Mechanism of oxidation of inorganic sulfur compounds by thiosulfate-grown *Thiobacillus thiooxidans*

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Abstract: *Thiobacillus thiooxidans* was grown at pH 5 on thiosulfate as an energy source, and the mechanism of oxidation of inorganic sulfur compounds was studied by the effect of inhibitors, stoichiometries of oxygen consumption and sulfur, sulfite, or tetrathionate accumulation, and cytochrome reduction by substrates. Both intact cells and cell-free extracts were used in the study. The results are consistent with the pathway with sulfur and sulfite as the key intermediates. Thiosulfate was oxidized after cleavage to sulfur and sulfite as intermediates at pH 5, the optimal growth pH on thiosulfate, but after initial condensation to tetrathionate at pH 2.3 where the organism failed to grow. *N*-Ethylmaleimide (NEM) inhibited sulfur oxidation directly and the oxidation of thiosulfate or tetrathionate indirectly. It did not inhibit the sulfite oxidation by cells, but inhibited any reduction of cell cytochromes by sulfur, thiosulfate, tetrathionate, and sulfite. NEM probably binds sulfhydryl groups, which are possibly essential in supplying electrons to initiate sulfur oxidation. 2-Heptyl-4-hydroxy-quinoline *N*-oxide (HQNO) inhibited the oxidation of sulfite directly and that of sulfur, thiosulfate, and tetrathionate indirectly. Uncouplers, carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP), inhibited sulfite oxidation by cells, but not the oxidation by extracts, while HQNO inhibited both. It is proposed that HQNO inhibits the oxidation of sulfite at the cytochrome *b* site both in cells and extracts, but uncouplers inhibit the oxidation in cells only by collapsing the energized state of cells, $\Delta\tilde{\mu}_{H+}$, required either for electron transfer from cytochrome *c* to *b* or for sulfite binding.

Key words: Thiobacillus thiooxidans, thiosulfate, oxidation, sulfite.

Résumé : La bactérie *Thiobacillus thiooxidans* a été cultivée sous un pH de 5 sur du thiosulfate en tant que source d'énergie afin d'étudier le mécanisme d'oxydation de composés organiques sulfurés, en mettant à profit l'effet d'inhibiteurs, les stoechiométries de la consommation d'oxygène et de l'accumulation de soufre, de sulfite ou de tétrathionate, et la réduction des cytochromes par les substrats. Des cellules intactes de même que des extraits acellulaires ont été utilisés dans cette étude. Les résultats s'accordent bien à la voie métabolique ayant le soufre et le sulfite comme intermédiaires clés. Le thiosulfate a été oxydé après avoir été clivé en intermédiaires soufre et sulfite à un pH de 5, le pH optimal pour la croissance sur du thiosulfate, mais seulement après la condensation initiale en tétrathionate à un pH de 2,3, conditions dans lesquelles l'organisme n'a pu se développer. Le N-ethylmaleimide (NEM) a directement inhibé l'oxydation du soufre en thiosulfate et indirectement celle menant au tétrathionate. Il n'a pas inhibé l'oxydation du sulfite par les cellules, mais a inhibé toute réduction de cytochromes cellulaires par le soufre, le thiosulfate, le tétrathionate et le sulfite. Le NEM s'est probablement lié aux groupements sulfhydryl qui sont de toute évidence des sources d'électrons essentiels pour amorcer l'oxydation du soufre. Le HQNO (2-heptyl-4-hydroxy-quinoline *N*-oxyde) a directement inhibé l'oxydation du sulfite et indirectement celle du soufre, du thiosulfate et du tétrathionate. Des découpleurs, le CCCP (carbonyl cyanure-m-chlorophénylhydrazone) et le DNP (2,4-dinitrophénol) ont inhibé l'oxydation du sulfite par les cellules sans pour autant inhiber l'oxydation par les extraits, alors que le HQNO a inhibé les deux. Nous proposons que le HQNO inhiberait l'oxydation du sulfite au stade du cytochrome *b* à la fois dans les cellules et dans les extraits, alors que les découpleurs inhiberaient l'oxydation dans les cellules seulement en faisant chuter l'état énergétique des cellules, $\Delta\tilde{\mu}_{H+}$, nécessaire soit pour pour le transfert des électrons du cytochrome *c* à *b*, soit pour la liaison au sulfite.

Mots cles : Thiobacillus thiooxidans, thiosulfate, oxydation, sulfite.

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Introduction

Inorganic sulfur compounds are widespread in nature, and microorganisms are intimately involved in the sulfur cycle. A number of bacteria and archaea are responsible for the oxidation of inorganic sulfur compounds. It is not always easy to separate the reactions carried out by microorganisms from purely chemical reactions of reactive sulfur compounds. Often microbial reactions mimic chemical models and follow the main oxidation pathway of $S^2 \rightarrow S^0 \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$, although the intermediates can chemically interact with each other and make the pathway complex (Suzuki 1974, 1999). Inhibitors for $S^0 \rightarrow SO_3^{2-}$ and $SO_3^{2-} \rightarrow SO_4^{2-}$ are useful in obtaining expected stoichiometries and in establishing the oxidation pathway, particularly in the oxidation of thiosulfate and tetrathionate (Suzuki 1999). As shown in Fig. 1, thiosulfate can be oxidized to sulfate ($S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$) either through the initial cleavage (pathway A): [1] $S\text{–}SO_3^{2-} + 2H_2O \rightarrow S + H_2SO_3 + 2OH^-$; followed by the oxidation of sulfur: [2] $S + O_2 + H_2O \rightarrow H_2SO_3$; and sulfite: [3] $2H_2SO_3 + O_2 \rightarrow 2SO_4^{2-} + 4H^+$; or the initial condensation (pathway B): [4] $2S_2O_3^{2-} + ½O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$; followed by hydrolysis of tetrathionate: [5] $S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + S + SO_4^{2-} + 2H^+$; and the oxidation of sulfur (reaction [2]) and sulfite (reaction [3]). 2-Heptyl-4 hydroxy-quinoline *N*-oxide (HQNO) inhibits the oxidation of sulfite, and *N*-ethylmaleimide (NEM) inhibits the oxidation of sulfur. The use of these inhibitors and stoichiometries of O_2 consumption and sulfite or sulfur accumulation should provide critical information on which pathway is used by a microorganism (Suzuki 1999).

Thiobacillus thiooxidans (*Acidithiobacillus thiooxidans*, Kelly and Wood 2000) is an acidophilic chemolithotroph that uses inorganic sulfur compounds as energy sources. The sulfur oxidation mechanism has been studied with sulfur-grown cells to show the pathway of oxidation: $S^{2-} \rightarrow S^0 \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$. Although resting cells of the organism grown on sulfur can oxidize thiosulfate at pH 2.3 (Chan and Suzuki 1994), the acidic pH used for growth on sulfur where thiosulfate is chemically more reactive, the organism cannot grow on thiosulfate at this pH. Growth can be achieved only at a higher pH of 4.5–5.0 (Barton and Shively 1968; Nakamura et al. 1990). In the current study *T. thiooxidans* was grown at pH 5 on thiosulfate, and the oxidation mechanism was studied under the conditions where the organism can obtain energy for growth (pH 5) as well as at pH 2.3 where it cannot grow on thiosulfate. Stoichiometry and inhibitor studies described above were carried out at both pH values to determine the pathway used. The thiosulfate-grown cells contained higher amounts of cytochromes than the sulfur-grown cells and were more suitable for the electron transfer studies, particularly during the oxidation of sulfite, the key energy-generating reaction. The sulfite oxidation system in this acidophilic bacterium involves membrane-bound cytochrome *b*, the site of HQNO inhibition (Tano et al. 1982), and membrane-bound sulfite oxidase (Nakamura et al. 1995) in addition to cytochromes *c*, *a*, and *d*, unlike the neutrophilic *T. novellus* (*Starkeya novella*, Kelly et al. 2000) where the oxidation of sulfite is simple: sulfite → sulfite oxidase (sulfite: cytochrome *c* oxidoreductase) → cytochrome *c* → cytochrome a_3 oxidase → O_2 (Charles and Suzuki 1966; Yamanaka 1996; Kappler et al. 2000). Therefore, the response of cytochromes, of thiosulfate grown *T. thiooxidans* cells and extracts to sulfite and other inorganic sulfur compounds, was studied to elucidate the electron transfer pathway and energy metabolism.

Materials and methods

Chemicals

Chemicals were the highest grade commercially available. Sodium thiosulfate pentahydrate and precipitated sulfur powder were obtained from British Drug Houses (BDH) Inc. Toronto, Ont. All inhibitors were obtained from Sigma Chemical Co. St. Louis, Mo.

Organism and media

Thiobacillus thiooxidans ATCC 8085 was used throughout this study.

The basal 9K medium (Silverman and Lundgren 1959) contained per litre of glass-distilled water (in g): 3.0 $(NH_4)_2SO_4$, 0.1 KCl, 0.5 K_2HPO_4, 0.5 $MgSO_4 \cdot 7H_2O$, and 0.014 $Ca(NO_3)_2 \cdot 4H_2O$. The pH was adjusted to 5.0 with concentrated sulfuric acid. Ten millilitres of a Millipore-filtered 18 mg/L $FeSO_4 \cdot 7H_2O$ solution was added to the sterilized medium.

Starkey's no. 1 medium (Starkey 1925) contained (in g/L): 0.3 $(NH_4)_2SO_4$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.018 $FeSO_4 \cdot 7H_2O$, 3.5 KH_2PO_4, and 0.25 $CaCl_2$.

Starkey's no. 2 medium (Starkey 1934) contained (in g/L): 4.0 KH_2PO_4, 4.0 K_2HPO_4, 0.3 $(NH_4)_2SO_4$, 0.019 $CaCl_2$, 0.05 $MgSO_4 \cdot 7H_2O$, 0.014 $MnSO_4 \cdot H_2O$, and 0.02 $FeCl_3 \cdot 6H_2O$.

Adaptation from sulfur to thiosulfate

Thiobacillus thiooxidans was grown on powdered sulfur (10 g/L), spread on the surface in Starkey's no. 1 medium adjusted to pH 2.3 with sulfuric acid (2.5% inoculum) for 4 days at 28°C without shaking.

The sulfur-grown cells were collected and washed three times in the 9K medium at pH 5 in a microcentrifuge (IEC, Micro-MB centrifuge 3615, 14 000 rpm for 3–5 min) to remove excess sulfuric acid. The washed cells (10% inoculum, 10 mL) were inoculated into 90 mL 9K medium at pH 5 containing 1 g $Na_2S_2O_3 \cdot 7H_2O$ and two drops of 0.5% bromphenol blue pH indicator (pH 4.6→3.0 color changing from yellow to blue violet). The 250-mL Erlenmeyer flask was kept static for 2–4 days at 28°C before placing it on a rotary shaker (150 rpm). Disappearance of thiosulfate and acid production indicated growth.

Growth on thiosulfate in a reactor

The flask experiment showed an optimal growth pH on thiosulfate of 5 in the 9K medium. The flask culture was used as an inoculum (10%) for a 400-mL reactor equipped with an automated titrator (Radiometer, titrator 11 and pH meter 28, Copenhagen) to maintain the pH at 5 with 5% K_2CO_3. It was continuously aerated through a glass sparger and a magnetic stirrer. Sodium thiosulfate (1%) was added as substrate. The grown culture was transferred to a 4-L reactor to obtain 4 L of thiosulfate-grown cells after 4–5 days at 28°C. The cultivation was carried out on a semi-continuous basis leaving 400 mL culture as the 10% inoculum for new 9K medium at pH 5 to start another 4 L culture.

Cell harvesting and washing

After 4–5 days when thiosulfate was nearly gone, the culture was centrifuged at 8000 × *g* for 10 min. Cells were washed twice with milli-Q water with centrifugation in between (10 000 × *g* for 10 min). Washed cells were suspended in 0.1 M sodium citrate (pH 6) at a concentration of 50 mg wet cells/mL. The cell suspension could be stored for 2 weeks at 4°C without losing much of the

Fig. 1. Possible thiosulfate oxidation pathways and reactions involved. Reaction [1] takes place only when reaction [2] or [3] is operating. Reaction [2] is inhibited by NEM, and reaction [3] is inhibited by HQNO. Stoichiometries expected in the presence of these inhibitors are shown.

Thiosulfate oxidation

$(S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+)$

Pathway A

[1] $S\text{-}SO_3^{2-} + 2H_2O \rightarrow S + H_2SO_3 + 2OH^-$

[2] $S + O_2 + H_2O \rightarrow H_2SO_3$

[3] $2H_2SO_3 + O_2 \rightarrow 2SO_4^{2-} + 4H^+$

HQNO: $S_2O_3^{2-} + O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2OH^-$

NEM: $S_2O_3^{2-} + ½O_2 \rightarrow S + SO_4^{2-}$

Pathway B

[4] $2S_2O_3^{2-} + ½O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$

[5] $S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + S + SO_4^{2-} + 2H^+$

[2] $S + O_2 + H_2O \rightarrow H_2SO_3$

[3]x½ $H_2SO_3 + ½O_2 \rightarrow SO_4^{2-} + 2H^+$

HQNO: $S_2O_3^{2-} + 1½O_2 + H_2O \rightarrow H_2SO_3 + SO_4^{2-}$

NEM: $S_2O_3^{2-} + ½O_2 \rightarrow S + SO_4^{2-}$

oxidation activities. If the cells were suspended in the growth medium, 9K medium at pH 5, the activity gradually decreased with time. For most of the experiments, therefore, 0.1 M sodium citrate at pH 6 (Nakamura et al. 1990) was used for cell suspension and storage unless otherwise indicated.

Preparation of cell-free extracts

The cells suspended in 0.1 M sodium citrate were washed once in 0.1 M Tris–HCl (pH 7.5) buffer and suspended in the same buffer at a wet-cell concentration of 200 mg/mL. The suspension was passed through a French pressure cell three times at 110 MPa to break the cells and was centrifuged at 10 000 × *g* for 10 min to obtain cell-free extracts. The supernatant was translucent with a reddish-brown color.

Oxidation of thiosulfate and other substrates

Oxidation was followed by measurement of oxygen consumption at 25°C in a thermostated vessel equipped with a Clark oxygen electrode (Gilson Medical Electronics, Oxygraph). A full scale in the chart recorder corresponded to 310 nmol O_2, solubility of O_2 in 1.2 mL. The substrates for oxidation: 10 mM or 0.1 M sodium thiosulfate, 0.1 M sodium sulfite in 50 mM EDTA, 10 mM sodium tetrathionate, and 15 mM sulfur in dimethyl sulfoxide (DMSO) were added in microlitre amounts to reaction mixtures containing cells (2.5 mg wet cells or 50 µL of 50 mg/mL) or extracts (0.1 mL, 2 mg protein) in a specified buffer and pH to start the reaction in a total volume of 1.2 mL. Control experiments without cells or extracts were carried out to confirm the observed oxidation was not purely chemical. There was no measurable chemical oxidation during the short experimental periods.

To study the stoichiometry of oxidation, 0.1 µmol of thiosulfate was used unless otherwise specified so that the total O_2 consumption could be determined in the Oxygraph (0.3 µmol O_2 solubility in 1.2 mL at 25°C). The amounts of potential oxidation intermediates were also selected on the same basis.

Determination of thiosulfate and tetrathionate

Thiosulfate was determined by the method of Sörbo (Sörbo 1957, 1958) during the growth of organism. Tetrathionate formed during thiosulfate oxidation was determined by the same cyanolysis method without $CuCl_2$ using a 1-mL reaction mixture. When sodium citrate was the buffer, 0.625 mL of 1 M instead of 0.2 M NH_4OH was added to raise the pH high enough for 30 min cyanolysis with 0.156 mL of 0.1 M KCN. The mixture was centrifuged to remove cells. Addition of concentrated HNO_3 (0.1 mL) after 0.156 mL ferric nitrate reagent (10% $Fe(NO_3)_3 \cdot 9H_2O$ in 13% HNO_3) was required to lower the pH for 15 min of color development (brown), which was measured at 460 nm.

Determination of sulfite

The pararosaniline method of West and Gaeke (1956) was used. One millilitre of reaction mixture was mixed with 5 mL of 0.1 M sodium tetrachloromercurate II and was centrifuged to remove cells. To the supernatant, 0.5 mL of 0.2% formaldehyde and 0.5 mL of HCl-bleached pararosaniline (0.04%) were added with mixing in between. After 30 min, the intensity of the color (red–violet) was determined at 554 nm. When sodium citrate was used as buffer, it was necessary to add 20 µL of concentrated HCl before pararosaniline addition to avoid the formation and precipitation of dark blue material, possibly coming from the interaction of Hg^{2+} and polysulfides on the cell surface.

Determination of elemental sulfur

Elemental sulfur formed was determined according to Bartlett and Skoog (1954) as modified by Chan and Suzuki (1993).

Cytochrome studies

Cytochromes both in cells and extracts were studied in a Shimadzu multipurpose recording spectrophotometer (MPS-50L) with a 1-cm light path at room temperature. Cells were in 0.1 M sodium citrate at pH 5.0 (5 mg wet cells/mL) and extracts were diluted 10 times in 0.1 M Tris–HCl (pH 7.5).

Difference spectra were obtained between a sample with a substrate (sample cuvette) and another without a substrate (reference cuvette). A scanning speed was 3 min from 400 to 800 nm, and scanning was repeated many times to follow the spectral change with time. A complete reduction of cytochromes was achieved by the addition of sodium dithionite (hydrosulfite). The spectra of cells and extracts were also studied with the refraction method, the technique used by Tano et al. (1982), but the high concentration of cells or extracts required made the analysis of results more difficult, since endogenous substrates reduced the cytochromes.

Results

Growth of *Thiobacillus thiooxidans* on thiosulfate

Thiobacillus thiooxidans ATCC 8085 did not grow on thiosulfate at pH 2.3, but did grow at pH 5.0. In shake-flask growth experiments, sulfur-grown cells grew on thiosulfate after a lag period of 3 days in 9K medium, 3.5 days in Starkey's no. 1 medium, and 4 days in Starkey's no. 2 medium, when measured in thiosulfate utilization. Thiosulfate consumption was complete in 5 days in 9K, 6 days in Starkey's no. 1, and 7 days in Starkey's no. 2 medium. The 9K medium was selected as the growth medium, and a 4-L reactor was used to obtain cells grown at a constant pH of 5.0 on thiosulfate. With 1% sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) the average cell yield was 0.425 g wet cells/L. The cells were brownish with more cytochromes than sulfur-grown cells, which were grayish.

Table 1. Effect of pH and salts on thiosulfate oxidation by *T. thiooxidans* cells at different thiosulfate concentrations.

		O_2 consumption (nmol O_2/min) in			
pH	$Na_2S_2O_3$ (μmol)	Na-citrate	9K medium	Na_2SO_4	K-P_i
2.3	0.1	3	68	75	0
	1.0	3	59	68	0
	10.0	6	25	32	5
5.0	0.1	23	6	6	10
	1.0	80	22	23	27
	10.0	76	48	40	43
7.0	0.1	8	3	7	3
	1.0	10	5	19	4
	10.0	20	20	20	9

Note: The O_2 consumption was followed in a Gilson oxygraph at 25°C with 1.2 mL reaction mixtures of 2.5 mg wet cells, and $Na_2S_2O_3$ as indicated in various salt solutions at the pH indicated. Concentration of sodium citrate, sodium sulfate, or potassium phosphate is 0.1 M.

Thiosulfate oxidation activity

The rate of thiosulfate oxidation by thiosulfate-grown cells was greatly affected by pH, salts, and thiosulfate concentrations. The optimum pH for growth on sulfur (pH 2.3), the optimum pH for growth on thiosulfate (pH 5.0), and the neutral pH where no growth on either substrate occurred (pH 7.0) were selected for the oxidation studies. As shown in Table 1, the optimum pH for oxidation in 0.1 M sodium citrate buffer (the buffer used by Nakamura et al. 1990) was 5.0 at three different concentrations of thiosulfate (the same optimum pH was obtained with additional data at pH 2.3, 3.0, 4.5, 5.0, 5.5,. 6.0, and 7.0 with 1 μmol $Na_2S_2O_3$ in 1.2 mL in the citrate buffer). In 9K medium or sodium sulfate, however, the acidic pH of 2.3 and the lowest thiosulfate concentration produced the highest activity. The activity in potassium phosphate was low, but optimal at pH 5 similar to sodium citrate. When the concentration of thiosulfate was increased from 0.1 to 10 μmol in 1.2 mL, the rate of O_2 consumption increased generally, except in 9K medium and sodium sulfate at pH 2.3 where it decreased. Thiosulfate was oxidized at pH 2.3 only in 9K medium or sodium sulfate. At pH 5, the growth pH, thiosulfate oxidation was fastest in sodium citrate, with 1 or 10 μmol thiosulfate in 1.2 mL. In 9K medium, increasing concentrations of thiosulfate decreased the activity at pH 2.3 and increased the activity at pH 5, so that the activity in 9K (growth medium) showed an optimal pH of 5 with 10 μmol thiosulfate in 1.2 mL, similar to growth pH. With 50 μmol thiosulfate, the activity at pH 5 in 9K medium was even higher and nearly the same as in sodium citrate. Thus the K_m values for thiosulfate are affected by pH and buffer. At pH 5 the apparent K_m was estimated as 0.25 mM in sodium citrate and 3 mM in 9K medium, while at pH 2.3 it was estimated as 0.03 mM in 9K medium (data not shown). Thus, *T. thiooxidans* cells require high concentrations of thiosulfate (1% or 40 mM) to grow at pH 5 in 9K medium. Because the organism cannot grow at pH 2.3 on thiosulfate, the mechanism of oxidation at the two pH values must be different. Because citrate is a metal chelator, the high thiosulfate oxidation activity in the citrate buffer at pH 5.0 could be related to its metal chelation effect. Ethylenediamine tetraacetate (EDTA), a metal chelator, at 1 mM stimulated the oxidation rate in citrate by 50% at pH 5. Chelators for ferrous iron, 2,2′-dipyridyl and *o*-phenanthroline, stimulated it by 20%, and tiron (4,5-dihydroxy-1,3-benzene disulfonic acid), a chelator for ferric iron, had little effect at the same concentration.

Oxidation of tetrathionate, sulfur, and sulfite

The oxidation by cells of possible thiosulfate oxidation intermediates was also affected by salts and pH. Tetrathionate (60 nmol) was oxidized in citrate buffer with the highest rate at pH 5 (56 nmol O_2/min) similar to thiosulfate (Table 1). Oxidation of tetrathionate in 9K medium was fast (75 nmol O_2/min) at pH 2.3 and slow at pH 5, similar to thiosulfate. Sulfur (150 nmol in DMSO) was oxidized by these cells either in citrate buffer or 9K medium at rates comparable to the optimal thiosulfate oxidation rates (60–70 nmol O_2/min) irrespective of the assay pH between pH 5 and 8. The activity at pH 2.3 was slightly lower. The oxidation of sulfite (0.2 μmol) was much slower with an optimum at pH 5 (9–12 nmol O_2/min) either in citrate or 9K medium. The oxidation in citrate was inhibited by 1 mM EDTA by 60%, *o*-phenanthroline by 40%, 2,2′-dipyridyl by 32%, and tiron by 25% at pH 5, a similar order to the stimulation effect of these metal chelators on thiosulfate oxidation.

Effect of azide and cyanide on substrate oxidation

The citrate buffer at pH 5 and the 9K medium at pH 2.3 were chosen as the conditions for inhibitor studies to investigate the difference in the oxidation mechanisms at two pH values. As shown in Table 2, azide and cyanide at 1 mM as the inhibitors of terminal electron transfer through cytochrome oxidases inhibited the oxidation of thiosulfate, tetrathionate, and sulfur more strongly in the 9K medium at pH 2.3 than in citrate buffer at pH 5.0, and azide was a much stronger inhibitor. Cyanide had little effect at pH 5 and even stimulated the oxidation under certain conditions, e.g., with higher thiosulfate concentration (1 μmol in 1.2 mL, not shown) or sulfur oxidation. Sulfite oxidation at pH 5 was inhibited by both azide and cyanide, but more strongly by azide in citrate than in 9K medium. Cyanide catalyzed the chemical oxidation of sulfite in the absence of EDTA. This reaction may have been responsible for cyanide

Table 2. Effect of inhibitors on the rate of oxidation.

			% Activity of the control without inhibitors as 100					
Substrate	pH	Buffer	KCN	NaN_3	NEM	HQNO	CCCP	DNP
$Na_2S_2O_3$	5	Citrate	90	40	25	20	70	94
	2.3	9K medium	50	10	45	67	50	55
$Na_2S_4O_6$	5	Citrate	94	40	5	50	50	70
	2.3	9K medium	60	10	20	40	15	15
S^0	5	Citrate	110	50	5	64	30	—
	2.3	9K medium	50	5	—	—	—	—
Na_2SO_3	5	Citrate	20	6	100	9	5	4
	5	9K medium	62	59, 20[a]	—	—	—	—

Note: The O_2 consumption was followed as in Table 1 in 1.2 mL reaction mixtures. Substrate amount: 0.1 μmol $Na_2S_2O_3$, 60 nmol $Na_2S_4O_6$, 150 nmol S^0 in DMSO, or 1 μmol K_2SO_3 (0.5 μmol EDTA). Inhibitor concentration: 1 mM KCN or NaN_3, 0.2 mM NEM, 4 μM HQNO, and 20 μM CCCP or 2,4-DNP.

[a]Initially 59, then 20 after 2 min.

Fig. 2. Possible tetrathionate oxidation pathways and reactions involved. Stoichiometries expected in the presence of HQNO or NEM are shown.

Tetrathionate oxidation

$(S_4O_6^{2-} + 3½O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+)$

Pathway C

[6]	$S_4O_6^{2-} + 2e^- \rightarrow 2S_2O_3^{2-}$
[1]x2	$2S_2O_3 + 4H_2O \rightarrow 2S + 2H_2SO_3 + 4OH^-$
[2]x2	$2S + 2O_2 + 2H_2O \rightarrow 2H_2SO_3$
[7]	$H_2SO_3 + H_2O \rightarrow SO_4^{2-} + 4H^+ + 2e^-$
[3]x1½	$3H_2SO_3 + 1½O_2 \rightarrow 3SO_4^{2-} + 6H^+$
HQNO	$S_4O_6^{2-} + 2O_2 + 3H_2O \rightarrow 3H_2SO_3 + SO_4^{2-}$
NEM	$S_4O_6^{2-} + ½O + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+$

Pathway D

[5]x2	$2S_4O_6^{2-} + 2H_2O \rightarrow 2S_2O_3^{2-} + 2S + 2SO_4^{2-} + 4H^+$
[4]	$2S_2O_3^{2-} + ½O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$
[2]x2	$2S + 2O_2 + 2H_2O \rightarrow 2H_2SO_3$
[3]	$2H_2SO_3 + O_2 \rightarrow 2SO_4^{2-} + 4H^+$
HQNO	$S_4O_6^{2-} + 2½O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2SO_4^{2-} + 2H^+$
NEM	$S_4O_6^{2-} + ½O_2 + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+$

stimulation of the oxidation of sulfur compounds which produce sulfite as an intermediate.

Effect of other inhibitors and uncouplers on substrate oxidation

N-Ethylmaleimide (NEM), a thiol-binding agent, inhibited the oxidation of sulfur, and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), an inhibitor of cytochrome *b*–c_1 region, inhibited the oxidation of sulfite as reported earlier (Suzuki et al. 1992, 1993, 1994). Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) are known uncouplers or protonophores. The oxidation of 0.1 μmol thiosulfate in 1.2 mL citrate buffer was sensitive to the inhibition at pH 5: 75% by NEM, 80% by HQNO, 30% by CCCP, and 6% by DNP (Table 2). With 1 μmol thiosulfate in 1.2 mL citrate, a higher concentration of HQNO (40 μM) was required for a strong inhibition, but CCCP was more inhibitory (not shown).

The oxidation of thiosulfate in 9K medium was most sensitive at pH 2.3 to the inhibition. Uncouplers were more inhibitory at pH 2.3 in 9K medium than at pH 5 in citrate medium (Table 2). The oxidation in 0.1 M sodium sulfate was similarly inhibited at pH 2.3 (not shown).

The oxidation of tetrathionate was inhibited in citrate buffer at pH 5 most strongly by NEM (95%), the same extent as sulfur oxidation. In 9K medium at pH 2.3, DNP and CCCP were also strongly inhibitory. Sulfite oxidation was not affected by NEM, but was strongly inhibited not only by HQNO, but also by uncouplers CCCP and DNP (Table 2). Sulfur oxidation was strongly inhibited by NEM as expected but was also inhibited by HQNO and CCCP.

Stoichiometry of oxidation

Stoichiometric studies of sulfur, thiosulfate, and tetrathionate oxidation in the presence of HQNO, the inhibitor of sulfite oxidation, or NEM, the inhibitor of sulfur oxidation, were carried out to elucidate the mechanism and pathway of oxidation (Figs. 1 and 2).

As shown in Table 3 sulfur oxidation to sulfate ($S + 1½O_2 + H_2O \rightarrow H_2SO_4$) was changed by HQNO to sulfite formation ($S + O_2 + H_2O \rightarrow H_2SO_3$) with a good stoichiometry at pH 5. At pH 2.3, however, the oxidation rate was much reduced by HQNO, and the sulfite recovery was lower, in agreement with the results with sulfur-grown cells (Suzuki et al. 1992).

Oxidation of thiosulfate ($S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$) can be affected by HQNO differently, depending on the pathway of oxidation followed (Fig. 1). In pathway A, both sulfur atoms should be converted to sulfite (reaction[1] + reaction [2]: $S_2O_3^{2-} + O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2OH^-$) and, in pathway B, only one sulfur atom is converted to silfite (reaction [4] + reaction [5] + reaction [2]: $S_2O_3^{2-} + 1½O_2 + H_2O \rightarrow H_2SO_3 + SO_4^{2-}$). The results in Table 3 suggest that pathway A may be followed at pH 5 (160 nmol sulfite from 100 nmol thiosulfate), but at pH 2.3 pathway B may be dominant (only 84 nmol sulfite in 9K medium).

Tetrathionate oxidation ($S_4O_6^{2-} + 3½O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+$) could be initiated either by reduction (pathway C): [6] $S_4O_6^{2-} + 2e^- \rightarrow 2S_2O_3^{2-}$; followed by reactions in pathway A for thiosulfate oxidation (reactions [1], [2], and [3]) or by hydrolysis (pathway D); reaction [5] followed by reactions [4], [2], and [3] (Fig. 2, Suzuki 1999). Electrons

Table 3. Stoichiometry of oxidation and sulfite accumulation.

Substrate	pH	Buffer	Inhibitor	O_2 (nmol)	Sulfite (nmol)	Oxidation rate (nmol O_2/min)
Sulfur (150 nmol)	5	Citrate	—	195	21	56
			HQNO	145	123	36
	5	9K medium	—	180	3	55
			HQNO	125	137	29
	2.3	9K medium	—	185	16	54
			HQNO	140	55	14
	2.3	Na_2SO_4	—	190	0	54
			HQNO	150	69	16
$Na_2S_2O_3$ (100 nmol)	5	Citrate	—	180	19	34
			HQNO	150	160	7
	2.3	9K medium	—	200	1	49
			HQNO	150	84	24
	2.3	Na_2SO_4	—	195	0	76
			HQNO	180	44	50
$Na_2S_4O_6$ (60 nmol)	5	Citrate	—	170	39	61
			HQNO	150	96	22
	2.3	9K medium	—	160	0	66
			HQNO	130	57	37
	2.3	Na_2SO_4	—	160	0	74
			HQNO	125	55	31

Note: Inhibitor, 4 nmol HQNO in 1.2 mL reaction mixture.

required for reaction [6] could be generated during sulfite oxidation in reaction [3] which consists of sulfite oxidase: [7] $H_2SO_3 + H_2O \rightarrow SO_4^{2-} + 4H^+ + 2e^-$ and cytochrome oxidase: [8] $4e^- + O_2 + 4H^+ \rightarrow 2H_2O$. In the presence of HQNO which inhibits reaction [3] tetrathionate should be oxidized in pathway C ($S_4O_6^{2-} + 2O_2 + 3H_2O \rightarrow 3H_2SO_3 + SO_4^{2-}$) or in pathway D ($S_4O_6^{2-} + 2½O_2 + 3H_2O \rightarrow 2H_2SO_3 + 2SO_4^{2-} + 2H^+$), assuming that HQNO does not inhibit reactions [6] and [7]. The results in Table 3 show substantial accumulation of sulfite, but insufficient recovery for either pathway, presumably because of its instability. The pH 5 results may be considered to be close to those expected of pathway D (120 nmol sulfite), but the results cannot distinguish the two pathways.

Table 4 shows that the oxidation of thiosulfate changes in the presence of NEM, the inhibitor of sulfur oxidation, to: $S_2O_3^{2-} + ½O_2 \rightarrow S + SO_4^{2-}$, either at pH 5 or at pH 2.3. There was some sulfur production at pH 2.3 even in the absence of NEM, presumably because of increased reactivity of thiosulfate at the acidic pH (Roy and Trudinger 1970) and the decreased rate of sulfur oxidation. Both pathway A and pathway B can account for the results (Fig. 1). In the presence of both HQNO and NEM the oxidation of both sulfur and sulfite should be inhibited resulting in total inhibition of thiosulfate oxidation in pathway A. The results in pH 5 citrate buffer are consistent with the prediction. In pathway B two thiosulfate ions should be oxidized to one tetrathionate (reaction [4]). The results in Table 4 indicate the accumulation of tetrathionate from thiosulfate at pH 2.3 in the presence of both HQNO and NEM. Tetrathionate hydrolysis (reaction [5]) is probably inhibited under those conditions.

Tetrathionate oxidation in the presence of NEM should follow the equation: $S_4O_6^{2-} + ½O_2 + H_2O \rightarrow 2S + 2SO_4^{2-} + 2H^+$, in either pathway C or D (Fig. 2). The results in Table 4 generally agree with this interpretation (30 nmol O_2 and 120 nmol sulfur from 60 nmol tetrathionate). Although not shown, the oxidation of 120 nmol of $Na_2S_4O_6$ in pH 5 citrate produced 200 nmol sulfur with 100 nmol O_2 consumption in the presence of NEM.

Study of cytochromes

Difference spectra of dithionite-reduced minus oxidized cells (5 mg/mL) of *T. thiooxidans* grown on thiosulfate showed a number of absorption peaks (Fig. 3) tentatively identified as the peaks of cytochromes *c*, *b*, *a*, and *d*. The difference spectra of cell-free extracts (from 20 mg cells/mL) were similar. Spectra of reduced and oxidized cells were also obtained by the refraction method using a 50-mg/mL cell suspension. The oxidized cells showed 415 nm (*c* oxidized) and 655 nm (*d* oxidized) peaks and reduced ones 420 (c_γ), 430 (b_γ), 440 (a_γ), 523 (c_β), 530 (b_β), 553 (c_α), 562 (b_α), 607 (a_α), and 635 nm (d_α) peaks, corresponding to the difference spectra (Fig. 3). Cytochromes of the *c*, *b*, *a*, and *d* types were reported also in *T. thiooxidans* grown on sulfur in a fermentor with forced aeration (Tano et al. 1982), where concentrations of cytochromes *b* and *d* increased during growth, while those of cytochromes *c* and *a* decreased.

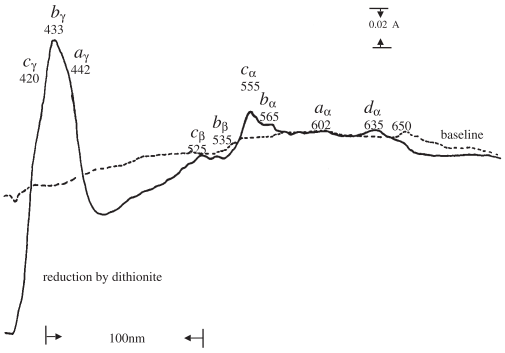
Difference spectra showed a nearly complete reduction of all the cytochromes within 5–10 min upon addition of substrates (1 mM $Na_2S_2O_3$, $Na_2S_4O_6$, K_2SO_3, or 0.3 mM S^0) when all the O_2 was consumed, but the time course spectral change clearly indicated the initial reduction of *c* type cytochrome followed by the reduction of *b*, *a*, and *d* cytochromes. When air was introduced by shaking the cuvette, *d* and *b* cytochromes were oxidized immediately, sometimes showing characteristic double peaks at 420 and 440 nm for *c* and *a* Soret (γ) absorption (missing the 430-nm peak for *b*). Vigorous shaking oxidized all the cytochromes, thus the re-

Table 4. Stoichiometry of oxidation and sulfur or tetrathionate accumulation.

Substrate	pH	Buffer	Inhibitor	O_2 (nmol)	Sulfur or **tetrathionate** (nmol)	Oxidation rate (nmol O_2/min)
$Na_2S_2O_3$ (100 nmol)	5	Citrate	—	200	0	43
			NEM	50	96	13
	2.3	9K medium	—	190	56	26
			NEM	50	108	16
	2.3	Na_2SO_4	—	190	12	26
			NEM	50	116	30
$Na_2S_2O_3$ (100 nmol)	5	Citrate	—	200	**0**	62
			HQNO+NEM	30	**0**	4
	2.3	9K medium	—	200	**0**	40
			HQNO+NEM	50	**57**	12
	2.3	Na_2SO_4	—	190	**0**	63
			HQNO+NEM	100	**73**	22
$Na_2S_4O_6$ (60 nmol)	5	Citrate	—	180	0	20
			NEM	20	68	3
	2.3	9K medium	—	140	0	50
			NEM	40	132	14
	2.3	Na_2SO_4	—	170	44	51
			NEM	60	124	22

Note: Inhibitor, 1 μmol NEM ± 4 nmol HQNO in 1.2 mL reaction mixture. Amounts of tetrathionate formed are shown in bold.

Fig. 3. Difference spectrum (reduced/oxidized) of *T. thiooxidans* cells (solid line). Numbers indicate the wavelengths (in nm) of absorption peaks which are identified for specific cytochromes. The broken line is the baseline before the addition of dithionite to the sample cuvette.



duction of cytochrome experiments were repeated when substrate was still available. When HQNO was present at 40 μM, cytochrome *c* was the only cytochrome reduced, suggesting the inhibition of cytochrome *b* reduction by reduced cytochrome *c*. In the presence of 1 mM NEM, cytochrome reduction by these substrates was never initiated. When 25 μM CCCP was present, the substrates reduced only cytochrome *c* similarly to HQNO. DNP at the same concentration had a similar but weaker effect than CCCP, delaying the reduction of other cytochromes.

The refraction method required a higher concentration of cells (50 mg/mL) and the endogenous metabolism was strong enough to consume all the O_2 within a few minutes. As a result, all the cytochromes were reduced without any addition of substrates.

Study of cell-free extracts

When cells were broken by passage through a French press, the crude extracts without centrifugation or cell-free extracts obtained after centrifugation could oxidize only sulfite but not thiosulfate or tetrathionate. Sulfur oxidation required the addition of reduced glutathione (Suzuki 1965). The oxidation of sulfite was strongly inhibited by HQNO, but uncouplers failed to inhibit the oxidation (Table 5) unlike the oxidation by intact cells. Cyanide and azide were less inhibitory in the extracts, but NEM seemed to show some inhibition, possibly because of chemical interaction with sulfite (Jocelyn 1987). The oxidation of sulfite by cells was similarly inhibited at this higher concentration of NEM (1 μmol in 1.2 mL). There was no sulfite oxidation in sodium citrate at pH 5 by extracts, although it was a favoured buffer for the intact cells. Potassium phosphate was also inhibitory. Tris–HCl, 9K medium, or K_2SO_4 was not inhibitory. EDTA was inhibitory in 9K medium at pH 5 (46% at 1 mM and 70% at 2 mM), but not in 0.1 M K_2SO_4 at either pH 5 or 7.

Cytochromes were fully reduced by 1 mM K_2SO_3 (0.5 mM EDTA) within 10 min when 0.1 mL extract was in 0.1 M Tris–HCl at pH 7.5 (total volume 1 mL). In the presence of 4 μM HQNO or 1 mM NEM, only the *c*-type cytochrome was reduced. CCCP (25 μM) or DNP (25 μM) had no effect on the reduction of cytochromes in the extracts, contrary to the results with cells. With undiluted extracts prepared in 0.1 M Tris–HCl and using the refraction method, all the cytochromes were reduced by 1 mM K_2SO_3 within 1–5 min. Introduction of air oxidized all the cytochromes, which were reduced again after 5 min. In the presence of 4 μM HQNO, only the *c*-type cytochrome was initially reduced, but later other cytochromes were also reduced since the inhibition was not total. Carbon monoxide shifted the reduced peak of cytochrome *d* from 635 to 640 nm as it did

with whole cells, in agreement with other *d*-type cytochromes (Yamanaka 1992).

Discussion

Thiobacillus thiooxidans grew on thiosulfate at pH 5, but not at pH 2.3, which was the optimal pH for growth on elemental sulfur. The optimal pH for growth on thiosulfate was similar to the optimal pH for thiosulfate oxidation in 0.1 M sodium citrate buffer, pH 5 (Table 1). The sodium citrate buffer used by Nakamura et al. 1990 turned out to be the best buffer for thiosulfate oxidation and also for stability of cell suspensions. The citrate effect could be related to its metal-chelating ability, since EDTA and other chelators also increased the rate of thiosulfate oxidation by cells. These chelators may be binding some metals that are deleterious for normal thiosulfate oxidation by cells. Decreased oxidation rate in 9K medium at pH 2.3 when thiosulfate concentration was increased from 0.1 to 10 µmol per 1.2 mL could also be due to a possible binding of some metals (e.g., Cu^{+1}, Dean 1985) by thiosulfate which might affect the oxidation of thiosulfate under acidic conditions.

Tetrathionate oxidation was also fastest at pH 5 in the citrate buffer and at pH 2.3 in 9K medium. Sulfur oxidation was not much affected by pH, although the rate was lower at pH 2.3. Sulfite oxidation was slower with an optimum at pH 5, and the rate was reduced further by metal chelators.

Known inhibitors of cytochrome oxidase, azide and cyanide, acted differently (Table 2). Azide consistently inhibited the oxidation of thiosulfate, tetrathionate, sulfur, and sulfite. Cyanide, however, showed a weaker inhibition or even stimulation. Because cyanide, at the high concentration (1 µmol in 1.2 mL), catalyzed the chemical oxidation of sulfite in the absence of EDTA, its stimulatory effect on thiosulfate, tetrathionate, or sulfur oxidation under certain conditions was probably due to its ability to form complex ions of some metals (e.g., Cu^{+1}, Dean 1985) to stimulate the chemical oxidation of sulfite formed.

Both NEM and HQNO (Table 2) inhibited the oxidation of sulfur, thiosulfate, and tetrathionate, supporting the pathway of oxidation involving sulfur oxidation and sulfite oxidation. Uncouplers, CCCP and DNP, inhibited the oxidation of all the substrates tested, but the strongest inhibition was observed with sulfite oxidation, similar to the inhibition by HQNO.

The stoichiometry experiments (Tables 3 and 4) support a pathway of sulfur oxidation with sulfite as an intermediate, and that pathway A operates at pH 5, and pathway B operates at pH 2.3 in thiosulfate oxidation (Fig. 1). Tetrathionate oxidation stoichiometry was complex, perhaps because of possible chemical interaction of sulfite with polythionates (Suzuki 1999). Thus, based on our data, it is not possible to decide on the pathway followed during the oxidation of tetrathionate initiated either by reduction (pathway C) or hydrolysis (pathway D) of tetrathionate (Fig. 2). Nevertheless the involvement of sulfur and sulfite oxidation in the oxidation of tetrathionate is the same in either pathway.

Cytochrome components of cells agreed with the report by Tano et al. (1982). Because in our experiments, cytochromes *b* and *d* were oxidized faster with air than were cytochromes *c* and *a* (in agreement with the known higher affinity for O_2 of *d* than *a* (Yamanaka 1992)), it is understandable why the concentrations of cytochromes *b* and *d* increased during the growth of their cells when O_2 concentration must have decreased because of limited aeration rate and increased cell numbers. It is possible to consider a branched pathway of electron transfer after cytochrome *c*- to *a*-type cytochrome oxidase or to *b*–*d* cytochromes (Yamanaka 1992), similar to the *Azotobacter* cytochrome system (Jones and Redfearn 1966).

Cytochromes of *T. thiooxidans* cells (Fig. 3) were all reduced by substrates thiosulfate, tetrathionate, sulfur, or sulfite, but the *c*-type cytochrome was reduced first. NEM prevented the reduction of any cytochrome, and HQNO, CCCP, or DNP allowed the reduction of cytochrome *c* only. Thus, it does seem that HQNO stopped the reduction of cytochromes *b* and *d* by reduced cytochrome *c*, and the same inhibition by uncouplers suggests that it is an energy-dependent step in these cells.

When the cells were disrupted, the extracts oxidized only sulfite and, with the addition of reduced glutathione, sulfur. Sulfite oxidation (Table 5) was still inhibited by HQNO, but uncouplers were ineffective. Cytochromes were all reduced by sulfite, but in the presence of HQNO or NEM only

Table 5. Sulfite oxidation by cell-free extracts.

Assay buffer	pH	Additions	Oxidation rate (nmol O_2/min)
Tris–HCl	7.5	—	26
	7.5	NEM	16↓
	7.5	HQNO	3
	7.5	KCN	22
	7.5	NaN_3	21
	7.5	CCCP	29
	7.5	DNP	25
Tris–HCl	7.5	—	34
9K medium	5.0	—	48
Na-citrate	5.0	—	0
K_2SO_4	7.0	—	49
	5.0	—	32
	2.3	—	0
	5.0	NEM	20↓
	5.0	HQNO	5
	5.0	KCN	26
	5.0	NaN_3	24
	5.0	CCCP	32
	5.0	DNP	31
K-P_i	7.0	—	20
	5.0	—	6

Note: Cells washed in water and suspended in 0.1 M Na-citrate (pH 6.0) were collected by centrifugation, suspended in 0.1 M Tris–HCl (pH 7.5), after washing, suspended in the same Tris buffer to 200 mg wet weight cells/mL. The suspension was passed through a French pressure cell at 110 MPa three times, and cell-free extracts were obtained by removing cell debris by centrifugation at 10 000 × *g* for 10 min. The oxidation was measured in the buffer or salts (0.1 M) at pH indicated with 0.1 mL of the extract and 1 µmol K_2SO_3 plus 0.5 µmol EDTA with or without additions in 1.2 mL total volume. ↓ indicates the rate slowed down to zero in 3–5 min. Additions: NEM, KCN, NaN_3, 1 µmol; HQNO, 4 nmol; CCCP, DNP, 25 nmol.

cytochrome *c* was reduced. CCCP or DNP, however, had no effect on the reduction of cytochromes in the extracts.

HQNO, therefore, is the only inhibitor of both cell and extract sulfite oxidations, inhibiting the *b* region of the electron transfer system. Uncouplers were possibly destroying the electrochemical potential essential for sulfite oxidation in intact cells. It is possible to imagine that the electron transport system in intact cells is affected by $\Delta\tilde{\mu}_{H+}$, and the entry of electrons from sulfite through a mobile carrier, cytochrome *c*, into the *b* region requires $\Delta\tilde{\mu}_{H+}$ or energized states of cells. A preliminary study indicates both $\Delta\psi$ and ΔpH are essential for sulfite oxidation by intact cells, being strongly inhibited by 1 mM KSCN or KF at pH 5, but not at pH 7.5 (Suzuki and Oh 2000, unpublished data). A previous study with sulfur-grown cells indicates the inhibition of sulfur oxidation by these compounds at an acidic pH (Suzuki et al. 1999). Sulfite oxidation by *T. thiooxidans* cells is believed to be in its fully protonated form, H_2SO_3 or SO_2 gas, according to the pH-sulfite concentration response of its oxidation (Takeuchi and Suzuki 1994). Sulfite oxidase (sulfite: cytochrome *c* oxidoreductase, Suzuki 1994) of *T. thiooxidans* is located in the cell membrane with a large molecular mass (400 kDa) consisting of three different subunits (Nakamura et al. 1995). H_2SO_3 or SO_2 should be permeable through membranes, but perhaps the sulfite-binding site is affected by $\Delta\tilde{\mu}_{H+}$ when the enzyme is in intact cells. Inhibition by high H_2SO_3 concentrations is consistent with this idea. In agreement with the current work, cell-free sulfite oxidation lost the unusual pH–sulfite concentration response of cells (Takeuchi and Suzuki 1994). Thus, in this case the cell-free sulfite oxidation system is physically exposed without constraints of closed cell membranes, and sulfite can be oxidized without any restriction and with or without uncouplers.

The inhibition of cytochrome reduction by NEM in the presence of sulfur, thiosulfate, and tetrathionate observed in this study suggests a possible mechanism where NEM binds some sulfhydryl groups essential for sulfur binding or activation. Sulfur-binding protein of flagella of sulfur-grown *T. ferrooxidans* (*Acidithiobacillus ferrooxidans*, Kelly and Wood 2000) has also active sulfhydryl groups. (Ohmura et al. 1996). In the refraction method with a higher concentration of cells, endogenous substrates reduced all the cytochromes within a few minutes, upon exhaustion of O_2. Bacon and Ingledew (1989) showed the production of H_2S from elemental sulfur by Fe^{2+}-grown *T. ferrooxidans* cells (electrons presumably coming from endogenous metabolism) supporting the idea of initial reductive opening of S_8 ring to produce a linear polysulfane sulfide ($^-S–S_6–S–$enzyme) before the oxidation, similar to the proposed opening of S_8 ring by reduced glutathione (GSH) in the sulfur-oxidizing enzyme (Suzuki 1965, 1999). It is tempting to speculate the possibility that NEM inhibition of cytochrome reduction may be related to the inhibition of sulfur oxidation by inhibiting the electron flow from endogenous metabolism to the sulfur-binding or activation site.

The oxidation pathway of inorganic sulfur compounds was previously studied in various thiobacilli with inhibitors, but not always as thoroughly as the present study or with the view of specific reactions inhibited, i.e., sulfur to sulfite, or sulfite to sulfate. Kodama and Mori (1968) showed a strong inhibition of sulfur oxidation by NEM in sulfur-grown *T. thiooxidans* cells without any inhibition of sulfite oxidation. The NEM-treated cells oxidized thiosulfate to tetrathionate only (Chan and Suzuki 1994). Hazeu et al. (1988) showed the inhibition of the oxidation of sulfide, thiosulfate, and tetrathionate by NEM at the level of sulfur in *T. ferrooxidans* cells grown on tetrathionate. Sulfur was stoichiometrically oxidized to sulfite by sulfur-grown *T. thiooxidans* cells in the presence of HQNO (Suzuki et al. 1992) and sulfide was stoichiometrically oxidized to sulfur in the presence of NEM (Suzuki et al. 1993). In tetrathionate-grown *T. caldus* (*Acidithiobacillus caldus*, Kelly and Wood 2000, a moderately thermophilic acidophile) NEM inhibited the oxidation of sulfur and HQNO inhibited the oxidation of sulfite, but CCCP inhibited the oxidation of both sulfur and sulfite (Hallberg et al. 1996). Thus, it may be concluded that the mechanism of oxidation of inorganic sulfur compounds is very similar among these acidophilic sulfur-oxidizing microorganisms. In *T. ferrooxidans* grown on Fe^{2+}, the oxidation of sulfur is inhibited by HQNO, but not the Fe^{2+} oxidation (Corbett and Ingledew 1987). In *T. versutus* (*Paracoccus versutus*), *T. tepidarius* (*Thermithiobacillus tepidarius*, Kelly and Wood 2000), *T. novellus*, and *T. denitrificans* (Beffa et al. 1992*a*, 1992*b*, 1993*a*, 1993*b*) sulfur oxidation was inhibited by HQNO or myxothiazol. In *T. tepidarius* and *T. denitrificans* CCCP also inhibited sulfur oxidation. Because HQNO and CCCP also inhibited sulfite oxidation in some of these studies, it is necessary to show the stoichiometry to establish the specific inhibition of sulfur oxidation to sulfite and not of sulfite to sulfate to consider them as specific or direct inhibitors of sulfur oxidation. Accumulated sulfite can inhibit sulfur oxidation under certain conditions (Suzuki et al. 1992). Also, the hydrophilic sulfur used is supposed to be composed of mainly long-chain polythionates according to Steudel et al. (1988) and the inhibition of its oxidation may not be identical to that of elemental sulfur. In *T. tepidarius* HQNO or FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an uncoupler) strongly inhibited the oxidation of sulfite or tetrathionate (Lu and Kelly 1988). Thus, among most of these sulfur-oxidizing microorganisms sulfite oxidation is probably the key reaction directly affected by HQNO and uncouplers. The inhibition of sulfur oxidation by NEM is also widespread.

Thus, the hypothesis is that sulfur oxidation is inhibited by NEM because of inhibition of sulfur binding or activation, and sulfite oxidation is inhibited by HQNO specifically at the cytochrome *b* site and by uncouplers at the sulfite binding site or electron transfer dependent on $\Delta\tilde{\mu}_{H+}$. A recent paper on the uphill electron transfer from cytochrome *c* to the bc_1 complex of *T. ferrooxidans* (Elbehti et al. 2000) presents interesting results relevant to the present work and supports the branched electron flow from cytochrome *c*. A further study is being carried out to elucidate the mechanism of sulfite oxidation and the control of electron flow in *T. thiooxidans.*

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