Oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*

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Received April 3, 1991/Accepted October 4, 1991

**Abstract.** Oxidation of reduced sulphur compounds by *Thiobacillus acidophilus* was studied with cell suspensions from heterotrophic and mixotrophic chemostat cultures. Maximum substrate-dependent oxygen uptake rates and affinities observed with cell suspensions from mixotrophic cultures were higher than with heterotrophically grown cells. pH Optima for oxidation of sulphur compounds fell within the pH range for growth (pH 2–5), except for sulphite oxidation (optimum at pH 5.5). During oxidation of sulphide by cell suspensions, intermediary sulphur was formed. Tetrathionate was formed as an intermediate during aerobic incubation with thiosulphate and trithionate. Whether or not sulphite is an intermediate during sulphur compound oxidation by *T. acidophilus* remains unclear. Experiments with anaerobic cell suspensions of *T. acidophilus* revealed that trithionate metabolism was initiated by a hydrolytic cleavage yielding thiosulphate and sulphate. A hydrolytic cleavage was also implicated in the metabolism of tetrathionate. After anaerobic incubation of *T. acidophilus* with tetrathionate, the substrate was completely converted to equimolar amounts of thiosulphate, sulphur and sulphate. Sulphide- and sulphite oxidation were partly inhibited by the protonophore uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) and by the sulphydryl-binding agent N-ethylmaleimide (NEM). Oxidation of elemental sulphur was completely inhibited by these compounds. Oxidation of thiosulphate, tetrathionate and trithionate was only slightly affected. The possible localization of the different enzyme systems involved in sulphur compound oxidation by *T. acidophilus* is discussed.

**Key words:** *Thiobacillus acidophilus* — Acidophiles — Sulphur metabolism — Sulphide — Elemental sulphur — Thiosulphate — Tetrathionate — Trithionate — Sulphite — Hydrolytic polythionate cleavage

The recent literature on sulphur oxidation by various neutrophilic and acidophilic thiobacilli clearly demonstrates that there is no uniformity in the pathways employed by different *Thiobacillus* species and that, in fact, vast differences may be found in the pathways involved (Kelly 1985 and 1989). This explains why many previous attempts to formulate a unifying route for the observed biological reactions have failed.

The acidophilic thiobacilli can thrive in environments with pH values as low as pH 1.5. From bioenergetic considerations, it can be expected that at least some of the reactions involved in the oxidation of reduced sulphur compounds will take place extracytoplasmically (Hooper and DiSpirito 1985), implying that the enzymes involved should be able to function while exposed to low pH values. Studies of sulphur compound oxidation by these bacteria are useful to obtain a better insight in their physiology. Such fundamental knowledge may contribute to an increased understanding of the role of these organisms in their natural environments and to the further development of biological leaching operations (Norris and Kelly 1988).

*Thiobacillus ferrooxidans*, an obligately chemolithoautotrophic organism, has been used frequently as an organism to study the physiology of growth in acidic environments (for a review see Ingledew 1982). However, during biochemical studies large amounts of biomass are often required. Due to the low energy availability per mol of substrate and limitation of substrate concentration, due to solubility and/or osmotic stress, biomass concentrations in *T. ferrooxidans* cultures grown on ferrous iron or reduced sulphur compounds are low.

To overcome the practical problems associated with the use of *T. ferrooxidans*, the facultative autotroph *Thiobacillus acidophilus* was introduced in our studies on sulphur oxidation by acidophiles. This organism was initially isolated as a contaminant of a ferrous iron-grown culture of *T. ferrooxidans* (Guay and Silver 1975). Like *T. ferrooxidans*, *T. acidophilus* is an acidophilic bacterium with a pH optimum of approximately 3. Growth substrates for autotrophic growth include elemental sulphur (Guay and Silver 1975), tetrathionate (Norris et al. 1986), thiosulphate and trithionate (Mason et al. 1987). Carbon
sources supporting heterotrophic growth include a number of monosaccharides, TCA-cycle intermediates and some amino acids (Guay and Silver 1975; Pront et al. 1990a).

Aim of the present study was to investigate the kinetics and mechanism of sulphur compound oxidation by T. acidophilus.

Materials and methods

Organism and growth conditions

Thiobacillus acidophilus DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen as a liquid culture grown on glucose and maintained as described previously (Pront et al. 1990a).

Chemostat cultivation

Mixotrophic chemostat cultures of T. acidophilus were grown in the mineral medium described by Pront et al. (1990b) at 30 °C, at pH 3.0 and at a dilution rate of 0.05 h⁻¹. The medium contained 10 mM Na₂SO₄, 5 H₂O and 2.5 mM glucose as the growth-limiting substrates. Heterotrophic cultures were grown in the same mineral medium (without sodium thiosulphate), adjusted to pH 3.0 with H₂SO₄, with glucose as the growth-limiting substrate. High cell density (5 g dry wt⁻¹) mixotrophic chemostat cultures of T. acidophilus were used for anaerobic incubations of cell suspensions with tetrathionate. These cultures were fed from two separate medium vessels. One contained 100 mM sodium thiosulphate and the other a mineral medium containing per liter of demineralized water: (NH₄)₂SO₄, 20 g; KH₂PO₄, 6.0 g; Na₂SO₄, 2.8 g; MgSO₄·7 H₂O, 1.0 g; CaCl₂·2 H₂O, 0.55 g; EDTA, 0.15 g; ZnSO₄·7 H₂O, 45 mg; CoCl₂·2 H₂O, 3 mg; CuSO₄·5 H₂O, 3 mg; NaMoO₄·2H₂O, 4 mg; FeSO₄·7 H₂O, 55 mg; H₂BO₃, 8 mg; KI, 1 mg; nitrotriacetic acid, 25 mg; silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 0.2 ml. The mineral medium was adjusted to pH 3.0 with concentrated H₂SO₄ and autoclaved at 120 °C. Glucose was autoclaved separately at 110 °C and added to the mineral medium at a concentration of 200 mM. These mixotrophic cultures were grown at 30 °C, at pH 3.0 and at a dilution rate of 0.04 h⁻¹. The dissolved oxygen concentration was over 80% air saturation in all chemostat cultures used. The pH of the chemostat cultures was maintained at pH 3.0 by automatic titration with 4 M KOH.

The purity of the chemostat cultures was checked by phase contrast microscopy and immunofluorescence as described by Pront et al. (1990b). The dry weight of cell suspensions was determined with dried nitrocellulose filters as described previously (Pronk et al. 1990b).

Measurement of substrate-dependent oxygen consumption

Respiration rates of cell suspensions were assayed polarographically in a Biological Oxygen Monitor with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio,USA) at 30 °C. Assays were performed with cell suspensions (0.25 g dry wt⁻¹) taken directly from substrate-limited chemostat cultures. Calculations were made on the basis of an oxygen concentration of 236 µM in air-saturated water at this temperature. The values presented have been corrected for the (low) endogenous respiration rates.

Oxidation of elemental sulphur by T. acidophilus was studied as oxygen uptake after the addition of solutions of elemental sulphur (S₀) in acetone. The acetone concentration in the reaction mixture did not exceed 1% (v/v). Control experiments showed that this acetone concentration influenced neither endogenous nor glucose-dependent oxygen uptake by T. acidophilus.

The oxygen uptake rates at different substrate concentrations were used for calculation of the Kₚ (apparent substrate saturation constant) and Vₚmax (maximum substrate-dependent oxygen uptake rate) from a reciprocal plot according to Hanes (1932).

For adjusting the pH of cell suspensions, 2 N solutions of H₂SO₄ and KOH were used.

Oxygen uptake rates in the presence of inhibitors were assayed after 5 min incubation of the inhibitor with the cell suspension.

Anaerobic incubations of cell suspensions with trithionate

Cell suspensions (1.25 g dry wt⁻¹) of mixotrophically grown T. acidophilus were flushed with argon for 15 min at 30 °C in a 10-ml thermostated reaction chamber. After addition of 1 mM trithionate, 0.5-ml samples were taken at desired time intervals and immediately mixed with 5 µl 1 M KOH to stop the reaction. Samples were then rapidly centrifuged in an Eppendorf bench-top centrefuge (5 min at 13,000 × g) before determinations of substrate concentrations were performed.

Anaerobic incubations of cell suspensions with tetrathionate

To remove sulphate, dense cell suspensions of mixotrophically grown T. acidophilus were centrifuged (10 min at 12,000 × g), washed twice with 25 mM potassium phosphate, pH 4.0, and resuspended in 11 of the same buffer. Biomass concentration in the resulting suspension was 30 g dry wt⁻¹. This cell suspension was transferred to a fermenter equipped with norprene tubing, stirred at 1250 rpm and flushed with nitrogen gas (1 l min⁻¹) throughout the experiment. The dissolved oxygen concentration was monitored with a polarographic electrode and the pH was maintained at pH 4.0 by automatic titration of 1 M KOH. After addition of 10 mmol tetrathionate, through a septum, samples were taken at desired time intervals. Concentrations of thiosulphate, tetrathionate and sulphate were determined in the sample supernatant after centrifugation (5 min at 48,000 × g). For sulphur analysis, 0.25-ml samples were centrifuged at 48,000 × g for 5 min. The pellet was washed with 25 mM potassium phosphate, pH 4.0, re-centrifuged and extracted overnight with acetone. KOH consumption was monitored with a buret.

Determination of sulphur compound concentration

Thiosulphate, tetrathionate and trithionate were determined according to the method of Sorbø (1957) as modified by Kelly et al. (1969).

Sulphide and sulphite were determined according to Trüper and Schlegel (1964). Production of long-chain intermediary sulphur compounds from different substrates was investigated by monitoring the OD₄₅₀ in a Hitachi double-beam spectrophotometer (model 100–60, Hazeu et al. 1988). The same suspension of cells without substrate was placed in the reference cuvette.

Solutions of elemental sulphur in acetone were assayed by cyanolysis (Sorbø 1957).

Sulphate and thiosulphate were assayed with a Waters HPLC.
using a Machery-Nagel anion exchange column (Nucleosil, 250 x 4 mm), eluted with 0.04 M sodium salicylate, pH 4.0 (flowrate 1 ml min\(^{-1}\) at 20 °C). Detection was performed with an RI detector (Waters 410, detection limits approximately 0.05 mM for sulphate and 0.5 mM for thiosulphate).

**Chemicals**

Trithionate was prepared as described by Wood and Kelly (1986). Carbonyl cyanide m-chlorophenylhydrazone and N-ethylmaleimide were obtained from Aldrich (Aldrich Chemie n.v.,s.a., Brussels, Belgium). 2,4-Dinitrophenol was obtained from Baker (J. T. Baker Chemicals, Deterver, Holland). All other chemicals were reagent grade and obtained from commercial sources.

**Results**

**Kinetics of sulphur compound oxidation**

From previous work on the oxidation of reduced sulphur compounds, it is well-known that, at higher substrate concentrations, many chemical interactions between the sulphur compounds may occur (Roy and Trudinger 1970). Control experiments indicated that at the substrate concentrations used in this study (generally lower than 500 μM), chemical oxidation of the sulphur compounds did not contribute significantly to the observed substrate conversion rates (data not shown).

Substrate inhibition was not observed with any of the sulphur compounds tested, except for sulphite. Sulphite oxidation was strongly inhibited at concentrations above 50 μM. Thiosulphate-, tetrathonate-, trithionate- and sulphite-dependent oxygen uptake by *Thiobacillus acidophilus* exhibited the stoichiometries expected for complete oxidation to sulphate. The rates of sulphur and sulphide oxidation decreased to values only slightly above the endogenous respiration rates before the oxygen uptake (i.e. substrate oxidation and electron transfer to oxygen), corresponding to complete conversion to sulphate, had been reached.

Sulphide-dependent oxygen uptake by *T. acidophilus* followed a biphasic pattern. Oxygen uptake rates during the first phase were higher than in the second phase. Initial sulphide-dependent oxygen uptake rates, observed with cells from mixotrophic chemostat cultures, were approximately two-fold higher than the \( V_{max} \) of heterotrophically grown cells (Table 1). The apparent \( K_s \) values of cell suspensions from heterotrophic and mixotrophic cultures were similar.

The maximum rate of elemental sulphur-dependent oxygen uptake observed with heterotrophically grown cells was lower than with cells from mixotrophic cultures. In addition, the latter cells exhibited a lower \( K_s \) for elemental sulphur (Table 1).

Cell suspensions of *T. acidophilus* grown in mixotrophic chemostat cultures exhibited a biphasic pattern of thiosulphate- and trithionate-dependent oxygen uptake. As with sulphide oxidation, oxygen uptake rates during the first phase were higher than in the second phase. Because of the small amount of oxygen consumed during the first phase of thiosulphate and trithionate oxidation, initial rates of oxygen uptake could only be determined accurately at substrate concentrations above 50 μM. This resulted in higher \( K_s \) values than the \( K_s \) values observed with heterotrophically grown cells (Table 1), which exhibited a monophasic oxidation pattern with thiosulphate and trithionate. The \( V_{max} \) values of heterotrophically grown cells were, for both substrates, significantly lower than those of mixotrophically grown cells (Table 1).

The maximum tetrathonate-dependent oxygen uptake rates observed with cells from mixotrophic and heterotrophic chemostat cultures were similar. However, the \( K_s \) of mixotrophically grown cells appeared to be slightly lower than that of heterotrophically grown cells (Table 1).

Mixotrophically grown cells of *T. acidophilus* exhibited very low rates of sulphite oxidation under physiological conditions (30 °C, pH 3) (Table 1, Fig. 1). In theory, this observed maximum oxygen uptake rate for extracellularly added sulphite could account for only 35% of the flux of sulphur atoms to sulphate in the mixotrophic cultures. Of course, these data are not necessarily representative for the oxidation rate of intracellularly formed sulphite. Sulphite oxidation rates of heterotrophically grown cells were too low to allow accurate calculation of kinetic parameters (data not shown).

With the exception of sulphite, the pH optima for the

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**Table 1. Apparent substrate saturation constants (\( K_s \)) and maximum oxygen uptake rates (\( V_{max} \)) for oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus*. Cells had been grown mixotrophically on 2.5 mM glucose and 10 mM thiosulphate (0.25 g dry wt. \(-1\)) or heterotrophically on 10 mM glucose (6.6 g dry wt. \(-1\)), in substrate-limited chemostat cultures at a dilution rate of 0.05 h\(^{-1}\). Substrate-dependent oxygen consumption rates were determined in a Biological Oxygen Monitor**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mixotrophically grown cells</th>
<th>Heterotrophically grown cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_s ) (μM)</td>
<td>( V_{max} ) (nmol O(_2) min(^{-1}))</td>
</tr>
<tr>
<td>Sulphide</td>
<td>5</td>
<td>268 (mg dry wt.)(^{-1}))</td>
</tr>
<tr>
<td>Elemental sulphur</td>
<td>2</td>
<td>94 (mg dry wt.)(^{-1}))</td>
</tr>
<tr>
<td>Thiosulphate</td>
<td>13(^*)</td>
<td>310 (mg dry wt.)(^{-1}))</td>
</tr>
<tr>
<td>Tetrathonate</td>
<td>1</td>
<td>56 (mg dry wt.)(^{-1}))</td>
</tr>
<tr>
<td>Trithionate</td>
<td>72(^*)</td>
<td>175 (mg dry wt.)(^{-1}))</td>
</tr>
<tr>
<td>Sulphite</td>
<td>13</td>
<td>17 (mg dry wt.)(^{-1}))</td>
</tr>
</tbody>
</table>

\( \ast \) determined at relatively high substrate concentrations (see text)
oxidation of sulphur compounds by *T. acidophilus* all fell within the pH range for growth (pH 2–5). Oxidation of sulphide had a very broad pH optimum, whereas the pH optimum for oxidation of thiosulphate was much sharper (Fig. 1). Surprisingly, the pH optimum for sulphite oxidation was higher than the pH optima for the oxidation of the other sulphur compounds (Fig. 1).

**Formation and metabolism of tetrathionate**

Oxidation of tetrathionate by cell suspensions of *T. acidophilus* was sulphate-dependent. Tetrathionate-dependent oxygen uptake rates decreased strongly upon washing of cell suspensions in a sulphate-free solution. However, 25 mM potassium sulphate could replace this need for sulphate totally.

The stoichiometry of oxygen uptake during the distinct phases of thiosulphate and trithionate oxidation may give an indication of the identity of intermediary products. During the first, rapid phase of thiosulphate and trithionate oxidation, 0.23 ± 0.02 mol O₂ and 0.28 ± 0.05 mol O₂ were consumed per mol of substrate, respectively. The lower oxygen uptake rates during the second phase of both compounds corresponded with the oxygen uptake rates observed with tetrathionate as a substrate. These observations were consistent with the formation of tetrathionate as an intermediate during the oxidation of both thiosulphate and trithionate. Indeed, product analysis revealed an almost quantitative conversion of thiosulphate and trithionate to tetrathionate during the first phase of oxidation.

In the neutrophiles *Thiobacillus tepidarius* (Lu and Kelly 1988) and *Thiobacillus neapolitanus* (Trudinger 1964a) trithionate is initially hydrolysed to give thiosulphate and sulphate. In these organisms thiosulphate is subsequently oxidized to tetrathionate (Wood and Kelly 1986; Trudinger 1961). During anaerobic incubation of *T. acidophilus* with trithionate, thiosulphate was identified as the main product (Fig. 2). A small amount of tetrathionate was also produced (Fig. 2), probably as a result of a chemical reaction between thiosulphate and trithionate, yielding tetrathionate and sulphite (Naito et al. 1975). Indeed, when mixtures of low concentrations of thiosulphate and trithionate were incubated in the absence of cells, formation of small amounts of tetrathionate was also observed (data not shown).

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**Fig. 1.** pH Optima for the oxidation of sulphide, thiosulphate and sulphite by intact cells. *Thiobacillus acidophilus* was grown mixotrophically as described in the legend to Table 1. Substrate concentrations were 50 μM sulphide, 250 μM thiosulphate and 40 μM sulphite. ●: sulphide; ▲: thiosulphate; ■: sulphite

**Fig. 2.** Formation of thiosulphate and tetrathionate during anaerobic incubation of a *Thiobacillus acidophilus* cell suspension (1.25 g dry wt. -1-1) with 1 mM trithionate. *T. acidophilus* was grown mixotrophically as described in the legend to Table 1. Thiosulphate and tetrathionate were analysed by cyanoanalysis and trithionate concentration was calculated by subtracting thiosulphate and tetrathionate concentrations from total sulphate sulphur concentration (Sorbø 1957; Kelly et al. 1969). ▲: thiosulphate; ●: tetra- thionate; ■: trithionate

**Fig. 3.** Formation of products and KOH consumption during incubation of 10 mM tetrathionate in an anaerobic cell suspension (30 g dry wt. -1-1) of *Thiobacillus acidophilus*. The organism was grown mixotrophically on 100 mM glucose and 50 mM thiosulphate in a substrate-limited chemostat culture at a dilution rate of 0.04 h⁻¹. ●: tetrathionate; ▲: thiosulphate; ■: sulphur; ●: sulphate; ▼: KOH
During anaerobic incubation of tetrathionate in cell suspensions of *T. acidophilus*, tetrathionate was stoichiometrically converted to thiosulphate, sulphate and some form of sulphur (Fig. 3). Formation of sulphide and sulphur could not be detected. KOH consumption revealed that two moles of H⁺ were released per mol of tetrathionate metabolized (Fig. 3). These results suggest that tetrathionate metabolism is initiated by a cleavage reaction according to the equation:

\[
\text{S}_4\text{O}_{6}^{2-} + \text{H}_2\text{O} \rightarrow 2\text{S}_2\text{O}_3^{2-} + \text{S} + \text{SO}_4^{2-} + 2\text{H}^+ \quad (1)
\]

Formation of sulphur could also be monitored as an increase of \( \text{OD}_{430} \) when tetrathionate was incubated with anaerobic cell suspensions and when thiosulphate, tri- thionate or tetrathionate was incubated in aerobic cell suspensions with 100 µM NEM.

**Formation of intermediary sulphur from sulphide**

As mentioned above, sulphide-dependent oxygen uptake exhibited a biphasic pattern. Biphasic oxygen uptake was particularly evident with cells from heterotrophic cultures. The oxygen consumption during the first phase of sulphide oxidation by cells from such cultures corresponded to 0.57 ± 0.08 mol per mol sulphide. With cell suspensions from mixotrophic cultures, it was more difficult to determine the transition from first to second phase and oxygen uptake during the first phase tended to be larger.

Oxidation of sulphide by *T. acidophilus* was accompanied by a transient increase of the \( \text{OD}_{430} \) of cell suspensions (Fig. 4). The time spans over which the increase and decrease of the \( \text{OD}_{430} \) occurred, corresponded with the first and second phase observed during oxygen uptake experiments, respectively. The decrease of the \( \text{OD}_{430} \) during the second phase of sulphide oxidation was much slower with cells from heterotrophic cultures than with mixotrophically grown cells (Fig. 4). The biphasic oxygen uptake pattern observed during sulphide oxidation, combined with the transient increase of the optical density of the cell suspensions, suggests that, as anaerobic incubation of tetrathionate, some intermediary sulphur compound was formed. The lower rates of oxygen uptake during the second phase of sulphide oxidation would then reflect the complete oxidation of this intermediate to sulphate. The stoichiometry observed during the first phase of sulphide oxidation by cells from heterotrophic cultures indicates that sulphide is initially oxidized to the redox level of elemental sulphur. The larger amount of oxygen consumed during the first phase of sulphide oxidation by cells from mixotrophic cultures is probably due to simultaneous oxidation of sulphide to sulphur and intermediary sulphur to sulphate.

As has been observed with the obligately autotrophic acidophile *T. ferrooxidans* (Hazeu et al. 1988), the oxidation of intermediary sulphur, formed during sulphide oxidation, was completely inhibited by the sulphhydril-binding agent N-ethylmaleimide (NEM) (Fig. 4). This compound also completely inhibited the oxidation of elemental sulphur and, to a lesser extend, the oxidation of sulphide and tetrathionate by *T. acidophilus* (Fig. 4, Table 2). NEM had a profound effect on the stoichiometry of sulphide-dependent oxygen uptake. In the presence of the inhibitor, only approximately 0.5 mol of oxygen was consumed per mol of sulphide added, corresponding to the incomplete oxidation of sulphide to the redox level of elemental sulphur. Oxidation of other sulphur compounds was not substantially inhibited by NEM (Table 2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DNP (%)</th>
<th>CCCP (%)</th>
<th>NEM (%)</th>
</tr>
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<tbody>
<tr>
<td>Sulphide</td>
<td>18</td>
<td>21</td>
<td>61</td>
</tr>
<tr>
<td>Elemental sulphur</td>
<td>0</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Thiosulphate</td>
<td>119</td>
<td>106</td>
<td>98</td>
</tr>
<tr>
<td>Tetrathionate</td>
<td>119</td>
<td>67</td>
<td>76</td>
</tr>
<tr>
<td>Tri thionate</td>
<td>101</td>
<td>65</td>
<td>92</td>
</tr>
<tr>
<td>Sulphite</td>
<td>62</td>
<td>60</td>
<td>91</td>
</tr>
</tbody>
</table>

**Effects of uncouplers on sulphur compound oxidation**

To investigate whether energy-requiring reactions (e.g. active transport or activation of substrate molecules) are involved in the metabolism of any of the sulphur compounds tested, the effects of the protonophore uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were studied. The concentrations of the uncouplers used in the experiments completely inhibited glucose-dependent oxygen uptake.

The oxidation of the sulphur oxyanions thiosulphate, thiothionate and tetrathionate was not inhibited by DNP.
(Table 2). CCCP caused a significant decrease of the trithionate and tetrathionate oxidation rates (Tables 2).

Oxidation of sulphide was strongly inhibited by uncoupers (Table 2). The uncoupers also affected the stoichiometry of sulphide-dependent oxygen uptake. In the presence of DNP or CCCP, oxygen consumption corresponded to the amount required for the incomplete oxidation of sulphide to elemental sulphur. Also the oxidation of exogenously added elemental sulphur was completely inhibited by uncoupers (Table 2).

Sulphite-dependent oxygen uptake rates were inhibited by about 40% after addition of uncoupers (Table 2).

Discussion

Kinetics of sulphur compound oxidation

Thiobacillus acidophilus grown in mixotrophic chemostat cultures exhibited very low apparent substrate saturation constants for the sulphur compounds studied (Table 1). These kinetic parameters for oxidation of reduced sulphur compounds by *T. acidophilus* were comparable with those determined with its obligately autotrophic counterpart, *Thiobacillus ferrooxidans* (Hazeu et al. 1986). This, together with the constitutive nature of oxidation of reduced sulphur compounds in *T. acidophilus* (Table 1), may be advantageous in the competition of *T. acidophilus* with obligate autotrophs in environments where both inorganic sulphur compounds and organic substrates are available.

Oxidation of sulphide, thiosulphate and trithionate by cell suspensions of mixotrophically grown *T. acidophilus* proceeded in two phases, reflecting the transient accumulation of intermediary sulphur compounds. It should be realized that the occurrence of biphasic oxygen uptake patterns depends on the substrate concentration added and on the grown conditions. For example, biphasic oxygen uptake patterns were not observed with very low concentrations of thiosulphate or during tiosulphate oxidation by cells from heterotrophic cultures. Apparently the rate of tetrathionate degradation by these cell suspensions was equal to the rate of tetrathionate formation from thiosulphate and trithionate. Significant accumulation of intermediary sulphur compounds did not occur under substrate-limited growth conditions in chemostat cultures (Prönk et al. 1990b).

Localization of sulphur compound-oxidizing enzyme systems

The pH optima for the oxidation of sulphur compounds by *T. acidophilus* cells fell within the pH range for growth (pH 2–5) of this acidophile (Fig. 1). A notable exception was sulphite, which was hardly oxidized at pH 3, the pH at which the cells had been grown (Fig. 1). Maximum sulphite oxidation rates were observed at pH 5.5. A high pH optimum for sulphite oxidation has also been reported for the acidophilic chemolithoautotroph *Thiobacillus thiooxidans* (Kodama and Mori 1968). The uncoupler sensitivity of sulphite oxidation (Table 2) may indicate that an energized membrane is required, possibly for energy-dependent uptake of sulphite. If it is assumed that the carrier protein here involved in the uptake of sulphite is, in fact, a sulphate carrier, the sulphite has to be in the dissociated form (SO$_4^{2-}$) to be recognized by the carrier protein. Considering the dissociation constants of H$_2$SO$_3$ (pK$_1$ = 1.77; pK$_2$ = 7.21) a pH optimum for sulphite uptake is then expected around pH 7. However, in view of the decreased metabolic activity at this neutral pH, the lower pH optimum for sulphite-dependent oxygen uptake (i.e. sulphite uptake and sulphite oxidation) is not surprising.

The oxidation of thiosulphate, trithionate and tetrathionate was not inhibited by DNP (Table 2). The inhibition of tetrathionate and trithionate oxidation by CCCP is therefore probably a pleiotropic effect. The stimulation of thiosulphate oxidation by DNP and CCCP can be explained by uncoupling of respiratory control. The insensitivity of the oxidation of the sulphur oxyanions towards uncoupers suggests that the oxidation of these compounds does not require membrane energization. Our present hypothesis is therefore, that, with the exception of sulphite, initial oxidation of the sulphur oxyanions occurs extracytoplasmically.

The oxidation of sulphide and elemental sulphur by cell suspensions was strongly inhibited by uncoupers (Table 2), suggesting that sulphide is actively transported across the membrane and oxidized in the cytoplasm.

Pathways of sulphur compound oxidation in *Thiobacillus acidophilus*

*T. acidophilus* employs the metabolic sequence from trithionate via thiosulphate to tetrathionate (Fig. 5). We recently purified trithionate hydrolase and thiosulphate oxidoreductase (Meulenbergs, unpublished). These enzymes catalyse the hydrolysis of trithionate to thiosulphate and the oxidation of thiosulphate to tetrathionate, respectively.

Oxidation of trithionate via thiosulphate to tetrathionate has previously been demonstrated in a variety of obligately autotrophic thiobacilli (*T. thiooxidans*: Okuzumi 1966, Okuzumi and Kita 1965; *T. tepidarius*: Lu and Kelly 1988; *T. neapolitanus*: Trudinger 1961 and 1964a; *T. ferrooxidans*: Sinha and Walden 1966; for a review see Prönk et al. 1990c). Thiocyanate metabolism in *T. acidophilus* differs significantly from that in the neutrophil facultative autotroph *Thiobacillus versus*: Oxidation of thiosulphate by the latter organism does not involve tetrathionate as an intermediate (Lu and Kelly 1984).

The formation of equimolar amounts of thiosulphate, sulphur and sulphate from tetrathionate under anaerobic conditions (Fig. 3 and 5) has also been demonstrated with the obligately autotrophic neutrophil *T. neapolitanus* (Trudinger 1964b). A hydrolytic cleavage of tetrathionate, yielding disulphane monosulphonatic acid (S$_2$O$_4^{2-}$) and sulphate has recently been suggested for
During aerobic incubation of cell suspensions of *T. acidophilus* with tetrathionate, no formation of intermediary sulphur could be detected. This suggests that, during oxidation of tetrathionate, the rate of sulphur oxidation equals the rate of sulphur formation. Increase of OD$_{430}$ due to formation of intermediary sulphur, could be detected when further oxidation of sulphur was inhibited by NEM or exclusion of oxygen.

Transient accumulation of intermediary sulphur was observed during oxidation of sulphide by *T. acidophilus* (Fig. 4). Similar results have been reported for *T. ferrooxidans* (Hazeu et al. 1988). The observed stoichiometries of sulphide-dependent oxygen consumption indicated that the intermediary sulphur is of the same redox level as elemental sulphur. The subsequent oxidation of intermediary sulphur exhibited a number of similarities with the oxidation of exogenously added elemental sulphur: both processes were uncoupler-sensitive and could be inhibited by NEM (Table 2). These similarities suggest that the oxidation pathways of intermediary sulphur and elemental sulphur share a common intermediate (Fig. 5).

The almost complete inhibition of elemental sulphur oxidation by uncouplers has previously been described for other sulphur-oxidizing bacteria. From their work on *T. ferrooxidans*, Bacon and Inglede (1989) recently suggested that an energized membrane is required for activation of the chemically inert S$_8$ ring structure. Alternatively, the uncoupler sensitivity of elemental sulphur oxidation may reflect the involvement of active transport mechanisms.

At present, it is unclear whether or not sulphite is an intermediate during the oxidation of sulphur compounds by *T. acidophilus*. Accumulation of sulphite during the oxidation of any of the sulphur compounds tested was not observed (results and shown). Moreover, the sulphite oxidation rates observed at physiological pH values were not observed (results not shown). Moreover, the sulphite sulphur atoms during sulphur compound oxidation. However, it is possible that the observed oxygen uptake rates give an underestimate of the oxidation capacity for intracellularly generated sulphite.

Further research will be focused on the enzymes involved in the metabolism of the inorganic sulphur compounds by *T. acidophilus* and on the intermediates formed during the oxidation of sulphine and elemental sulphur.

**Acknowledgements**. We thank Prof. Dr. R. Steudel (Department of Inorganic and Analytical Chemistry, University of Technology, Berlin, FRG) and Dr. J. P. van Dijk (Delft University of Technology) for stimulating discussions. We are grateful to Peter de Bruijn (of our department) for help with the HPLC analyses.

**References**


Hanes CS (1932) Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of paraffine oil. Biochem J 26:1406–1421


