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Effect of pH on Sulfite Oxidation by *Thiobacillus thiooxidans* Cells with Sulfurous Acid or Sulfur Dioxide as a Possible Substrate

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The oxidation of sulfite by *Thiobacillus thiooxidans* was studied at various pH values with changing concentrations of potassium sulfite. The optimal pH for sulfite oxidation by cells was a function of sulfite concentrations, rising with increasing substrate concentrations, while that by the cell extracts was unaffected. The sulfite oxidation by cells was inhibited at high sulfite concentrations, particularly at low pH values. The results from kinetic studies show that the fully protonated form of sulfite, sulfurous acid or sulfur dioxide, is the form which penetrates the cells for the oxidation.

Thiobacilli oxidize inorganic sulfur compounds to sulfuric acid to obtain energy for growth. Sulfite is considered the key intermediate in the oxidation (8, 20) since it is the product of sulfur-oxidizing enzyme systems (17, 23) and the substrate for the sulfite-oxidizing enzyme systems (1, 4, 14, 16). Sulfite in fact accumulates during the oxidation of thiosulfate by *Thiobacillus novellus* cells under certain conditions (5) and of sulfur by *Thiobacillus ferrooxidans* when the pH is raised (15, 18). Sulfur is nearly quantitatively oxidized to sulfite by *Thiobacillus thiooxidans* cells when the oxidation of sulfite is inhibited by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (21).

Sulfite oxidation by thiobacilli is easily demonstrated in neutrophilic thiosulfate-grown *T. novellus* cells (3), but is difficult to show in acidophilic thiobacilli, e.g., *T. thiooxidans* (9, 11, 21) and *Thiobacillus acidophilus* (15), without raising the pH above the acid growth pH. An exception is *T. ferrooxidans*, which has a unique sulfite:Fe^{3+} oxidoreductase system coupled to the Fe^{2+} oxidation system (16) and whose cells oxidize sulfite under acidic growth conditions (15–18). Furthermore, sulfite inhibits the oxidation of sulfur by *T. thiooxidans* cells only under acidic conditions (21).

Sulfite ion (SO_3^{2-}) can be protonated to bisulfite ion (SO_3^{2-} plus $H^+ \leftrightarrow HSO_3^-$, $K_{a_2} = 1.02 \times 10^{-7}$ or $pK_{a_2} = 7.0$) and further to sulfurous acid (HSO_3^- plus $H^+ \leftrightarrow H_2SO_3$, $K_{a_1} = 1.54 \times 10^{-2}$ or $pK_{a_1} = 1.81$). We have studied the oxidation of varied concentrations of potassium sulfite by *T. thiooxidans* cells at different pH values to determine which form of sulfite is used by the cells for oxidation. Our results show that the K_m values for total sulfite (SO_3^{2-}, HSO_3^-, H_2SO_3) concentrations increase with increasing pH, but the K_m values calculated for sulfurous acid (H_2SO_3) remain relatively unchanged while the maximal rate of oxidation (V_{max}) is not affected by the assay pH. These results suggest a possibility that the fully protonated sulfurous acid may be the actual substrate used by the cells. Since the cell-free sulfite-oxidizing system has an optimal pH around 7 in agreement with the reported value (9, 10) and K_m values for total sulfite are largely unaffected by pH, it is concluded that either sulfurous acid or sulfur dioxide ($H_2SO_3 \leftrightarrow SO_2$ plus H_2O) may be the only form which penetrates the cells but once inside loses the preference.

Sulfite oxidation by *T. thiooxidans* cells is inhibited by high total sulfite concentrations at acidic conditions probably because the lower pH increases the sulfurous acid concentration beyond the substrate inhibition level. Thus, the optimal pH for sulfite oxidation by *T. thiooxidans* increases with increasing total sulfite concentration. The reported optimal pH values are higher than growth pH simply because it is technically impossible to measure the oxidation of sulfite at very low noninhibitory sulfite concentrations required at acidic growth pH.

Sulfite oxidation assays. *T. thiooxidans* (ATCC 8085) was grown stationary for 4 days at 28°C in Starkey's medium 1 adjusted to pH 2.3 with H_2SO_4 with elemental sulfur spread on the surface as described previously (19, 24).

Cells were filtered through Whatman 1 paper under suction to remove sulfur and collected by centrifugation at 10,000 × *g* for 10 min. 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 weight) of cells per ml for storage at 4°C. The washing processes took around 1 h at 4°C. The cells were used normally on the same day for experiments.

Cell extracts were prepared by breaking the cells with passage through an Aminco French pressure cell (three times) at a pressure of 20,000 lb/in^2 (138 MPa) of a cell suspension (200 mg [wet weight] of 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 min with gentle stirring and then with trypsin inhibitor for 5 min (1.5 μg/mg [wet weight] of cells for either bovine pancreatic trypsin or soybean trypsin inhibitor [Sigma]). The trypsin-treated cells (2) were ruptured more easily, but untreated cells also produced extracts with similar sulfite oxidation properties. The cell extracts were obtained as supernatants by centrifugation of the broken cell suspension at 10,000 × *g* for 10 min.

Sulfite oxidation was monitored in a Gilson oxygraph with a Clark oxygen electrode at 25°C, and the initial linear rate of O_2 consumption (nanomoles per minute) was used as the oxidation rate. The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH as specified), 1 mg [wet weight] of cells (20 μl of 50 mg/ml) or 50 μl of cell extracts (23 mg of protein per ml, determined with bovine serum albumin as standard according to reference 13), 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

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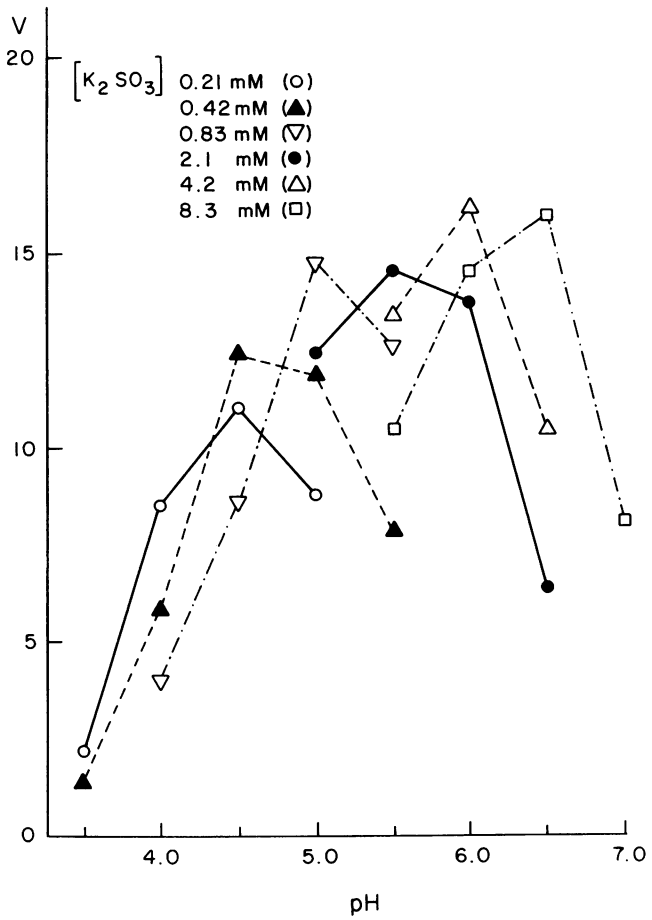

FIG. 1. Effect of potassium sulfite concentration on the optimal pH for sulfite oxidation by *T. thiooxidans* cells. The rate of O_2 consumption (v = nanomoles of O_2 per minute) was determined as described in the text with 1 mg (wet weight) of cells washed at pH 4.7 in 1.2-ml reaction mixtures with various pH values.

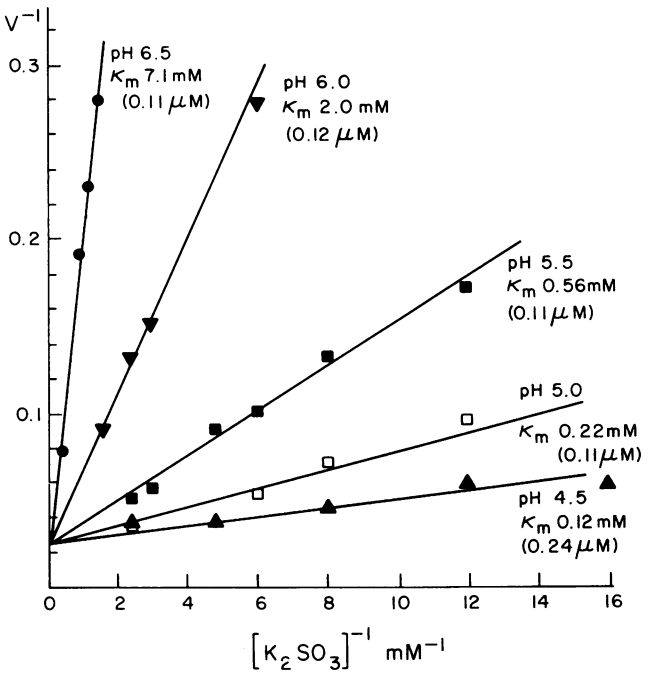

FIG. 2. Effect of pH on the double reciprocal plots of sulfite oxidation rates and sulfite concentrations. *T. thiooxidans* cells (1 mg [wet weight] of cells) washed at pH 2.3 were used to determine the rate at different pHs and sulfite concentrations as described for Fig. 1. K_m values for $[K_2SO_3]$ are shown for different pH experiments with the values calculated for $[H_2SO_3]$ in parentheses.

M K_2SO_3 solutions prepared in 50 mM disodium EDTA. Nonbiological oxidation of sulfite was insignificant under the conditions used in this study, but became considerable at sulfite concentrations higher than 10 mM.

Optimal pH for sulfite oxidation. *T. thiooxidans* cells oxidized sulfite at various pH values, but the pH optimum was a function of potassium sulfite concentration used as substrate as shown in Fig. 1. The optimal pH was low at low sulfite concentrations, but increased with increasing sulfite concentrations (nearly 2 pH units when the sulfite concentration was raised 40-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. 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-} plus one-half $O_2 \rightarrow SO_4^{2-}$ in O_2 consumption, but the rate of oxidation was a function of both substrate concentration and pH as shown in Fig. 2. At a fixed pH, the rate (v) increased with increasing substrate concentration [S] following normal Michaelis-Menten kinetics giving a linear Lineweaver-Burk plot (12), except at high inhibitory concentrations of substrate (data not shown in Fig. 2). The K_m values for potassium sulfite added as substrate increased sharply when the pH of the reaction mixture was increased as shown in Fig. 2 (as much as 60-fold when the pH was raised from 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 pK_{a_1} value of 1.81 and pK_{a_2} value of 7.0 at various pHs of the reaction mixture, however, the K_m values converged to a narrow range of very low values (0.11 to 0.24 μM) as shown in Fig. 2. Remarkably, all the lines in Fig. 2 intersected the y axis with the same V_{max} value of 42 nmol of O_2 per min, 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. Inhibition of enzyme reaction by high substrate concentrations can be treated (6) as the inhibition by the binding of a second substrate molecule with a dissociation constant of 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 (6). The experimental results (not given) 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 1) 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

TABLE 1. Effect of washing and assay pH on the sulfite oxidation by *T. thiooxidans* cells

pH for washing of cells	V_{max} (nmol of O_2/min)	K_i or K_m for potassium sulfite in mM (K_i or K_m for H_2SO_3 in μM) at assay pH[a]:							
		2.3	3.5	4.0	4.5	5.0	5.5	6.0	6.5
2.3		0.002 (0.5)*	0.035 (0.7)*	0.13 (0.8)*	0.38 (0.8)*				
	42				0.12 (0.24)	0.22 (0.14)	0.56 (0.11)	2.0 (0.12)	7.1 (0.11)
4.7	57				0.25 (0.50)		1.7 (0.31)	4.0 (0.24)	10.0 (0.15)
7.5	83				1.0 (2.0)	1.7 (1.0)	2.9 (0.55)	10.0 (0.61)	

[a] Asterisks denote values that are K_i; all other values are K_m.

20 μM. If the substrate concentration was converted to the concentration of sulfurous acid (H_2SO_3), however, the K_i values converged again to a very narrow range of 0.5 to 0.8 μM (Table 1), as in the case of K_m values (Fig. 2).

These results of pH dependency of sulfite oxidation by *T. thiooxidans* cells can be satisfactorily explained if the 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 extracts. The pH dependency study of sulfite oxidation by the cell extracts of *T. thiooxidans*, in contrast to those of intact cells (Fig. 1), showed little effect of substrate concentrations on the optimal pH (Fig. 3), and the rate of oxidation remained highest around pH 7 to 7.5 when the concentration of K_2SO_3 was varied 50-fold. The double-reciprocal plots (not shown) of cell extract activity versus K_2SO_3 concentrations did not show the characteristic pattern of Fig. 2 (intact cells) with changing pH. In fact the datum 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 extracts became progressively more turbid when the pH was lowered below 6.0, and the activity also decreased.

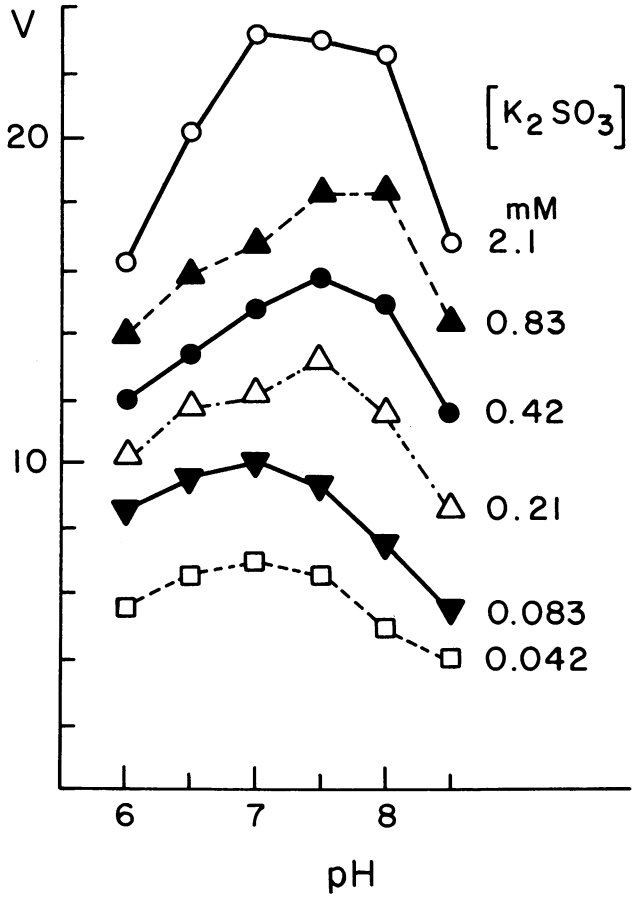

FIG. 3. Effect of sulfite concentration on the optimal pH for sulfite oxidation by cell extracts of *T. thiooxidans*. Conditions were the same as for Fig. 1 except that cell extracts (1.2 mg of protein) from 10 mg (wet weight) of cells were used instead of intact cells.

Effect of pH on washing cells. In the previous experiments whose results are shown in Fig. 2, *T. thiooxidans* cells were washed and suspended in 50 mM (pH 2.3) potassium phosphate. When the cells were washed at a higher pH, their response to sulfite oxidation at various pH values changed considerably with increasing V_{max} and K_m values (Table 1). The K_m values for K_2SO_3 still increased with increasing assay pH. The K_m values calculated for sulfurous acid, however, again remained relatively constant.

Implications. Acidophilic thiobacilli are believed to maintain the cytoplasmic pH close to neutrality even under external acidic conditions, resulting in a large pH difference across the cell membrane (7). The maintenance of neutral cytoplasmic pH is essential for the proper function of some *T. thiooxidans* enzymes (25). Gram-negative bacterial cells including thiobacilli possess a lipopolysaccharide-covered outer membrane which can, in addition to the cytoplasmic membrane, act as a permeability barrier (26). Since *T. thiooxidans* cells grow only under acidic conditions producing sulfuric acid, the cells must have acidic periplasmic space. Externally added sulfite must penetrate both the outer membrane and inner membrane to reach the neutral cytoplasm where it is oxidized. Experimental results in this paper indicate that sulfite enters the cells in the form of undissociated (or protonated) sulfurous acid or sulfur dioxide (SO_2). Sulfur dioxide as a gas will easily diffuse through the membranes. The situation is analogous to the ammonia oxidation by *Nitrosomonas europaea* in which NH_3 rather than NH_4^+ is used as substrate although at its optimal pH of 8 for the oxidation ammonia largely exists as NH_4^+ (22).

The results of washing experiments suggest that these cells are sensitive to the pretreatment conditions and warrant a study of the possible effect on the cell surface or intracellular pH.

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