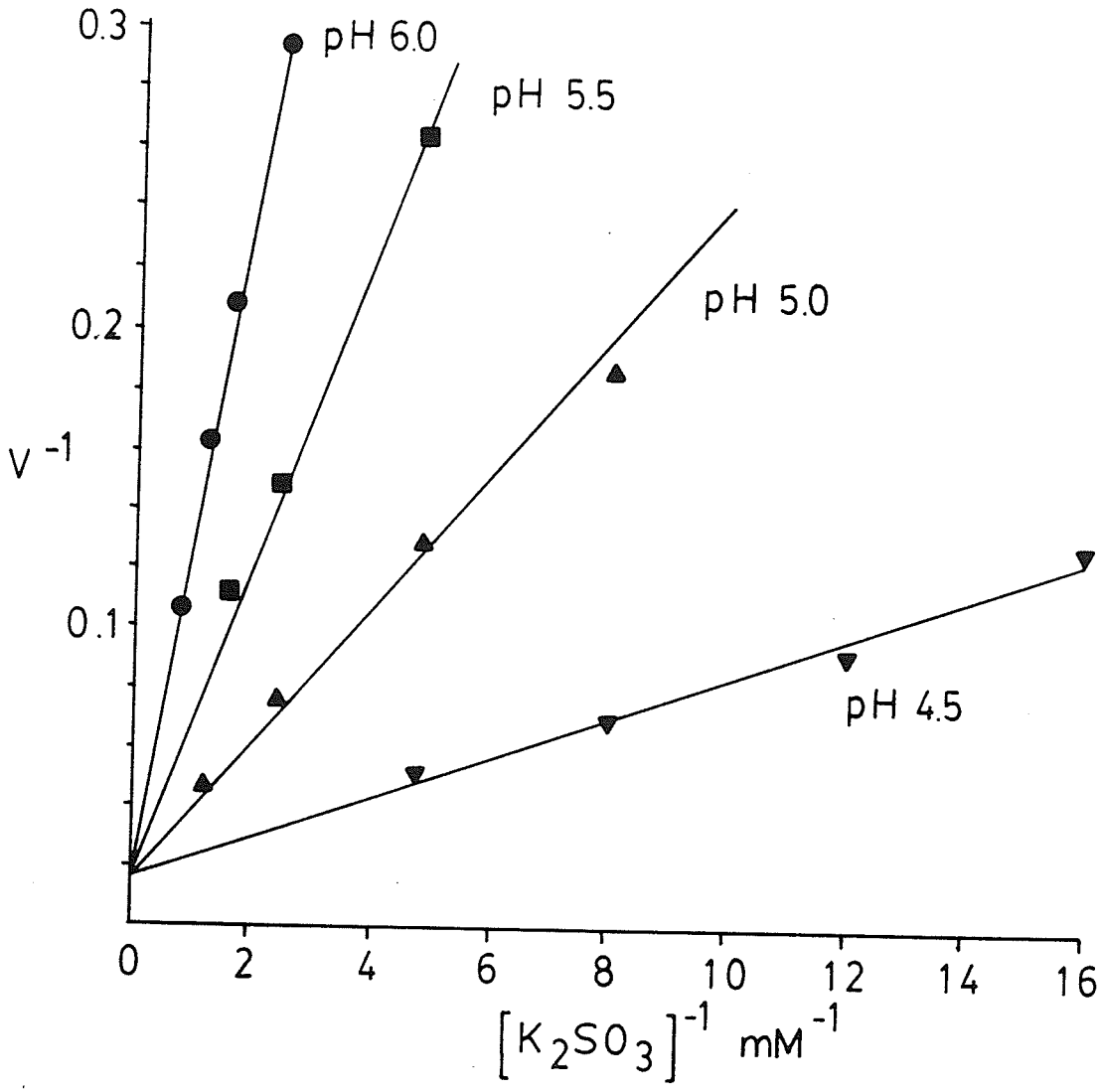

0.3
0.2
0.1
V -1
pH 6.0
pH 5.5
pH 5.0
pH 4.5
0
2
4
6
8
10
12
14
16
[K2SO3]-1 mM-1

Figure 18. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations by SM-7. SM-7 mine isolate (Lizama and Suzuki, 1987) cells (1 mg wet cells) were washed and assayed at 50 mM potassium phosphate buffer as described in Materials and Methods. K_m and V_{max} values are listed in Table 5.

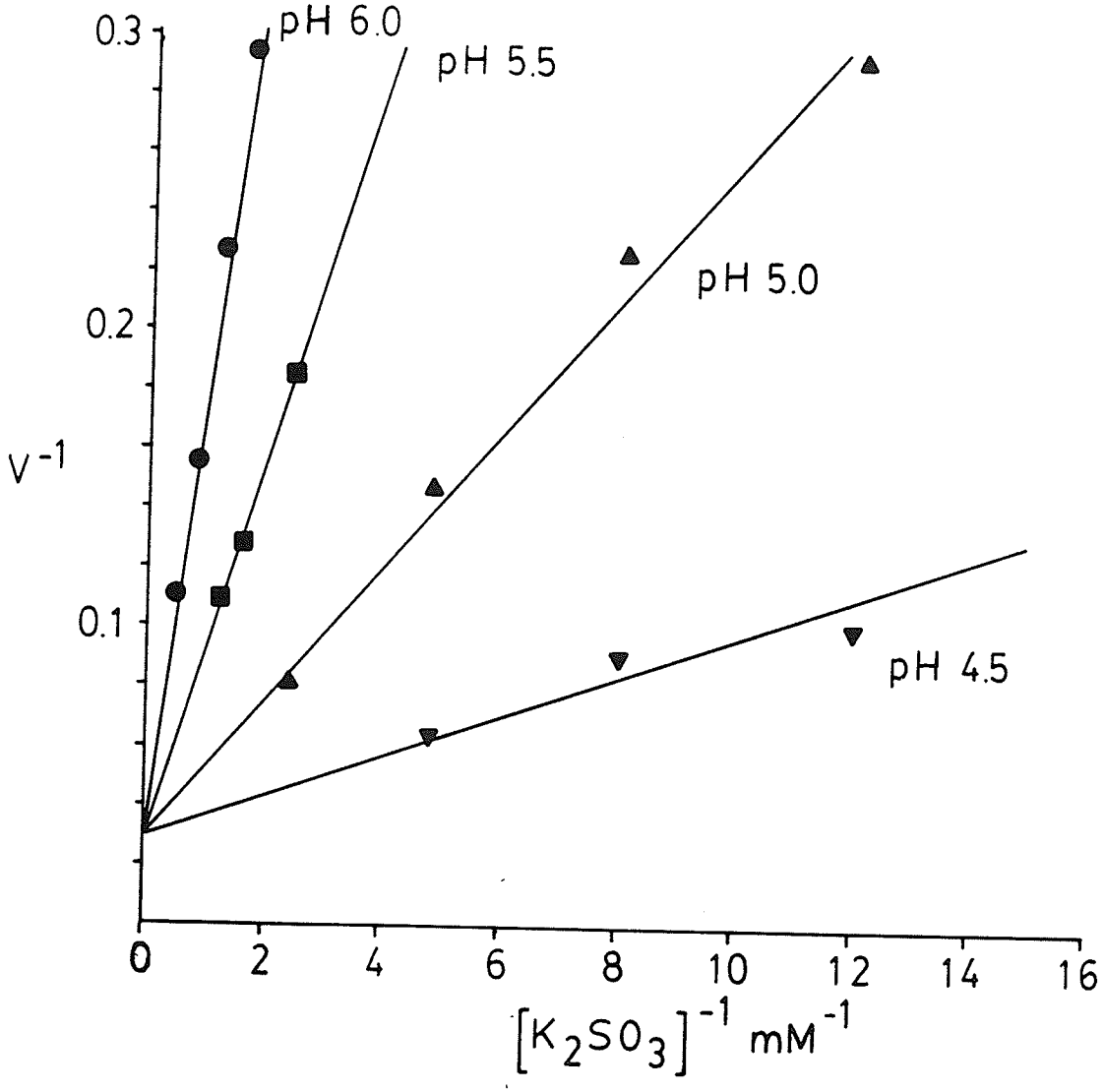
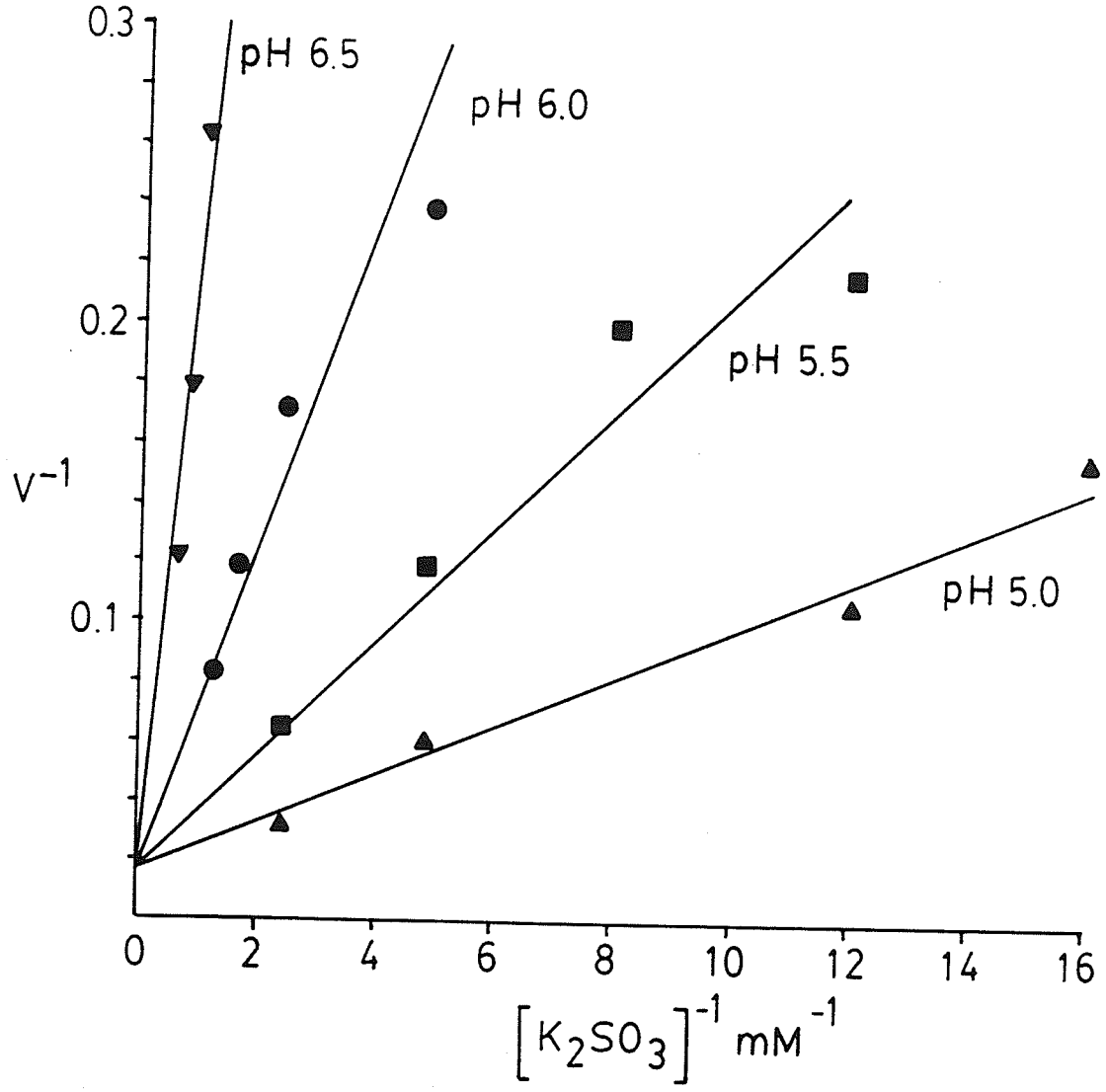

pH 6.0
pH 5.5
pH 5.0
pH 4.5
0.3
0.2
0.1
V -1
0
2
4
6
8
10
12
14
16
[K2SO3] -1 mM -1

Figure 19. Effect of pH on the double reciprocal plot of sulfite oxidation rates and sulfite concentrations by 301-S Iron Mountain mine isolate cells (1 mg wet cells) were washed and assayed at 50 mM potassium phosphate buffer as described in Materials and Methods. K_m and V_{max} values are listed in Table 5.


0.3
0.2
0.1
V⁻¹
pH 6.5
pH 6.0
pH 5.5
pH 5.0
0
2
4
6
8
10
12
14
16
[K2SO3]⁻¹ mM⁻¹

DISCUSSION

Acidophilic thiobacilli have been shown to have a cytoplasmic pH near neutral (Matin, 1990). Normal growth pH for the acidophilic thiobacilli ranges from 3.5 to less than 1.0 (Vishniac and Santer, 1957). This results in a large pH gradient across the cell membrane.

Gram negative bacterial cells including *Thiobacillus thiooxidans* possess lipopolysaccharide molecules in the outer membrane which can act as an additional permeability barrier for the cell. Since the bacteria oxidize sulfur at acidic pH to produce sulfuric acid, the periplasmic space must be acidic. When sulfite is externally added, it must penetrate both the outer and inner membranes to reach the neutral cytoplasm where it is oxidized by sulfite oxidase.

In experiments with intact cells, sulfite oxidation is difficult to show because of the low growth pH. Kodama et al. (1970) reported a pH optimum for sulfite oxidation between 6.0 and 6.5. It was found in this work that the sulfite oxidation optimum actually varied with sulfite concentration. At increasing sulfite concentrations, the pH optimum shifted towards neutral pH. In comparison, sulfite oxidation by cell free extracts had a constant pH optimum around 7 which agrees with previous reports of Kodama et al. (1969, 1970) who reported pH optimum values of 6.9 to 7.0 for cell free extracts.

Sulfite oxidation by *T. thiooxidans* may be considered similar to ammonia oxidation by *Nitrosomonas europaea* shown by Suzuki et al. (1974). The oxidation of NH_3, instead of NH_4^+, occurs because the uncharged species can readily enter the

cell. If the pK_a values for sulfite/bisulfite ion (pK_{a2} = 7.0) and bisulfite/sulfurous acid (pK_{a1} = 1.81) are considered, the uncharged sulfurous acid would compose most of the species present only below pH 1.8 (Fig.4). And yet it is this form that is oxidized by these cells even at a pH close to neutrality. Since the K_m values for sulfurous acid is so low (0.11-0.24 μM, Table 2) it is experimentally impossible to measure the oxidation at a very low pH, especially when the K_i values are not much higher (0.5-0.8 μM, Table 3). Under acidic conditions, uncharged species can enter the cytoplasm which has a pH of 6.5-7.0 (Matin, 1990). If sulfurous acid penetration rate is faster then its oxidation rate, then sulfurous acid accumulates and a shift in the species from sulfurous acid to bisulfite (a dissociation step producing protons) occurs and acidification of the cytoplasm results. This decrease in the pH causes sulfite to accumulate further since the optimal pH for sulfite oxidation in the cell-free system is close to neutrality (Fig. 7). At pH 2.3 sulfite inhibits sulfur oxidation by *T. thiooxidans* cells either when it was added externally or when it accumulated by an inhibitor of sulfite oxidation (Suzuki et al., 1992). Recently, Sugio et al. (1994) reported similar effects of sulfite on iron oxidation by *T. ferrooxidans* and *Leptospirillum ferrooxidans*.

The effect of washing cells under different conditions produced changes in the values of K_m and V_{max}. This is probably because the periplasmic space pH can be changed by wash procedures. This would alter the concentrations of sulfurous acid in the periplasmic space and affect the rate at which sulfurous acid enters the cytoplasm.

Assays were performed at high potassium phosphate concentration to test if osmotic pressure affected sulfite oxidation. Osmotic pressure changes are known to affect many different aspects of cell metabolism including the inhibition of sulfur oxidation. There was no change to the kinetic pattern suggesting that high concentrations of potassium phosphate did not affect the substrate availability. (It is interesting to speculate that the effects of osmotic pressure might have a role in the transport of sulfur into the cell).

Sulfite oxidation in other strains of *T. thiooxidans* and in *T. ferrooxidans* cells grown on elemental sulfur showed similar results for oxidation of externally added sulfite. That is, the double reciprocal plot of substrate concentration against rate showed a series of lines which cross the Y-axis at the same point. The values of K_m were varied and but were dependent upon pH, increasing with increasing pH. Thus the use of sulfurous acid as the substrate for oxidation by cells seems to be universal among acidophilic thiobacilli.

Sulfite oxidation with externally added sulfite available to the cell is a result of artificial conditions of the assay system. When the cell is oxidizing sulfur in a natural condition, sulfite would be produced internally (Suzuki et al., 1993) because of the pH optimum of the sulfur oxidizing enzyme (Suzuki, 1965) and oxidized internally.

Sulfite will accumulate only when the rate of sulfur oxidation is faster than the rate of sulfite oxidation. Under these conditions sulfite will come out of the cell as sulfurous acid and accumulate outside the cell as sulfite.

PART II:

HYDROPHOBICITY AND SULFUR OXIDATION

INTRODUCTION

One of the problems involved in sulfur and sulfide ore oxidation that remains to be explained is how the organism is able to attack the insoluble substrate for oxidation. Recent evidence has suggested that there is a role of the cell surface hydrophobicity or hydrophilicity in the attachment of cells during the oxidation of mineral ores.

Significant work has occurred in the area of bacterial attachment of cells in medical (Rosenberg and Doyle, 1990), oral (Kolenbrander and London, 1993) and food microbiology (Berkeley et al., 1980). Rosenberg developed a method for assaying cell surface hydrophobicity by determining the distribution of cells in aqueous and organic, hydrocarbon phases. This Bacterial Adhesion To Hydrocarbon or BATH method provided an easy and rapid system for determining the effects of washing and aqueous phases on cell surface hydrophobicity.

The results suggest that the washing and assay buffers have a significant role in the hydrophobicity of cells and suggest that this affects the ability of cells to attach to substrates such as sulfur. The study was carried out using three different methods: adhesion of cells to sulfur, hydrophobicity of cells, and finally oxidation of sulfur. The results indicate that low hydrophobicity or high hydrophilicity of cells favor their adhesion to, and oxidation of sulfur.

HISTORICAL

Bacterial adhesion

Bacterial adhesion to solid surfaces has been an area of intense study in recent years particularly the role of bacterial adhesion in economically feasible processes (example bioleaching), and in economically costly processes (example biofouling). Current knowledge of the adhesion of *Thiobacillus* species to mineral ores in bacterial leaching processes is limited. A full understanding of the adhesion process would allow for improvements in controlling the rate of metal solubilization. The adhesion of cells to or the surface contact of a cell with a second surface in general is complex and is based on colloid physical chemistry principles. Numerous parameters must be considered and assumptions made, when using the principles derived for colloids and applying them to living organisms attaching to surfaces.

Two important considerations exist when studying bacterial attachment. First, does a bacterial cell in contact with a second surface (the surface of a second cell or the surface of a material) adhere or associate to this second surface. Second, does one to assume that all of the surfaces involved are homogeneous in composition and static in net overall charge.

If we consider attachment as adhesion, we can define the process as a specific interaction occurring by means of a specialized structure (such as fimbriae or pili) which has an affinity for surfaces such that the interaction requires energy to separate the components from each other. If we consider attachment as an association, we define the interaction between a microorganism and a surface as occuring by a non-

specific mechanism (Rutter and Vincent, 1984).

In defining bacterial attachment as non-specific and specific, we further classify specific attachment into polymer bridging and polymer consolidation (Ward and Berkeley, 1980). Non-specific attachment, occurs as a result of forces such as surface charge. The role of surface charge raises the question as to whether or not the surface of the microorganism should be considered as homogeneous and static. From a physiological point of view, neither homogeneity nor stasis can be assumed because localized areas of charge and charge density exist on the bacterial surface and may be dependent upon cell energy levels, lipopolysaccharide and protein composition (which would determine the ions bound at the cell surface) and pH (which would affect the ionization states). Obviously, the quantitation of non-specific adhesion is more complex but equally challenging as the quantitation of appendages involved in specific adhesion.

The processes of specific and non-specific attachment of microorganisms to a surface can be better understood if we divide the attachment process into time dependent phases (Fletcher et al., 1980). The first stage would be best described as non-specific. The initial adsorption phase is largely dependent upon the surface charges of the microorganism and the charge of the second surface which the cell will approach. The second phase would be specific and involves the formation of polymer bridges to hold the organism to the surface. Although specific attachment suggests the use of appendages for attachment, the interaction by glycocalyx (Bryant et al., 1983) or bacterial capsule (Zobell, 1943) would be included in this phase. The

third phase is also specific and involves the growth and division of microorganisms resulting in the consolidation of the polymers. This phase is most readily seen as biofilm formation for many different organisms.

If physicochemical parameters are used to describe the initial stage of interaction, the Derjaguin and Landau and Verwey and Overbeek theory or DLVO theory is the most widely accepted (Pethica, 1980). The DLVO theory is used to calculate the interaction Gibb's energy between a particle and a surface as a function of the separation distance. The theory is applicable at distances greater than one nanometer. This is where the potential energy barrier has been overcome and specific interactions, as estimated by short-range polar forces can occur. The electrical double layer component depends upon the total ion concentration of the bulk solution (Rutter and Vincent, 1980). Although the DLVO theory was originally derived from colloid chemistry principles and modified for microorganisms, it arguably holds true given the assumptions of uniformity of cells it makes. Busscher and Weerkamp (1987) consider only van der Waal's forces to operate at distances greater than 50 nanometers and electrostatic repulsive forces are considered at distances between 10 and 20 nanometers. A distance greater than 50 nanometers is too large for opposing forces to recognize specific surface components but between 10 and 20 nanometers, short range-interactions can occur causing rearrangement of components on the cell surface.

The initial phase can be reversed and is considered by Boonamnuay Vitaya et al. (1991) to be a result of the long-range attractive forces acting. Reversible

adhesion was defined by Marshall (1984) as an interaction in which bacteria still show Brownian motion and can be removed by washing or bacterial motility. If the initial interaction becomes irreversible, Boonamnuay Vitaya et al. (1991) consider this a result of short range forces such as chemical bonds (electrostatic, hydrogen and covalent bonds), dipole interactions (dipole-dipole, dipole-induced-dipole and ion-dipole) or hydrophobic bonding. These forces hold the microorganisms to a surface and allow for extracellular adhesive materials such as the mucilagenous mass described by Zobell (1943) to accumulate and allow specific attachment via polymer formation (phase two) to occur.

The area of adhesion is very difficult to study because of the numerous parameters that must be assessed. Consider that bacterial cells are on average less than a few microns in diameter and are non-uniform with various charged groups protruding from the surface. Given that surface groups are ionizable, a change in pH would greatly affect the double layer thickness and in turn the calculations for the DLVO theory. Several methods have been attempted to predict the adhesive properties of cells to solid, liquid or gas phases. These include contact angle measurements, zeta potential measurements, and cell surface hydrophobicity characterization. Hydrophobicity has been considered as a means of predicting the adhesive properties because as Busscher and Weerkemp (1987) note, the role of hydrophobicity and hydrophobic cell surface components in bacterial adhesion will probably be its dehydrating effect of this water film, enabling short range interactions to occur.

Bacterial hydrophobicity can be measured in terms of hydrophobic properties of the outer cell surface or hydrophobicity in terms of adhesion (Rosenberg and Doyle, 1990). Much work has been done with heterotrophic organisms in medical, oral and food microbiology, but very few articles have appeared pertaining to the Thiobacilli.

Cell surface hydrophobicity is a term used to describe the physical interactions of cell surface components with aqueous phases (Duncan-Hewittt, 1990). As a result, one must consider the physicochemical properties of the solution, the cell and the sorbing surface when studying cell attachment (Bar-Or, 1990). Hydrophobicity in general has been implicated in playing a role in the folding of proteins, assembly of macromolecules into membranes and bilayers as well as the adsorption process.

The cell surface hydrophobicity is therefore governed by the composition of the cell surface by macromolecules such as proteins and lipopolysaccharides. Additionally, the charges contained in the surface of the cell affects the potential electrostatic attractive and repulsive interactions in bacteria with surfaces.

Certain aspects of the adhesion process must not be overlooked when quantitatively assaying the hydrophobicity of cells. Hydrophobic interactions between surfaces and microbes, properties of the aqueous phase, microbial culture conditions and assay conditions can directly affect the results of the experiments carried out (Rosenberg and Doyle, 1990). Given these aspects and the fact that a reliable method for standard procedures such as growth on solid media and serial dilutions are at best erratic for the thiobacilli, it is not surprising that few authors have been

able to generate sufficient experimental evidence for the Thiobacilli in general. Moreover, the theory of sulfur oxidation suggests that contact with the substrate is required for the organism to oxidize substrate (Takakuwa et al., 1979).

Sulfur attachment

Elemental sulfur is most stable in the S_8 ring form. This form of sulfur has a very low water solubility and is hydrophobic. This in fact is a means by which separation of sulfur from ores occur in the metal processing industry. The surface character of the thiobacilli has been shown to play a role in the attachment of cells and the oxidation of sulfur. The attachment of *Thiobacillus thiooxidans* to sulfur crystals was reported by Schaeffer et al. (1963) and Baldensperger et al. (1974). Although the exact molecular mechanism is not proven, either non-specific attachment or attachment mediated by an appendage could be supported by published data. Regardless of the mechanism, it is known that direct contact between cells and sulfur is required for sulfur oxidation (Takakuwa et al., 1979). This is further supported by results obtained during growth studies which showed that during the initial phase of growth, practically all of the cells were attached on the surface of sulfur particles and with progress of growth of the culture, an increasing number of cells appeared in the liquid phase.

Surface active agents or "wetting agents" have been isolated from culture filtrates (Cook, 1964 and Agate et al., 1969) and Jones and Starkey (1961) suggested that these agents allow the organism to attach to sulfur. Growth of *T. thiooxidans* has

also been shown to be stimulated by the addition of surface active agents such as Tween 80 (Agate et al., 1969 and Kingma and Silver, 1979). Vogler and Umbreit (1941) investigated the effect of surface active agents, particle size, and interposition of a dialyzing membrane on sulfur oxidation by *T. thiooxidans*, as well as the microscopic behaviour of these organisms in the presence of sulfur, and concluded that direct contact between sulfur and the bacteria was required for oxidation to proceed (Vogler and Umbreit, 1941). Despite intensive investigation of the mechanism of sulfur oxidation, very little information is available in regard to the mechanism of attack on the elemental sulfur particles by bacterial cells and the mobilization of this insoluble substrate to the essential enzyme systems. Upon contact the sulfur was thought to dissolve in a terminal fat globule and thus become susceptible to enzymatic oxidation in the bacterium (Knaysi, 1943). The requirement for physical contact implies that sulfur oxidation is favored in stagnant or slowly shaken cultures. Cook (1964) studied the growth of *T. thiooxidans* in shaken cultures in the presence of wetting agents. Starkey et al. (1956) have reported an increase in the rate of sulfur oxidation by *T. thiooxidans* on vigourous shaking. Another concept is that both the attachment of the organism to sulfur and solubilization of the sulfur particles by phospholipids and other extracellular compounds (wetting agents) released by the cells are necessary for subsequent oxidation (Cook, 1964). There is some experimental evidence for this theory and cells have been photographed clustered around eroded sulfur particles and a brief stationary phase after inoculation of a culture appears to be necessary for the production of phospholipids or other

cellular materials required for adhesion. Scanning electron micrographs (Baldsberger et al., 1974) have shown that the surface of sulfur particles were covered and eroded by *T. thiooxidans*. The cells can be released by the addition of carbon disulfide (Takakuwa, 1992 and Takakuwa et al., 1977) to the growth medium. This treatment was also used to show that the cell-sulfur contact was required for bacterial growth on sulfur. The attachment however does not involve pili as reported for other sulfur oxidizing organisms such as *Sulfolobus* (Weiss, 1973).

Electron micrographs of *T. thiooxidans* revealed a cell wall similar to that of other gram negative species (Mahoney and Edwards, 1966; Shively et al., 1970) with an additional barrier for sulfur entry into the cell. According to Knaysi (1943), bipolar fat globules reported previously, are granules which ordinarily contain volutin, and immediately after sulfur oxidation both volutin and sulfur. It is this stored colloidal sulfur which may undergo chemical reactions similar to those of lipids [for example staining and iodine absorption] and therefore give the impression of fat globules. In yeast, volutin has been identified as cyclic and linear polyphosphates. The accumulation of polymetaphosphate in *T. thiooxidans* has been tentatively confirmed by Barker and Kornberg (1954), who found large amounts of an easily hydrolyzable polyphosphate. Knaysi also observed a capsule which he thought should mediate direct contact between sulfur and the bacteria, agreeing with Umbreit et al. (1942) and later supported by work with a similar organism by Bryant et al. (1983).

The second possible means of attachment to sulfur is mediated by an appendage, such as pili, but has been shown only for *Sulfolobus* isolates by Weiss

(1973). Takakuwa et al. (1979) and his coworkers have done extensive studies on the cell-sulfur adhesion process and have reported that it was inhibited by thiol-binding reagents indicating that thiol groups which may be present in the cell envelope, are essential for this process. Both the adhesion process and oxygen uptake activity were shown to be significantly inhibited by some heavy metal chelators, electron transport inhibitors and anoxia indicating that the adhesion process must be energy dependent. The adhesion process also showed a pH dependency similar to sulfur oxidation with a pH optimum of 2 to 5 (Takukuwa et al., 1979). Specific binding sites may exist on the bacterial surface, because *T. thiooxidans* strain of Iwatsuka and Mori could not oxidize lenthionine, a chemical analogue of the elemental sulfur ring (Iwatsuka and Mori, 1960).

Diffusion and reduction to sulfide have both been suggested as mechanisms for the transport of sulfur across the cell wall of *T. thiooxidans*. Diffusion is likely to be too slow in view of the infinitesimal solubility of sulfur in aqueous media, although it may account for processes which require only small amounts of sulfur, such as fungicidal action (Beffa, 1993). The possibility of sulfur reduction followed by diffusion or active transport of the sulfide into the cell has been discussed by Starkey (1925) who considers it unlikely, partly because he did not detect a rapid production of H_2S by *T. thiooxidans* and partly because he believes that thermodynamic obstacles oppose it. Nevertheless, the common property of living cells to reduce sulfur may have given rise to a specialized transport mechanism. Glutathione or another sulfhydryl compound may serve as the functional group

(Suzuki, 1974) in these reactions.

As a whole our present knowledge on the oxidation of sulfur seems to indicate that thiol groups on the bacterial cell envelope form polysulfide complex with the sulfur before its oxidation to sulfate through sulfite as an intermediate. The various sulfur intermediate enzyme systems for oxidation reported by various investigators are simply caused by the presence or absence of membranous thiol groups in the preparations.

In this study, a correlation between cell surface hydrophobicity, cell attachment and substrate oxidation was made.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest grade commercially available. Soluble salts were obtained from Fisher Scientific (Fairlawn, New Jersey, U.S.A.) or Mallinkrodt Canada Inc. Pointe Claire, Quebec, Canada). Precipitated sulfur powder was obtained from British Drugs Houses (BDH) Limited (Toronto, Canada). Sodium sulfide ($Na_2S{\cdot}9H_2O$) was obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Potassium sulfite (K_2SO_3) was obtained from Matheson, Coleman and Bell, Norwood, Ohio and East Rutherford, New Jersey, U.S.A..

Growth Conditions. *Thiobacillus thiooxidans* (ATCC 8085) was grown in 2.8 l Fernbach flasks for 4 days unless otherwise indicated. The flasks [containing 1 l of sterile Starkey's medium No. 1 (0.3 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.018 g $FeSO_4{\cdot}7H_2O$, 1.75 g KH_2PO_4 and 0.25 g $CaCl_2{\cdot}2H_2O$) adjusted to pH 2.3 with H_2SO_4] were inoculated with 25 ml (2.5 %) of stock culture. Stocks were maintained at 4°C. Finely powdered sulfur (BDH, precipitated; 1 g/l) was spread evenly on the surface of the medium and a tissue placed on top of the flask in absence of any stopper. The flasks were incubated without shaking at 28° C.

Cell collection. The cultures were shaken and filtered through Whatman No. 1 paper under suction to remove sulfur. The filtrates were centrifuged at 10,000 x *g* for 10 min. The pellets were washed with buffers (50 mM pH 2.3 potassium phosphate buffer or other buffers as indicated) and the suspensions centrifuged at 10,000 x *g* for 10 min. The pellets were resuspended in the same buffer at a final concentration of

50 mg wet cell weight per ml of buffer. The cell suspensions were normally used within 24 hours.

Heat extract. Cells were filtered through Whatman No. 1 paper and centrifuged. Pellets were resuspended in glass distilled water and then centrifuged at 10,000 x *g* for 10 min. Cells were resuspended at 200 mg wet weight per milliliter glass distilled water. An aliquot of the cell suspension was shaken in a 15 milliliter Corex tube (No 8441) at 90°C for 10 min. The cells were removed by centrifugation at 10,000 X *g* for 10 min. The light yellow, clear supernatant was removed and stored at room temperature for up to 1 week.

Cell Adhesion Assay. The adhesion of cells to sulfur was measured by a modification to the method of Takakuwa et al. (1979). Cells (5 mg wet cell weight) in buffer (10 ml) were shaken at room temperature for 15 min at 200 rpm (Labline Orbit Shaker) in 50 ml Erlenmeyer flasks. Sulfur (powdered or suspension [32 g per 100 ml Millipore H_2O containing 500 ppm Tween 80]) was added and shaken for a further 15 min. The reaction mixture was then filtered through fluted Whatman No. 1 filter paper. The optical density (O.D.) of the filtrate was taken at 660 nm (A_{660}) in a Hewlett Packard 8452A Diode Array Spectrophotometer. The cells adsorbed to the sulfur particles were expressed as a percentage of the original cell suspension.

Hydrophobicity Assay. The hydrophobicity assay was a modification of the method of Rosenberg et al (1980). Three milliliters of buffer and microliter volumes of cell suspension were mixed in screw cap culture tubes (Pyrex No 9825). The O.D. of the dilute cell suspension was taken at 660 nm and 3.0 ml of n-hexadecane (Fisher

brand) was added. The mixture was vortexed for 30 s at maximum speed. The emulsion formed was allowed to separate for 60 s and the absorbance of the bottom aqueous phase was taken. The results were expressed as a percentage of the original O.D. of the dilute cell suspension.

Microsocopy. Phases from the hydrophobicity assay were analyzed by placing droplets of organic solvent phase on a glass well slide (12 x 1 mm diameter well) with a cover slip. The droplets could be seen under phase contrast (Nikon Labophot microscope) at 400x magnification with a phase 4 filter.

Photomicroscopy. The phases from the hydrophobicity assay were recorded using a Nikon Optiphot photomicroscope system. Film was exposed automatically using a Nikon AFX light sensing system and a green monochromatic filter. Images were recorded on Kodak X-Pan-Plus print film using a Nikon FX35 single lens reflex camera.

Oxidation assays. All water used was Millipore filtered. Sulfur was prepared by suspending 32 g BDH precipitated sulfur in 100 ml H_2O containing 500 ppm Tween 80. Sodium sulfide (20 mM) was prepared by adding 0.24 g washed crystals of $Na_2S{\cdot}9H_2O$ to 49.8 ml anaerobic H_2O. The solution was stored under N_2 gas at 10 psi in 100 ml Wheaton glass bottles sealed with aluminum caps. Potassium sulfite was made in 50 mM EDTA (disodium salt) at 1.0, 0.1 and 0.01 M concentrations. Sulfur dissolved in dimethylsulfoxide (DMSO)(15.8 mM) was also used.

Oxidation rates were measured using a Gilson oxygraph equipped with a Clarke oxygen electrode. The 1.2 ml assay chamber was maintained at 25° C.

Reactions were started by the addition of microliter volumes of substrate to mixtures containing buffer and cells (1 mg wet cells). Oxidation rates are expressed as ηmoles O_2 consumed per minute.

RESULTS

Adhesion Assay. *T. thiooxidans* cells attached to powdered sulfur in high proportions at low phosphate buffer concentrations, but increasing concentration of potassium phosphate inhibited the adhesion progressively (Table 1). When the adhesion data are plotted against the logarithm of potassium phosphate concentration, the inhibition plot becomes linear (Fig. 1). It levels off at a high concentration of 100 mM or so.

Since powdered sulfur is difficult to wet, a wetting agent or detergent, Tween 80, is normally used to make a suspension of sulfur. When such a sulfur suspension was tested a similar inhibition of cell adhesion to sulfur by increasing concentration of potassium phosphate was observed (Fig. 1). The extent of inhibition was sometimes affected by the batch of cells. The inhibitory effect of potassium phosphate in Table 2 (with Tween 80) was stronger than the experiments in Figure 1 although the semilog plot was still linear. Ammonium sulfate was also inhibitory, but at 50 or 100 mM less inhibitory than potassium phosphate (Table 2).

When the concentration of cells was increased at a fixed amount of sulfur, the cell adhesion percentage decreased as shown in Table 3, but the inhibitory effect of increasing phosphate concentration was obvious at three different cell concentrations. In 100 mM potassium phosphate the percentage of cells adhered were 33, 15 and 7 with 2.5, 5 and 10 mg cells, respectively. The results indicate that approximately equal numbers of cells were adhered to the surface of 0.4 g of powdered sulfur under these conditions. Table 4 shows the effect of varying amounts of sulfur with a fixed high cell amount of 20 mg. The adhesion percentage was nearly proportional to the

amount of sulfur indicating again a constant number of bacterial cells adhering to a unit weight of sulfur.

The effect of pH on the cell adhesion to sulfur is shown in Fig. 2. Although the percentage adhered was higher around pH 4.5-5.0 and again at pH 7.5, the effect was considered small compared to the large effect caused by potassium phosphate concentration.

As shown in Table 5 an inhibitor of oxidative phosphorylation, 2,4-dinitrophenol (DNP), and a thiol agent iodoacetate had very little, if any effect on the cell adhesion to sulfur in contrast to results by Takakuwa et al. (1979). Table 6 shows further experiments with inhibitors and boiled cells. Two other sulfhydryl binding agents, *p*-chloromercuribenzoate (*p*CMB) and *N*-ethylmaleimide (NEM), as well as DNP had no effect on the adhesion, and boiled cells (100°C for 5 min) adhered less to sulfur than live cells, again in contrast to Takakuwa et al.

Molybdate is a component of sulfite oxidase in *Thiobacillus novellus* (Southerland and Toghrol, 1983) and has been reported to increase the growth yield of *T. thiooxidans* (Takakuwa et al., 1977). Table 7 shows the adhesion experiments comparing the cells grown in the presence of molybdate with those without at pH 2.3 and 4.5 with three different cell concentrations. Molybdate grown cells had similar adhesion values as the control cells.

These results indicate that the adhesion of *T. thiooxidans* cells to sulfur can be almost completely controlled by the concentration of potassium phosphate and can range from a majority of cells adhering to sulfur at very low potassium phosphate

Figure 1. Effect of potassium phosphate concentration on the adhesion of *T. thiooxidans* cells-semilog plots.
Adhesion experiments were carried out as described in Materials and Methods with 5 mg wet cells with 0.32 g (A) or 0.4 g (B) powdered sulfur with (A) and without (B) Tween 80 in 10 ml potassium phosphate buffer of pH 2.3 at various concentrations.
A: with 50 ppm Tween 80
B: without Tween 80 (Table 1 data)

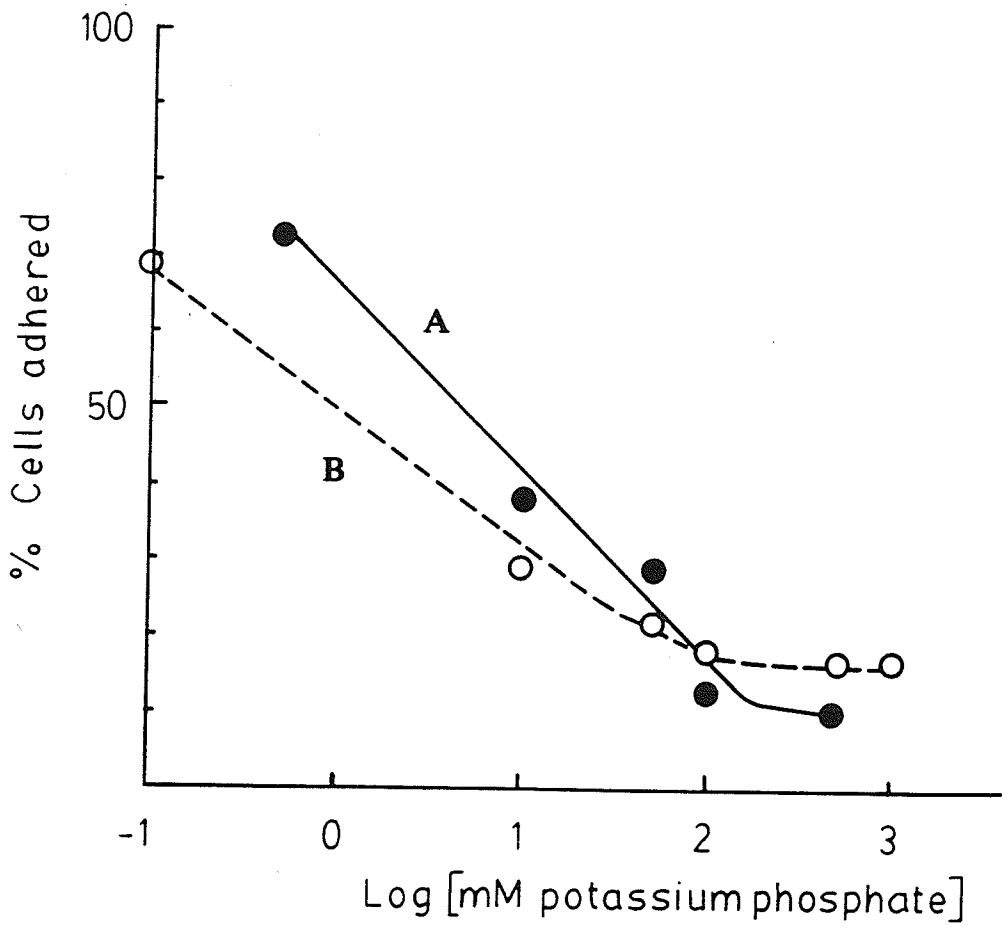

100
50
% Cells adhered
A
B
-1
0
1
2
3
Log [mM potassium phosphate]

Table 1. Adhesion of *T. thiooxidans* to sulfur at different concentration of potassium phosphate at pH 2.3

Potassium phosphate concentration	$A_{660\ nm}$ [a]	% Adhered
control cell suspension	0.192	0
0.1 mM[b]	0.060	69
0.01 M	0.137	29
0.05 M	0.150	22
0.10 M	0.158	18
0.50 M	0.160	17
1.0 M	0.160	17

[a] Adhesion assay was carried out as described in Materials and Methods with 5 mg wet *T. thiooxidans* cells (0.1 ml of 50 mg/ml in 10 mM potassium phosphate at pH 2.3) and 0.4 g powdered sulfur in 10 ml (pH 2.3) potassium phosphate (final concentration in the reaction mixture as shown)
Absorbance of filtered cell suspension was measured at 660 nm to obtain the percentage of cells adhered to sulfur
[b] Potassium phosphate introduced only as 0.1 ml of cell suspension in 10 mM potassium phosphate into 9.9 ml distilled water.

Table 2. Effect of potassium phosphate and ammonium sulfate on *T. thiooxidans* cell adhesion to sulfur in the presence of Tween 80[a]

Suspending medium		% Adhered[b]
Potassium phosphate (mM)	Ammonium sulfate (mM)	
0.25[c]	0	59
10	0	20
50	0	3
100	0	1
0.25	10	13
0.25	50	9
0.25	100	8

[a] Sulfur was added as 0.1 ml of a suspension in 500 ppm Tween 80 (320 g/l)

[b] Adhesion assay was carried out as described in Materials and Methods in 10 ml of pH 2.3 suspending medium as shown.

[c] Fifty microliters of *T. thiooxidans* cell suspension in pH 2.3 potassium phosphate were used in these experiments introducing 0. 25 mM phosphate in 10 ml water

Table 3. Effect of concentration of *T. thiooxidans* cells on adhesion to sulfur

Cells (mg)	Potassium phosphate (mM)	% Adhered[a]
2.5	0.25	84
	50	37
	100	33
	500	32
5.0	0.5	72
	50	24
	100	15
	500	9
10.0	1	21
	50	17
	100	7
	500	0

[a] Adhesion assay was carried out as described in Materials and Methods with 0.4 g sulfur and varied volumes of cell suspension (50 mg wet cells/ml pH 2.3, 50 mM potassium phosphate) in 10 ml pH 2.3 potassium phosphate.

Table 4. Effect of increasing sulfur amounts on adhesion of *T. thiooxidans* cells

Sulfur (g)	Adhesion[a] (%)
0	0
2	13
2	12
4	27
4	24
6	43
6	43

[a] Adhesion assay was carried out as described in Materials and Methods with 20 mg wet cells and amount of sulfur as indicated in 10 ml pH 2.3 potassium phosphate buffer, 50 mM.

Figure 2. Effect of pH on the adhesion of *T. thiooxidans* cells on sulfur. Adhesion assay was carried out as described in Materials and Methods with 5 mg wet *T. thiooxidans* cells with 0.4 g sulfur in 10 ml 50 mM potassium phosphate buffer of varied pH.

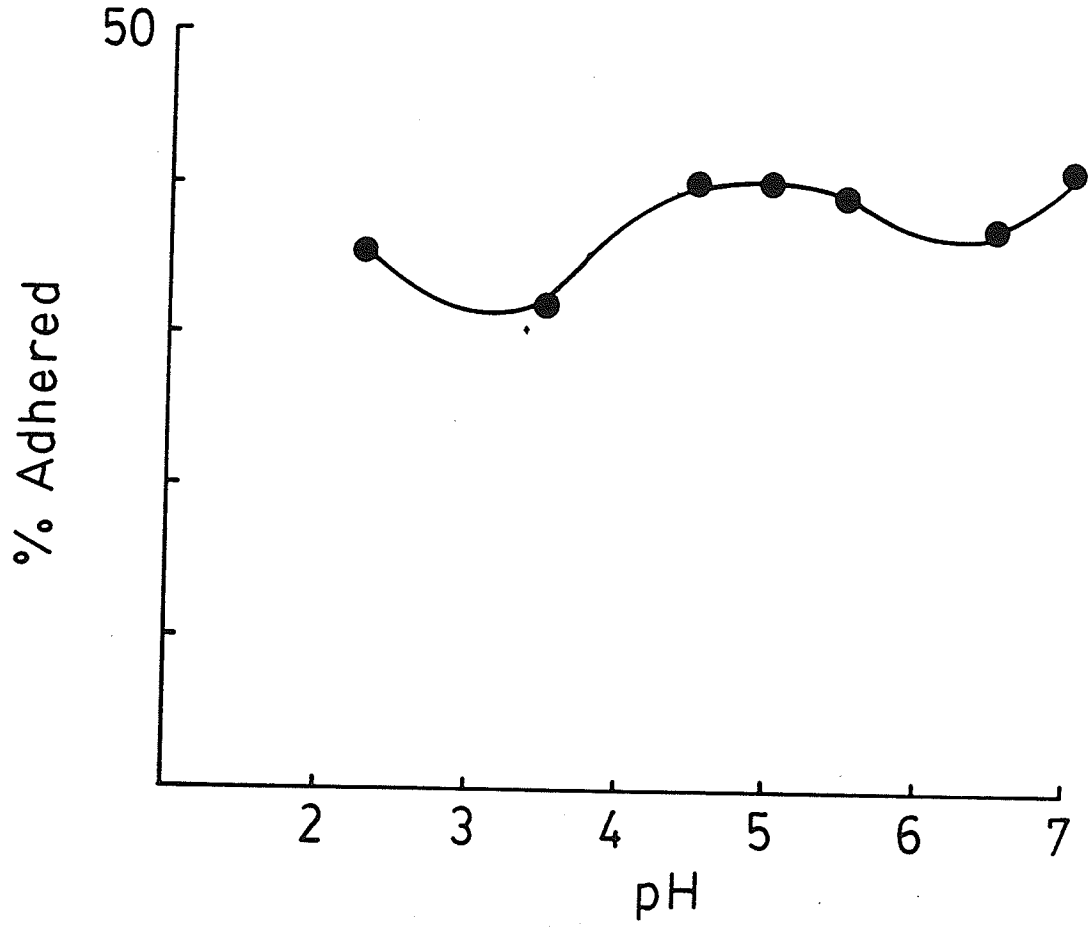

50
% Adhered
2
3
4
5
6
7
pH

Table 5. Effect of inhibitors on the adhesion of *T. thiooxidans* cells to sulfur at various cell and sulfur concentrations

Sulfur (g)	Cells (mg)	% Adhered[a]				
		No additions	DNP		Iodoacetate	
			20 M	11 mM	1 mM	1 mM
0.0	2	0	12		0	12
	4	0	6		3	3
	6	0	6		2	6
0.5	2	65	65	59	65	
	4	55	55	55	48	
	6	47	53	45	49	
1.0	2	71	82	76		59
	4		68	61		61
	6	68	66	62		45

[a] Adhesion assay was carried out as described in Materials and Methods with sulfur and cells as indicated in 50 mM potassium phosphate at pH 2.3 with and without inhibitors. When sulfur was absent the cells were still treated the same way with inhibitors and filtered. Control cell suspensions were not filtered. The loss of cell turbidity measured at 660 nm was due to fluctuations in filtration, not due to adhesion (there was no sulfur).

Table 6. Effect of various conditions on *T. thiooxidans* cell adhesion to sulfur

Potassium phosphate (mM)	Conditions	A_{660nm}	% Adhered[a]
50	Control	0.330	100
50	S° alone	0.173	48
500	S° alone	0.201	39
50	S° + 0.1 mM *p*CMB	0.161	51
50	S° + 0.1 mM DNP	0.172	48
50	S° + 1 mM NEM	0.171	48
50	Boiled cells + S°	0.238	27

[a] Adhesion assay was carried out as described in Materials and Methods with 5 mg wet cells and 0.4 g sulfur in 10 ml pH 2.3 potassium phosphate buffer under various conditions and the absorbance of filtered cell suspension (A_{660}) was compared to the control value. Inhibitors used were *p*-chloromercuribenzoate (*p*CMB), 2,4-dinitrophenol (DNP) and *N*-ethylmaleimide (NEM).

to a small minority of cells adhering to sulfur at high potassium phosphate concentrations.

Hydrophobicity assay. *T. thiooxidans* cell surface hydrophobicity increased with increasing potassium phosphate buffer concentration at pH 2.3 (Table 8). Young cells harvested after 4 days responded more dramatically to the concentration effect than older cells harvested at 8 days when growth went into a stationary phase. All subsequent experiments were carried out with 4 day cells. Ammonium sulfate at a high concentration had a similar effect as potassium phosphate making the cells very hydrophobic (95% of *T. thiooxidans* cells went into the *n*-hexadecane layer).

Ethylene glycol counteracted the effect of high phosphate concentration retaining more cells in the aqueous layer. The growth medium, Starkey No. 1, maintained a hydrophilic nature of cells. Figure 3 shows the result of experiments extending to lower phosphate concentrations.

The data seem to extrapolate to nearly 100% aqueous phase (no extraction by *n*-hexadecane) at zero potassium phosphate concentration. The semilog plot shows a linear relationship between the percentage aqueous observed and the log potassium phosphate concentration (Fig. 3 inset).

Distribution of *T. thiooxidans* cells between aqueous and *n*-hexadecane layers was confirmed by direct microscopic observation (Fig. 4). Droplets of *n*-hexadecane in 0.5 M potassium phosphate contained much more cells than those in 50 mM potassium phosphate.

The effect of pH on the hydrophobicity assay at 50 mM potassium phosphate

Table 7. Effect of molybdate in the growth medium on adhesion of *T. thiooxidans* cells to sulfur

Molybdate[a] (Mo, ppm)	Cells (mg)	Assay pH	% Adhered[b]
0	2	2.3	62
	4	2.3	50
	6	2.3	46
	2	4.5	87
	4	4.5	62
	6	4.5	56
300	2	2.3	62
	4	2.3	50
	6	2.3	39
	2	4.5	69
	4	4.5	52
	6	4.5	60

[a] Cells were grown with and without sodium molybdate.
[b] Adhesion assay was carried out as described in Materials and Methods with 0.5 g sulfur and varied amounts of cells at pH 2.3 and pH 4.5 in 50 mM potassium phosphate.

Figure 3. Hydrophobicity plot of *T. thiooxidans* cells against the concentrations of potassium phosphate buffer, pH 2.3. The log values of the phosphate concentrations were replotted (inset).

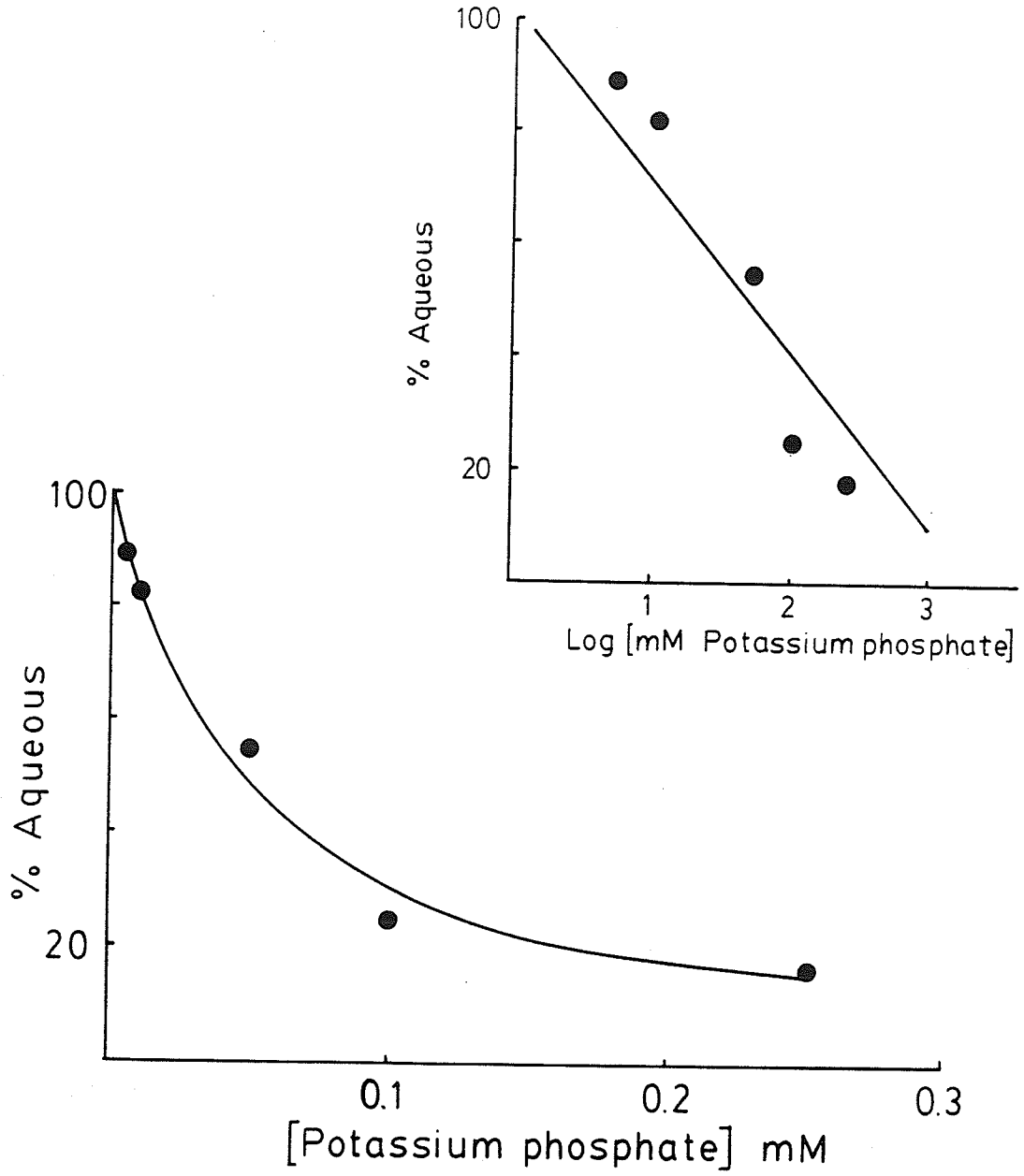
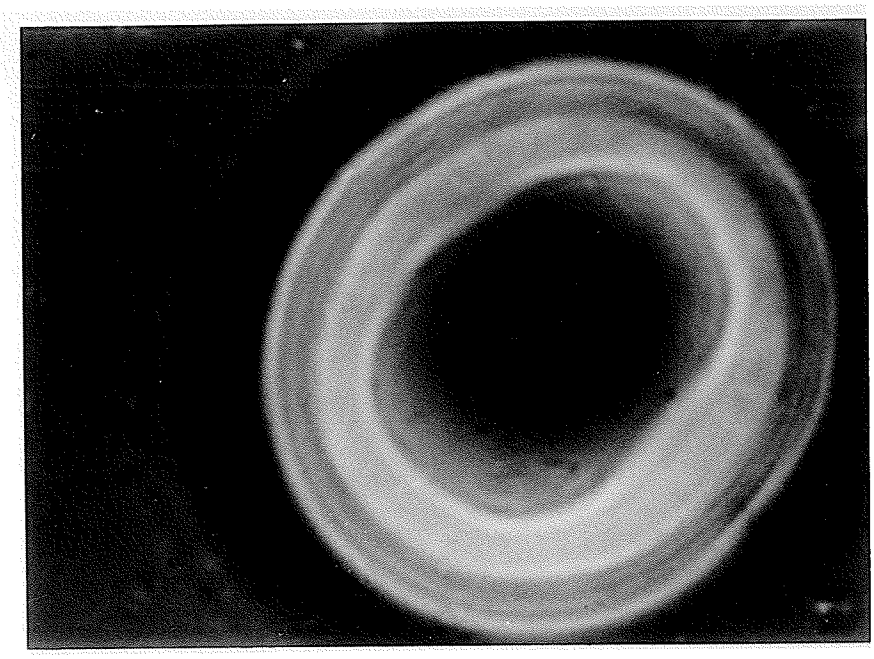

100
20
% Aqueous
1
2
3
Log [mM Potassium phosphate]
100
20
% Aqueous
0.1
0.2
0.3
[Potassium phosphate] mM

Figure 4. Photomicrographs of *T. thiooxidans* cells in n-hexadecane droplets in (a) 50 mM potassium phosphate buffer, pH 2.3 and (b) 0.5 M potassium phosphate buffer, pH 2.3. Hydrophobicity assays were performed as described in Materials and Methods. Magnification and exposure as described in Materials and Methods. Prints are magnified four times.

A.



B.

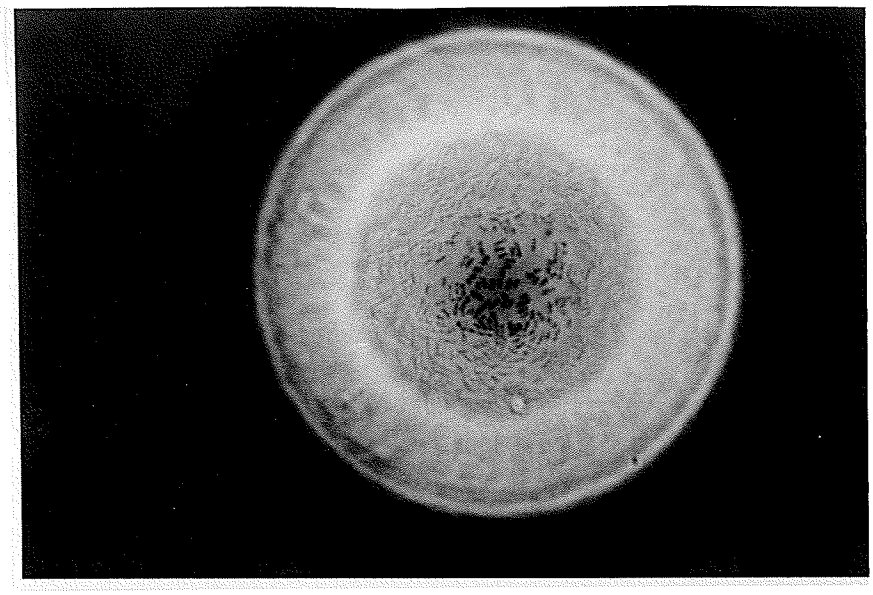


Table 8. Effect of potassium phosphate concentration on the cell surface hydrophobicity of *T. thiooxidans*

Suspending medium	Cells remaining in the aqueous layer[a] (%)	
	4 day cells	8 day cells
Potassium phosphate		
50 mM	78	55
0.1 M	37	39
0.5 M	8	25
1.0 M	8	
0.5 M + ethylene glycol[b]	58	
Ammonium sulfate		
1.0 M	5	
Starkey No. 1 medium	68	

[a] Hydrophobicity was determined as described in Materials and Methods by measuring $A_{660\,nm}$ before and after extraction with *n*-hexadecane. *T. thiooxidans* cells were grown for 4 or 8 days, washed and suspended in pH 2.3 50 mM potassium phosphate buffer (50 mg wet cells/ml). Fifty microliters of the cell suspension (2.5 mg cells) were mixed with 3 ml of the suspending medium for hydrophobicity assay.
[b] Ethylene glycol concentration: 10% (v/v).

is shown in Figure 5. The hydrophobicity was maximal around pH 5.0. High concentrations of potassium phosphate increased hydrophobicity of cells at pH 2.3 and pH 7.5 (Table 9). Metabolic inhibitors *p*CMB, DNP, and NEM did not affect the hydrophobicity of cells appreciably, but boiling of cells increased the hydrophobicity (Table 10). Heat extract of *T. thiooxidans* cells which often increased the sulfur oxidation rate of cells had a dramatic effect in converting cells from high hydrophobicity to low hydrophobicity (high hydrophilicity).

These results indicate that the extent of hydrophobicity of *T. thiooxidans* cells is a direct function of the concentration of potassium phosphate. At low potassium phosphate cells are extremely hydrophilic and only a small number of cells are distributed into the *n*-hexadecane phase, while at high potassium phosphate cells become very hydrophobic and most cells are extracted into the non-aqueous phase.

Oxygen uptake studies. Since high potassium phosphate concentrations inhibit cell adhesion to sulfur and increase hydrophobicity of cells, the effect on the oxidation of sulfur as well as sulfide and sulfite was investigated. Sulfide is oxidized to sulfur first and sulfite is the initial oxidation product of sulfur before its further oxidation to sulfate.

The oxidation of sulfur was inhibited by a high concentration of potassium phosphate in the assay (Figure 6 and Table 11). Increases in the concentration from 50 to 100 mM had little effect but 500 mM potassium phosphate was strongly inhibitory. Cells were normally washed and suspended in 50 mM buffer. When the cells were washed in 0.5 M and 1 M potassium phosphate buffer the sulfur oxidation

Figure 5. Effect of pH of 50 mM potassium phosphate buffer on the cell surface hydrophobicity of *T. thiooxidans*.

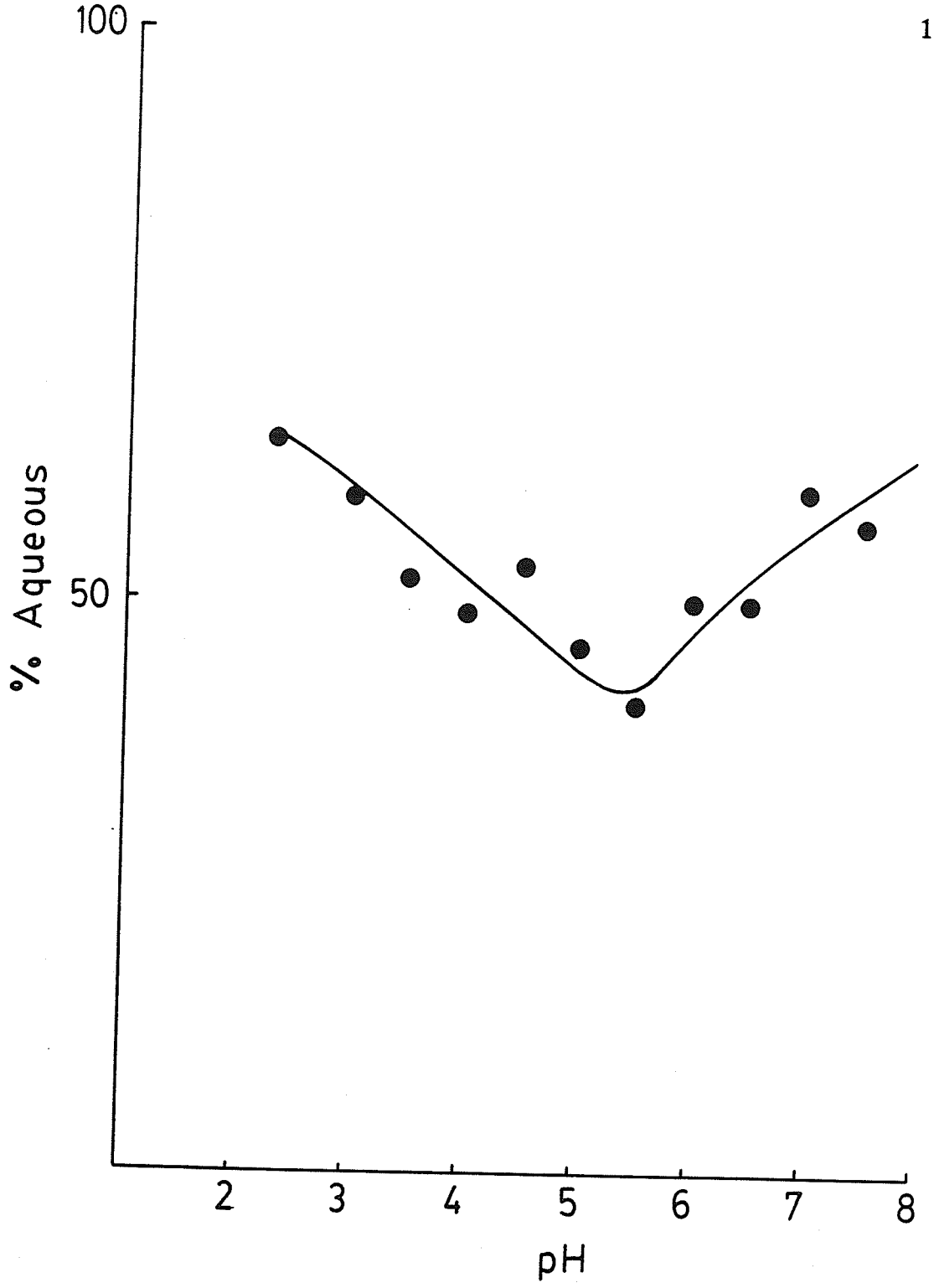

100
50
% Aqueous
2
3
4
5
6
7
8
pH

Table 9. Increase in hydrophobicity by increasing concentration of potassium phosphate at pH 2.3 and 7.5

pH	Potassium phosphate (mM)	% Aqueous phase[a]
2.3	50	41
	100	25
	500	20
7.5	50	52
	100	39
	500	17

[a] Experimental conditions were the same as in Table 8, except the pH of the suspending medium as indicated.

Table 10. Effect of various conditions on cell surface hydrophobicity of *T. thiooxidans*

Expt.	pH	Potassium phosphate (mM)	Conditions	% Aqueous phase[a]
I	2.3	50	Control	61
			+ 0.1 mM *p*CMB	55
			+ 0.1 mM DNP	56
			+ 1 mM NEM	54
			Boiled cells[b]	36
II	2.3	100	Control	18
			+ heat extract[c]	62
	4.5	100	Control	5
			+ heat extract	70

[a] Hydrophobicity was determined as in Table 8. Experiments I and II were separate experiments.
[b] Boiled cells were prepared by heating a cell suspension at 100°C for 5 min.
[c] Heat extract of *T. thiooxidans* cells at 90°C for 10 min in water as described in Materials and Methods. 300 μl used.

activities were similar to the 50 mM washed cells when assayed in 50, 100 or 500 mM buffer. In 500 mM assay buffer all three types of cells were inhibited in sulfur oxidation, but the 500 mM washed cells showed a slightly better activity than the other two (Table 11). Assay results in glass distilled water were unique in that the sulfur oxidation was affected by the concentration of wash buffer. The sulfur oxidation rates of cells washed in 50 mM buffer were progressively inhibited in water with incubation time (Fig. 6, Table 11), but cells washed in 500 or 1,000 mM buffer had little activity (less than 20% of the control cells washed and assayed in 50 mM buffer). Since the sulfur adhesion was maximal at very low potassium phosphate concentrations as shown earlier, the inhibition observed here was probably due to an osmotic shock causing disruption somewhere in the sulfur oxidation system. As shown later in this section sulfite oxidation is inhibited by these treatments and sulfite is a known inhibitor of sulfur oxidation at pH 2.3 (Suzuki et al., 1992). In experiments even with 50 mM potassium phosphate washed cells sulfur oxidation in water often slowed down (Fig. 6) after a few minutes (10-15 min in Table 11) and sulfite accumulation was detected by the pararosaniline method (West and Gaeke, 1956)(results not presented). Heat extract of *T. thiooxidans* cells which reduced the hydrophobicity of cells (Table 10) increased the rate of sulfur oxidation as much as two to three times under similar conditions at either pH 2.3 or 7.5 (the results not presented). The extent of stimulation, however, varied considerably with batches of cells and assay conditions. The oxidation of sulfur dissolved in dimethylsulfoxide (DMSO) was similarly inhibited by a high potassium phosphate concentration of 500

mM at all the pH values studied (Table 12). Interestingly, however, at pH 4.5-6.5 the inhibited cells in 500 mM buffer recovered some oxidation activity progressively with time. A similar time-dependent, progressive recovery of activity in cells was observed at pH 4.5 with powdered sulfur suspension in 500 mM buffer, but not at pH 2.3 (results not presented).

Sulfite oxidation by *T. thiooxidans* cells studied at pH 5.5 was affected more by potassium phosphate concentration of the washing buffer than that of the assay buffer (Table 13, Fig. 7). Washing in high concentrations of potassium phosphate decreased the activity of cells when assayed in 50 mM potassium phosphate (Fig. 7). It could be due to lowering of pH near the sulfite oxidation sites caused by high concentration of pH 2.3 phosphate buffer, opposite of the effect observed in pH 7.5 buffer washing in the first section of this thesis. Lowering of pH will lower the K_m and K_i for sulfite. The initial concentration of 0.5 μmol in 1.2 ml will become inhibitory. Later recovery of activity when sulfite is close to depletion agrees with this interpretation. An alternative interpretation is an osmotic shock from 500 to 50 mM potassium phosphate. A higher acitivity in 100 mM assay buffer may be explained as a smaller degree of osmotic shock (500 → 100 mM). The same result may also be explained by an accelerated recovery of cells from low pH by a higher (100 mM) concentration of pH 5.5 buffer. Progressive decline in rate observed with cells washed in 50 mM buffer or cells assayed in 500 mM buffer simply represents the rate decrease caused by the low initial sulfite concentration below K_m of 0.56 mM at pH 5.5.

Sulfide oxidation was affected by the concentration of potassium phosphate both in washing and assay as shown in Figure 8. When assayed in 50 mM buffer cells oxidized sulfide rapidly in one phase (Fig. 8 curves A and C) with nearly stoichiometric amount of O_2 for sulfide oxidation to sulfate ($H_2S + 2\ O_2 \rightarrow H_2SO_4$). When assayed in 0.5 M buffer the cells washed in 50 mM buffer consumed only 80 nmols or so O_2, then stopped (curve B). The cells washed in 0.5 M buffer, however, recovered after a lag and continued the O_2 consumption (curve D). These results are understandable if sulfide is first oxidized to sulfur, whose further oxidation is inhibited by high potassium phosphate concentration. The cells washed in 0.5 M buffer could recover some sulfur oxidizing activity in 0.5 M assay buffer (Table 11). High concentration of potassium phosphate inhibited not only sulfur oxidation but also the oxidation of sulfide to sulfur in a different batch of cells (Fig. 9). Assay in 0.1 M potassium phosphate (curve A) showed three distinct phases of O_2 consumption roughly corresponding to ($H_2S + 1/2\ O_2 \rightarrow S + H_2O$), ($S + O_2 + H_2O \rightarrow H_2SO_3$) and ($H_2SO_3 + 1/2\ O_2 \rightarrow H_2SO_4$). In 1 M potassium phosphate the initial phase was already inhibited to a considerable degree (curve B). Ammonium sulfate at 1 M inhibited the sulfide oxidation even more strongly (curve C). The same batch of cells used in Figure 9, when washed in water adjusted to pH 3 or 1.8 with H_2SO_4, oxidized sulfide rapidly in one phase in 0.1 M potassium phosphate, pH 2.3. Sulfide oxidation was still strongly inhibited (over 70%) in 1 M potassium phosphate with these cells (Table 14, Experiment II) similar to the inhibition (70%) shown in Figure 9 and Table 14 (last entry). The experiments in Figure 8 showed an inhibition only

50% or so (Table 14, Experiment I).

Figure 6. Sulfur oxidation by *T. thiooxidans* cells at different potassium phosphate concentrations. Assays were performed in (A) 50 mM potassium phosphate, pH 2.3, (B) glass distilled water, and (C) 0.5 M potassium phosphate buffer, pH 2.3. Cells (1 mg) were washed (50 mM potassium phosphate buffer, pH 2.3) and assayed as described in Materials and Methods with sulfur suspension (0.1 ml, 32 mg sulfur) as substrate.

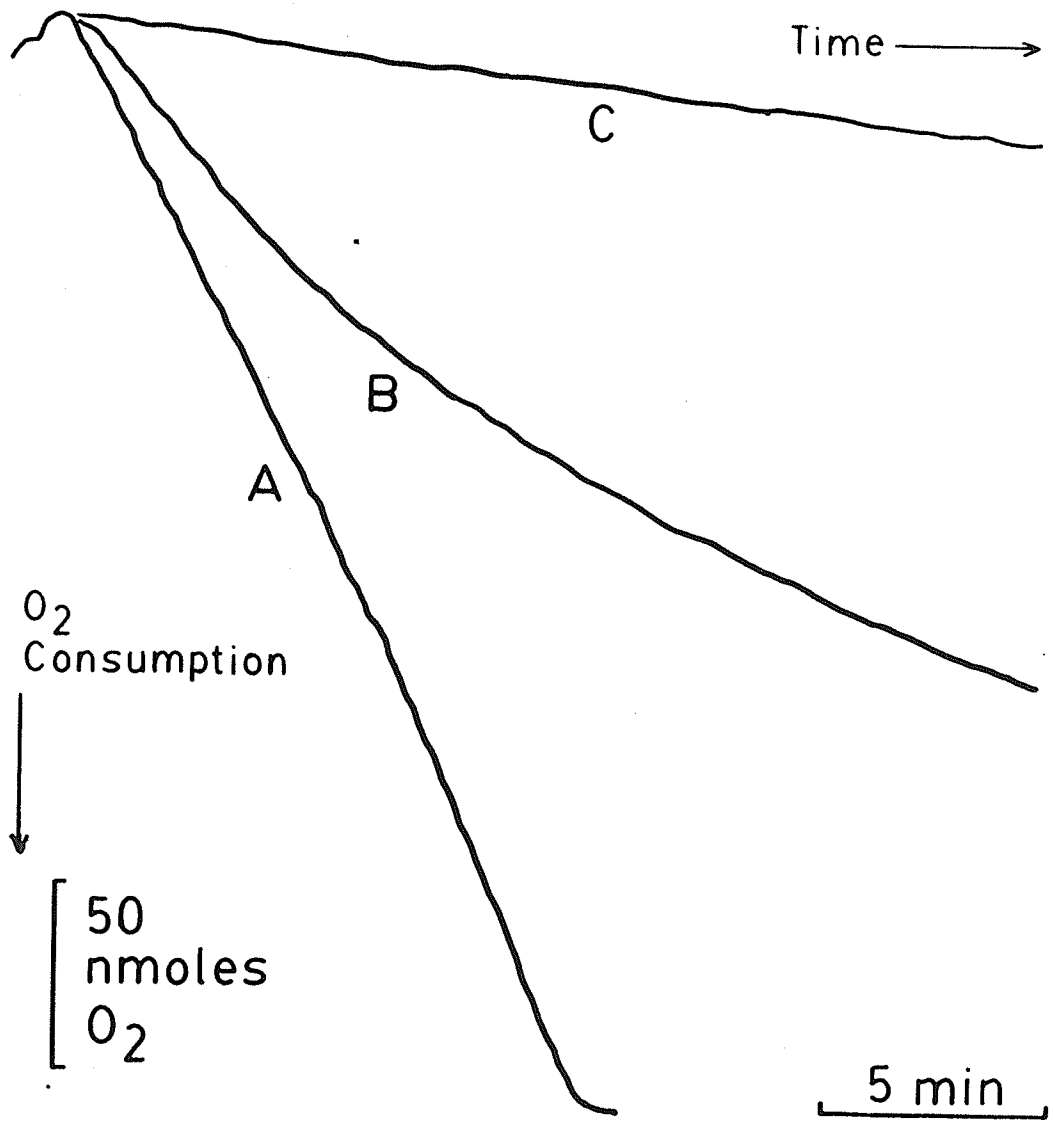

Time
C
B
A
O_2
Consumption
50
nmoles
O_2
5 min

Figure 7. Sulfite oxidation by *T. thiooxidans* cells washed in different concentrations of potassium phosphate at pH 2.3 (A) 50 mM, (B) 0.5 M and (C) 1 M. Sulfite oxidation was followed as described in Materials and Methods in 50 mM pH 5.5 potassium phosphate buffer with 1 mg cells and 0.5 μmol K_2SO_3.

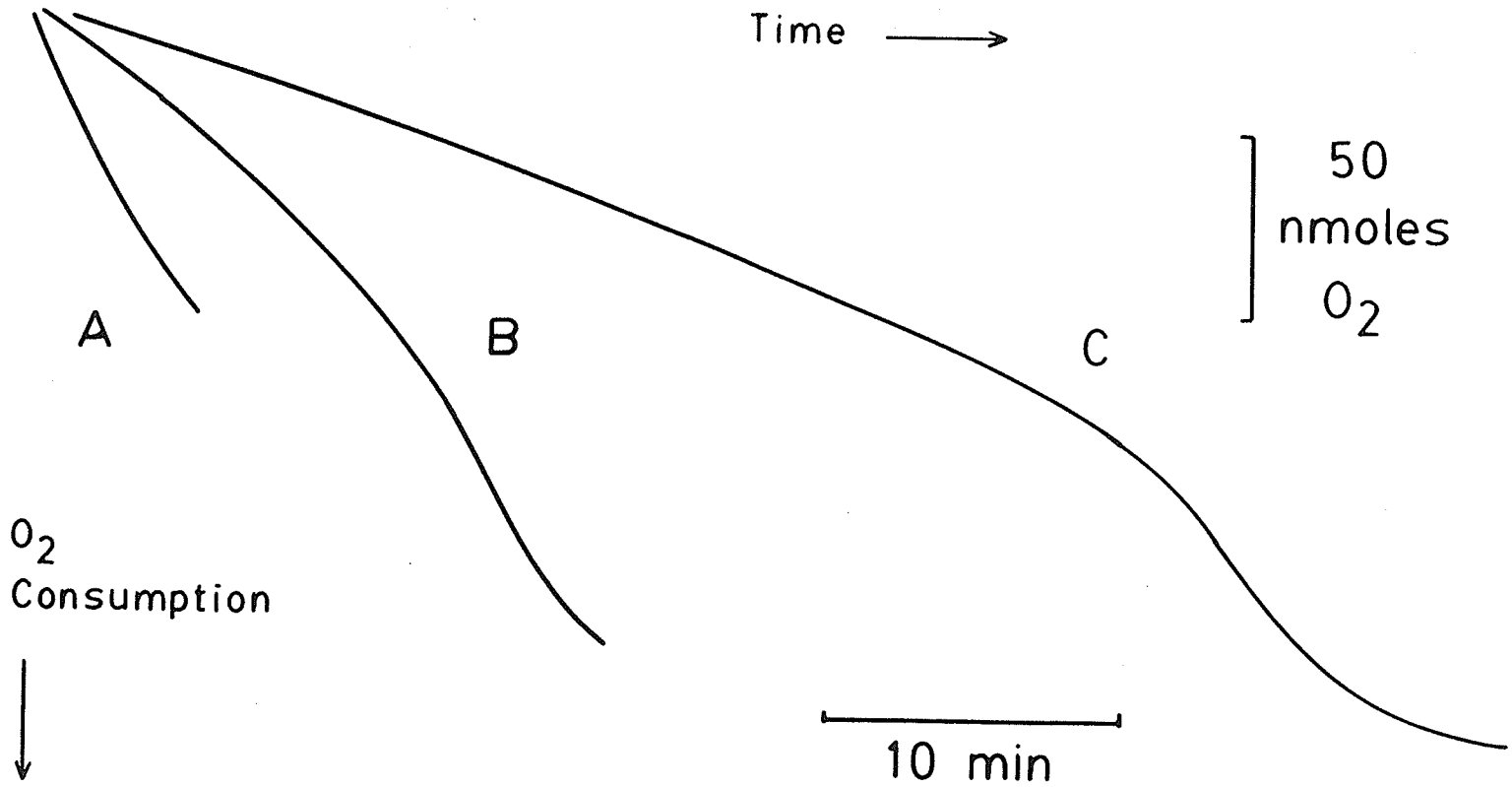

Time
50
nmoles
O_2
A
B
C
O_2
Consumption
10 min

Figure 8. Effect of potassium phosphate concentration on sulfide oxidation by *T. thiooxidans* in washing and assay. Sulfide oxidation was followed as described in Materials and Methods with 7.5 μl of 20 mM sodium sulfide to start the reaction with 1 mg wet cells. Cells were washed in pH 2.3 50 mM (A andB) or 500 mM (C and D) potassium phosphate and assayed in 50 mM (A and C) or 500 mM (B and D) potassium phosphate at pH 2.3.

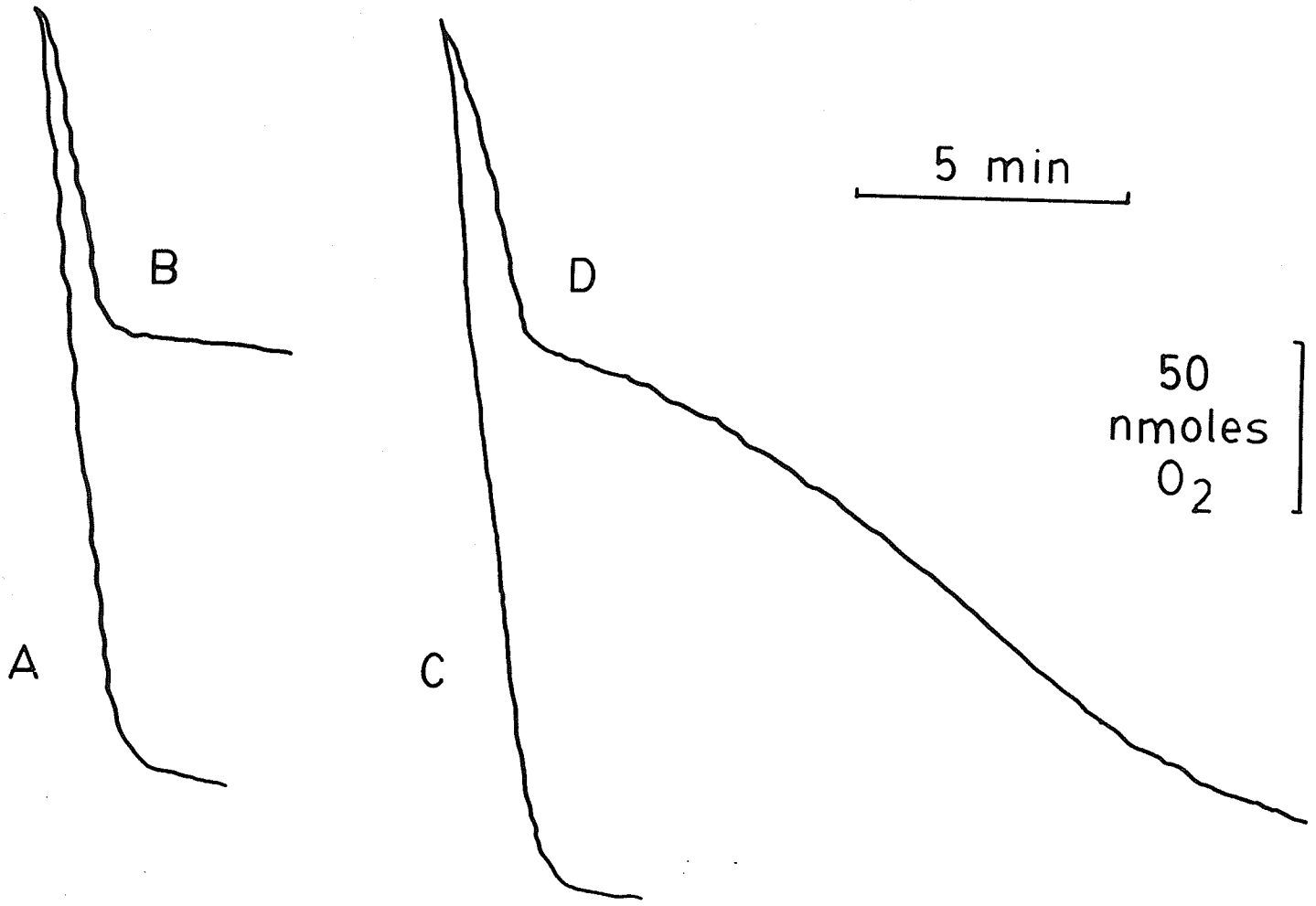

A
B
C
D
5 min
50
nmoles
O_2

Figure 9. Effect of high concentration of potassium phosphate and ammonium sulfate in sulfide oxidation by *T. thiooxidans*. Sulfide oxidation was followed as described in Materials and Methods and Fig. 8. Cells were washed in 50 mM potassium phosphate, pH 2.3.
A: assay in 100 mM potassium phosphate, pH 2.3
B: assay in 1 M potassium phosphate, pH 2.3
C: assay in 1 M ammonium sulfate, pH 2.3 with 10 N H_2SO_4.

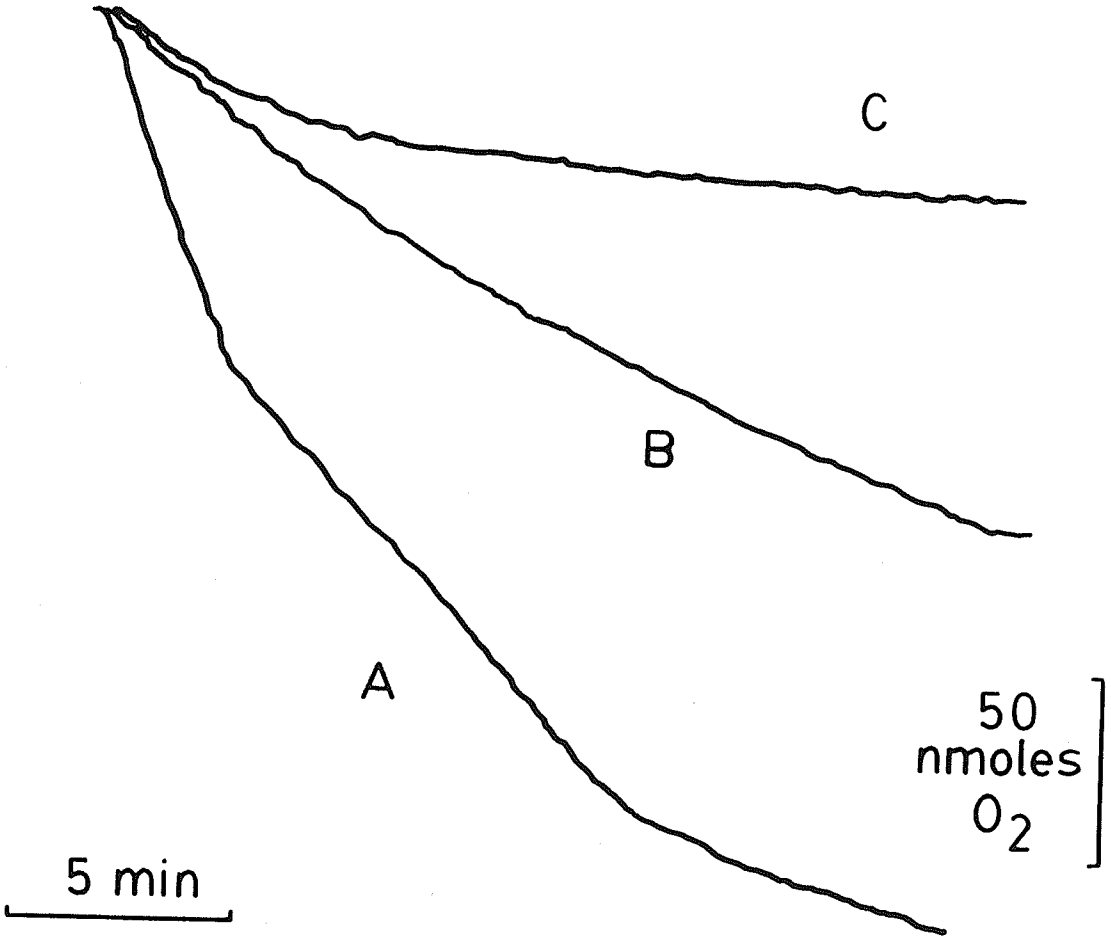

C
B
A
50
nmoles
O_2
5 min

Table 11. Effect of potassium phosphate concentration on powdered sulfur oxidation by *T. thiooxidans*

Potassium phosphate pH 2.3 (mM)		nmoles O_2 consumed at				Rate[a] (nmoles O_2/min)
Assay buffer	Wash buffer	2.5 min	5 min	10 min	15 min	
50	50	44	104	228	>290	28
	500	47	95	214	>290	25
	1,000	50	111	240	>290	27
100	50	44	104	228	>290	28
	500	33	88	190	>290	22
	1,000	40	96	209	>290	23
500	50	2	5	12	18	2
	500	2	6	18	35	8
	1,000	2	5	11	18	4
water	50	24	55	102	134	14
	500	7	20	38	52	5
	1,000	5	17	36		5

[a] Sulfur oxidation was assayed as described in Materials and Methods with 1 mg cells washed and suspended in buffer indicated (50 mg/ml) and assayed in the buffer shown with 0.1 ml sulfur (32 mg) suspension. O_2 consumption was recorded as shown at time intervals and the maximal rate observed on the chart paper before the complete depletion of O_2 (290 nmoles O_2 consumption) is shown as rate.

Table 12. Effect of potassium phosphate concentration on the oxidation of sulfur dissolved in DMSO by *T. thiooxidans*

Potassium phosphate		nmoles O_2 consumed					Rate[a] (nmol O_2/min)
Assay pH	(mM)	2.5 min	5 min	10 min	15 min	total	
2.3	50	60	131	142		142	32
	100	59	129	140		140	30
	500	1	3	6	10		1
4.5	100	41	97	142		142	24
	500	1	4	14	41	119	10
5.5	100	41	100	141		141	25
	500	1	3	14	36	105	8
6.5	100	51	120	132		132	27
	500	2	4	13	28	104	6
7.5	100	36	98	118		118	24
	500	3	6	14	23	70	2

[a] Oxidation was studied as described in Materials and Methods with 1 mg cells (washed and suspended in pH 2.3 50 mM potassium phosphate) in the assay buffer as indicated. The reaction was initiated by the injection of 7.5μl of sulfur in DMSO (100 nmol sulfur). The rate was the maximal rate observed.

Table 13. Effect of potassium phosphate concentration for washing *T. thiooxidans* cells and in sulfite oxidation assay

Potassium phosphate (mM)		nmoles O_2 consumed					Rate[a] (nmol O_2/min)
Assay buffer pH 5.5	Wash buffer pH 2.3	2.5 min	5 min	10 min	15 min	total	
50	50	37	73				16↓
	500	9	25	62	116	166	15
	1,000	2	8	22	38	190	10
100	50	25	54				13↓
	500	28	74	150	152	152	20
	1,000	6	19	53	100	166	16
500	50	20	49	90			12↓
	500	13	50	95			12↓
	1,000	14	46	89			12↓

[a] Sulfite oxidation was followed as described in Materials and Methods in pH 5.5 potassium phosphate with 1 mg cells (washed and suspended in pH 2.3 potassium phosphate). The reaction was initiated by injection of 5 μl of 0.1 M K_2SO_3. Rate was the maximal rate observed and "↓" indicates a progressive decline in rate.

Table 14. Effect of pH and buffer concentration on sulfide oxidation by *T. thiooxidans*

Expt.[a]	Washing[b]		Sulfide oxidation rate[c] (nmole O_2/min) in				
			Potassium phosphate				Ammonium sulfate
	pH	Buffer	50 mM	0.1 M	0.5 M	1 M	1 M
I	2.3	50 mM K-P*i*	105		66		
	2.3	0.5 M K-P*i*	105		52		
II	1.8	H_2O		160		43	40
	3.0	H_2O		108		27	31
	2.3	50 mM K-P_i		30		9	8

[a] Experiment I or II was with different batch of cells.
[b] Washing of cells was with potassium phosphate at 2.3 or with water acidified with H_2SO_4.
[c] Sulfide oxidation rate was the maximal rate observed (the initial fast phase). The assay was carried out as described in Materials and Methods with 1 mg cells and 150 nmol sodium sulfide in the pH 2.3 assay buffer of various concentrations as indicated.

DISCUSSION

The experimental results presented in this thesis cannot be explained by simple hydrophobicity-based adhesion of *T. thiooxidans* cells to sulfur for oxidation. In fact high concentrations of potassium phosphate which increased the hydrophobicity of cells inhibited the adhesion of cells to sulfur and the oxidation of sulfur by cells. Thus the cell adhesion mechanism operating here is obviously different from the standard hydrophobic interaction and D.L.V.O. theory used to explain microbial cell attachment (Doyle and Rosenberg, 1990).

In recent years, however, some reports have appeared on microbial adhesion in natural environments which do not follow the classical D.L.V.O. theory. Gordon and Millero (1984) using a common marine bacterium, *Vibrio alginolyticus*, showed that the D.L.V.O. theory was followed only at low electrolyte concentrations. Thus the adhesion increased with increasing salt concentrations below 0.1 M, but decreased with a further increase in salt concentration above 0.1 M, indicating the operation of a different mechanism. Their attachment affinity data against salt concentration plot above 0.1 M, when replotted against the logarithm of salt concentration, became linear as our sulfur adhesion plot or hydrophobicity plot. Zita and Hermannson (1994) confirmed the non-applicability of D.L.V.O. theory above an ionic strength of 0.1 in the bacterial adhesion to flocs in a wastewater activated sludge system.

Hydrophobicity was also considered in the study of *T. ferrooxidans* cell attachment to pyrite and other mineral surfaces. Ohmura et al. (1993) studied the

adhesion of *Escherichia coli* and *T. ferrooxidans* to pyrite, quartz, chalcopyrite and galena and concluded that *E. coli* adhered to mineral surface by hydrophobic interaction, but *T. ferrooxidans* selectively adhered to iron-containing minerals and when this selective adhesion was inhibited by ferrous ion the adhesion was controlled by hydrophilic interactions. Devasia et al. (1993) and Blake et al. (1994) studied the electrophoretic mobilities of *T. ferrooxidans* cells, sulfur and pyrite. Interestingly, *T. ferrooxidans* cells grown on ferrous ion, were more negatively charged (more negative zeta potential) at pH 2-3 than sulfur or pyrite grown cells and also sulfur or pyrite changed its electrophoretic mobility and zeta potential upon association with *T. ferrooxidans* cells. These results suggested to Blake et al. (1994) that the complex system cannot be explained by the simple D.L.V.O. theory since cells are not non-permeable objects of uniform surface topography and the outer layers are more like gel where various ionogenic groups are distributed at different levels, all contributing to the total surface charge.

Another interesting recent development is an attempt to interpret some of the salt or ionic effects in biological systems in terms of osmotic stress and decreased water activity (Rand, 1992). Colombo et al. (1992) showed that the oxygen binding to hemoglobin was affected by osmotic pressure caused by neutral solutes such as sucrose indicating the significance of chemical potential of water in the oxygen binding. Apparently 60 water molecules are bound to hemoglobin with 4 oxygen molecules in the oxygenation reaction. Douzou (1994) demonstrated in the familiar 70S ribosome formation from 30S and 50S subunits in the presence of Mg^{2+}, ethylene

glycol (35%) helps the association while monovalent cations such as Na^+, K^+ or NH_4^+ inhibits. The effect of ethylene glycol cannot be accounted for by electrostatic interactions. He considers ribosomes as a biopolymer gel influenced by osmotic pressure and water activity. A similar explanation may be possible for *T. thiooxidans* cells. Hydrophobicity of cells (Fig. 3) and adhesion of cells to sulfur (Fig. 1) both showed linear relationship to the logarithm of potassium phosphate concentration. These results agree with the increased osmotic pressure or decreased water activity increasing hydrophobicity of cells and decreasing the cell adhesion to sulfur. Ethylene glycol's counteracting effect in hydrophobicity increase in this study is similar to Douzou's ribosome work (1994), although it was not tested in the sulfur adhesion test because of possible solubility problem of sulfur. It is interesting to note that ethylene glycol is listed as an inhibitor of hydrophobic adhesion of a bacterium (Doyle and Rosenberg, 1990).

In a recent paper on the growth and sulfur oxidation in a batch reactor culture of *T. ferrooxidans* (Konishi et al., 1994) *T. ferrooxidans* cells adhered to sulfur following the Langmuir isotherm, i.e., the number of cells adsorbed increased linearly with increasing concentration of free bacterial cells at low cell concentrations, but levelled off to the maximum adsorption capacity at high concentrations, identical to the Michaelis-Menten saturation curve of enzyme kinetics. During growth on sulfur the concentration of free bacteria increased linearly with time, but the concentration of adsorbed cells gradually reached the limiting value, the maximum adsorption capacity. Both sulfur oxidation and growth were related to the number of cells

adsorbed and free cell concentration was related to those only through the adsorption equilibrium governed by the Langmuir isotherm. Thus the paper confirms the importance of cell adhesion to sulfur as the initial step in sulfur oxidation and growth in a theoretical and quantifiable manner.

A high concentration of potassium phosphate inhibited sulfur oxidation (Fig.6 and Tables 11 and 12) as expected from the hydrophobicity and cell adhesion studies. The results were complicated at very low phosphate concentrations because of indirect effect of accumulated sulfite on the rate of sulfur oxidation. It is interesting that sulfur dissolved in DMSO was oxidized by *T. thiooxidans* cells with the same response to the buffer inhibition as powdered sulfur. When the sulfur solution is injected into the aqueous system sulfur comes out as colloidal milky suspension, but apparently cells attack this sulfur the same way as the powdered sulfur suspension, i.e., through hydrophilic interactions. The effect of *T. thiooxidans* heat extract on cell hydrophobicity and sulfur oxidation suggest a possibility that *T. thiooxidans* cells have, on the surface, materials which can be extracted with heat and which can make cell surface hydrophilic. Obviously more work is required, but lipopolysaccharide is a possible candidate. If the material can act as a detergent, presumably hydrophobic sulfur can be made more hydrophilic and it is possible that hydrophilic interaction becomes the limiting step.

Sulfite oxidation by *T. thiooxidans* is strongly influenced by the pH of washing buffer as demonstrated in the section 1 of this thesis. Now the concentration of washing buffer has been shown to have a strong effect also (Fig. 7 and Table 13).

It is easy to understand that the sulfite oxidation system of *T. thiooxidans* is affected by the pH of the local micro-environment of gel-like membrane. Washing at pH 2.3 in high buffer concentrations could lower the local pH inhibiting the oxidation of sulfite (Table 13). Unlike sulfur oxidation, however, the assay buffer concentration did not much affect the oxidation of sulfite.

Oxidation of sulfide was inhibited by high potassium phosphate concentration in the assay medium primarily after the initial rapid oxidation of sulfide to sulfur (Fig. 8). With some cells this first phase was also inhibited by high concentrations of salts (Fig. 9). The oxidation of sulfide to sulfur should involve the electron transport system in the cell membrane which may be affected by the high osmotic stress. A partial slow recovery of oxidation by 0.5 M (but not 50 mM) buffer washed cells assayed in 0.5 M potassium phosphate (Fig. 8) may indicate that cells can readjust partially to the osmotic stress during long (one hour) washing process. A similar recovery, although less pronounced, was recorded also with sulfur as substrate (Table 11).

The study of oxidation of these sulfur compounds and the effect of potassium phosphate concentration are complex. The oxidation of elemental sulfur, a solid substrate, is clearly influenced by hydrophobicity or hydrophilicity of cells and adhesion of cells to sulfur. The oxidation of soluble substrates, sulfite and sulfide, is affected by the local pH or perhaps osmotic stress. Sulfide oxidation is further affected by the inhibition of sulfur oxidation and both sulfur and sulfide oxidations are affected by the inhibition of sulfite oxidation. Clearly more future work is

needed to resolve these complex relationships.

CONCLUSIONS

This work showed that the pH and concentration of potassium phosphate buffer used for cell washing and assay are important factors which affect the oxidation of externally added sulfite and sulfur. The oxidation rate of externally added potassium sulfite was a function of substrate concentration and pH. At a fixed pH, the rate of oxidation increased with increasing substrate concentration following normal Michaelis-Menten kinetics. An increase in the pH of potassium phosphate produced an increase in K_m for potassium sulfite but a narrow range of values for sulfurous acid (0.11 to 0.24 μM). An increase in values for V_{max} and K_m was shown for cells washed in 50 mM potassium phosphate, pH 7.5 as compared to cells washed in 50 mM potassium phosphate, pH 2.3. This may be the result of increased pH at localized areas of the cell which decreased the local concentration of sulfurous acid. All of the strains tested showed similar oxidation kinetics for externally added sulfite with increasing substrate inhibition at decreased pH values. Thus, the pH of the assay medium is an important factor in the oxidation of externally added potassium sulfite.

The concentration of the washing and assay buffers affected the adhesion to sulfur and the rate of sulfur oxidation. Cells washed in 50 mM potassium phosphate buffer showed decreased numbers of cells adhering to sulfur when assayed in higher concentrations of potassium phosphate buffer. A similar effect was shown when potassium phosphate was replaced by ammonium sulfate. This is probably the result

of changes to the gel-like outer surface of the cell wall. The cell surface hydrophobicity of cells washed in 50 mM potassium phosphate increased with increasing potassium phosphate concentrations. In turn, these changes to the cell affected the rate of sulfur oxidation. Sulfur oxidation of cells washed in 50 mM potassium phosphate buffer were strongly inhibited when assayed in 500 mM potassium phosphate buffer as compared to 50 mM or 100 mM potassium phosphate.

The chemical properties of the cell surface produced by washing and assay conditions affected the ability of *T. thiooxidans* cells to attach to and oxidize particulate sulfur. The information gained by this study may be considered in future studies on the oxidation of reduced sulfur compounds.

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