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Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli

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Key words: Sulphur metabolism; *Thiobacillus*; Acidophiles; Leaching

1. INTRODUCTION

Acidophilic sulphur-oxidizing bacteria were first isolated from acidic mine effluents [1], where they are the causative agents of the environmental problem acid mine drainage. Furthermore, acidophilic thiobacilli are at least partially responsible for the development of acid sulphate soils [2]. Over the past decades there has been a growing interest in the application of this type of organisms in the biological leaching of metal ores [3] and the biological desulphurization of coal [4,5].

The key reaction in the processes mentioned above is the biological oxidation of pyrite (FeS_2) to ferric sulphate and sulphuric acid. *Thiobacillus ferrooxidans* is widely used as a model organism to study the biological oxidation of pyrite. *T. ferrooxidans* is an obligately chemolithoautotrophic, aerobic, Gram-negative bacterium. Energy sources for autotrophic growth include ferrous iron and a number of reduced inorganic sulphur compounds [6]. Most studies into the physiology and bio-en-

ergetics of *T. ferrooxidans* have focused on the oxidation of ferrous iron (for a review, see [7]).

During the complete oxidation of pyrite, only one electron is derived from the ferrous iron part of the mineral, whereas 14 electrons are derived from the sulphur moiety (Fig. 1). Apart from this quantitative difference between the reduction equivalents derived from ferrous iron and sulphur, there is also a qualitative difference. The growth yield of *T. ferrooxidans* (expressed as the amount of biomass produced per mol of electrons) is much higher during growth on reduced sulphur compounds than during growth on ferrous iron (Table 1). Also the growth yield per mol of electrons on pyrite is significantly higher than the molar growth yield on ferrous iron [8]. This observation suggests that the sulphur part of the mineral is oxidized biologically to a significant extent, rather than exclusively via an indirect non-biological oxidation with ferric iron.

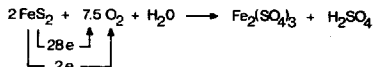


Fig. 1. Oxidation of pyrite. Note that for every 15 electrons available from the oxidation of pyrite, only one electron is derived from the ferrous iron part of the mineral.

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Table 1

Growth yields of *T. ferrooxidans* grown on ferrous iron, tetrathionate and pyrite [8]

Growth limitation	Mode of cultivation	Growth yield (g dry weight/mol electrons)
Ferrous iron	Chemostat ($D = 0.034 \text{ h}^{-1}$)	0.23
Tetrathionate	Chemostat ($D = 0.030 \text{ h}^{-1}$)	0.92
Pyrite	Batch	0.35-0.5

To facilitate comparison, growth yields are expressed as biomass produced per mol of electrons. Growth yields on pyrite were estimated from CO_2 and O_2 consumption rates during growth on pyrite in 10-l batch cultures [59] and represent a minimum value.

In view of the apparent importance of the reduction equivalents derived from the sulphur moiety of pyrite, work in our group is focused on the physiology and enzymology of the oxidation of reduced inorganic sulphur compounds by *T. ferrooxidans*.

Due to its autotrophic lifestyle, the biomass yields of *T. ferrooxidans* in batch and chemostat cultures are very low. This is a major disadvantage during biochemical studies, where large amounts of biomass are frequently required (e.g. for enzyme purification procedures).

To overcome the practical problems associated with the use of *T. ferrooxidans* as a model organism, we recently decided to introduce *Thiobacillus acidophilus* as a second model organism. This organism was initially isolated as a contaminant of a ferrous iron-grown culture of *T. ferrooxidans* [9]. Like *T. ferrooxidans*, *T. acidophilus* is an acidophilic bacterium with a pH optimum of approximately 3. Substrates for autotrophic growth include a number of reduced sulphur compounds, but not ferrous iron [6,9]. In contrast to *T. ferrooxidans*, the organism is also capable of heterotrophic growth on a number of simple organic compounds [9]. It has recently been demonstrated that growth of *T. acidophilus* on mixtures of glucose and reduced sulphur compounds leads to an increase both of the biomass yields and the specific oxidation rates with a number of reduced sulphur compounds ([10]; Pronk,

unpublished). These properties make *T. acidophilus* an attractive model organism to study the oxidation of reduced sulphur compounds in acidic environments.

From work on sulphur oxidation by various neutrophilic and acidophilic thiobacilli, it becomes increasingly clear 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 [11,12]. 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 [13], implying that the enzymes involved should be able to function at low pH values. Studies of sulphur compound oxidation by these bacteria would not only be useful from an academic and applied point of view, but also may yield useful information on the mechanisms underlying biological activity in this type of 'extreme environment'.

Aim of the present paper is to provide an overview of the current knowledge on the oxidation of a number of reduced inorganic sulphur compounds by three acidophilic thiobacilli: *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus*. The sulphur compounds that will be discussed are sulphide, elemental sulphur, thiosulphate, tetrathionate and sulphite. Apart from a review of the relevant literature, some as yet unpublished results from work in our own laboratory are discussed.

2. OXIDATION OF SULPHIDE

Sulphide is the most reduced species of the inorganic sulphur compounds. At concentrations in the millimolar range it reacts spontaneously and at significant rates with oxygen, in particular at pH values above 6 [14]. The spontaneous oxidation of sulphide may produce a number of products, including thiosulphate, sulphite and elemental sulphur [14,15]. Particularly at low sulphide

concentrations and in acidic environments, the biological oxidation of sulphide can compete effectively with the chemical process. The reactivity of sulphide is a major problem in physiological studies into the mechanism of sulphide oxidation. Perhaps for this reason, publications on sulphide oxidation by acidophilic thiobacilli are few and far between.

In contrast to the growth of acidophilic thiobacilli on insoluble sulphides such as ferrous sulphide and pyritic minerals, growth on free sulphides has received little or no attention [6], although it is known that *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus* readily oxidize sulphide ([16,17]; Meulenberg, unpublished). This is probably due to the experimental problems related to the high rate of spontaneous oxidation of sulphide in combination with its low solubility at low pH values. In our laboratory we have recently focused on the oxidation of sulphide by *T. ferrooxidans* and *T. acidophilus*. Both organisms exhibit a high affinity towards sulphide. The K_s for sulphide of both organisms is approximately $5 \mu\text{M}$ ([16]; Meulenberg, unpublished). Intact cells exhibit sulphide-oxidizing activity over a broad range of pH values (pH 1–6), with an optimum at pH 2–4 ([16]; Meulenberg, unpublished).

Oxidation of sulphide by cell-free extracts was first mentioned by London and Rittenberg [17]. Cell-free extracts of *T. concretivorus* (now *T. thiooxidans*) were reported to catalyse the complete oxidation of sulphide to sulphate with thiosulphate, trithionate and tetrathionate as intermediates. However, these observations should be interpreted cautiously, since high sulphide concentrations were used at near-neutral pH values. Furthermore, sample preparation involved heat-deproteinization.

A high affinity sulphide-oxidizing system ($K_s = 2 \mu\text{M}$) associated with the cell envelope of *T. thiooxidans* was studied by Moriarty and Nicholas [18,19,20]. Cell-free extracts catalysed the oxidation of sulphide with oxygen as an electron acceptor. Sulphide oxidation by cell-free extracts proceeded at high rates and could be coupled to the reduction of respiratory chain components and the phosphorylation of ADP. The initial step in sulphide oxidation was the formation of a con-

jugated sulphur compound, which was bound to a membrane fraction. Since this initial step led to the consumption of approximately one mole of oxygen for each 2 mol of sulphide oxidized, it was concluded that the initial product probably consisted of either elemental sulphur or long polysulphide chains. Copper-chelating compounds inhibited the initial phase of sulphide oxidation, suggesting a role of copper in the sulphide-oxidizing enzyme system. The sulphide-oxidizing system was active between pH 5 and pH 9. However, since oxygen was used as an electron acceptor in these studies, these properties cannot be attributed exclusively to a sulphide oxidoreductase enzyme, but may also reflect properties of the respiratory chain which couples the oxidation of sulphide to the reduction of molecular oxygen.

Also in *T. ferrooxidans* and *T. acidophilus* the oxidation of sulphide proceeds via some form of conjugated sulphur [21]. Formation of inter-

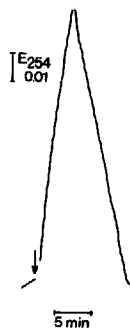


Fig. 2. Formation of long-chain intermediary sulphur compounds during the oxidation of sulphide by a *T. ferrooxidans* cell suspension. Sodium sulphide ($80 \mu\text{M}$) was added at the time indicated by the arrow. Formation of intermediary sulphur compounds was monitored by following the optical density at 254 nm. *T. ferrooxidans* was pregrown in thiosulphate-limited chemostat cultures ($D = 0.02 \text{ h}^{-1}$) [16].

mediary sulphur by cell suspensions can be monitored by following the optical density in the UV region (Fig. 2). Accumulation of significant amounts of intermediary sulphur also leads to an increase in the turbidity of cell suspensions. After staining with silver nitrate, formation of intermediary sulphur can be visualized by electron microscopy [21,22]. Close examination of thin sections of silver-stained *T. ferrooxidans* cells revealed that intermediary sulphur was mainly localized between the outer membrane and the cytoplasmic membrane. This observation suggests that in *T. ferrooxidans* the formation of intermediary sulphur from sulphide may be a periplasmic process.

Fresh cell suspensions of *T. ferrooxidans* oxidize sulphide completely to sulphate, with a transient accumulation of intermediary sulphur. Upon the addition of the inhibitors *N*-ethylmaleimide or CCCP, sulphide is incompletely oxidized with the uptake of approximately 1 mole of oxygen for each two moles of sulphide. A similar oxidation pattern is observed after freezing and thawing of cell suspensions [21]. These observations are compatible with the formation of intermediary sulphur consisting of elemental sulphur (mainly S_8) and higher polythionates, as found during the oxidation of tetrathionate by *T. ferrooxidans* [23]. Although the possibility that during sulphide oxidation polysulphides rather than polythionates are formed cannot be excluded, since in both cases the bulk of the compound should be in the state of elemental sulphur, this seems unlikely in view of the instability of polysulphides at low pH.

Also in *T. acidophilus* the oxidation of intermediary sulphur can be inhibited by the addition of NEM (Meulenberg, unpublished). Interestingly, the ability of *T. acidophilus* to oxidize sulphide to sulphate is also dependent on its growth history. Upon the addition of sulphide, cells from mixotrophic chemostat cultures rapidly produce sulphate with only a minor transient accumulation of intermediary sulphur which is subsequently oxidized to sulphate. In contrast, cells from heterotrophic cultures rapidly oxidize sulphide to intermediary sulphur, but the further oxidation to sulphate proceeds only at very low rates (Meulenberg, unpublished).

3. OXIDATION OF ELEMENTAL SULPHUR

Elemental sulphur occurs mainly in the form of S_8 rings and, in contrast to the inorganic sulphur anions, its water solubility (approximately $5 \mu\text{g/l}$) is very low [23,24]. In view of the low water solubility and hydrophobicity of elemental sulphur it seems likely that surface effects play a role in its biological oxidation. The presence of surface-active agents ('wetting agents') has been demonstrated in culture supernatants of *T. thiooxidans* and *T. ferrooxidans* grown on elemental sulphur [25,26]. Furthermore, growth of *T. thiooxidans* and *T. acidophilus* on elemental sulphur can be stimulated by the addition of surface-active agents to the growth media [25,27].

Although the accessibility of sulphur particles may be enhanced by the addition of surface-active agents or by increasing the area available for microbial action, the S_8 molecule as such is rather inert. Therefore, biological oxidation of elemental sulphur is likely to include an initial activation step, in which the S_8 structure is made susceptible to further microbial oxidation. In a recent publication, Bacon and Ingledew [28] have demonstrated that *T. ferrooxidans* releases small amounts of hydrogen sulphide during aerobic and anaerobic incubation with elemental sulphur, suggesting that the activation step may involve an initial reductive step.

Oxidation of elemental sulphur has been studied extensively in *T. thiooxidans*. Sulphur oxidation by intact cells occurs over a broad pH range, with an optimum at around pH 4 [29]. Oxidation of elemental sulphur is inhibited by freezing and thawing of cells, by inhibitors of respiration like cyanide, carbon monoxide and azide and by a number of metal-chelators, including diethyldithiocarbamate. Organic acids were found to inhibit sulphur oxidation when added at pH values below their pK_a [30].

Intact cells of *T. ferrooxidans* and *T. acidophilus* oxidize elemental sulphur over a broad range of pH values (Fig 3). In *T. ferrooxidans*, the rate of elemental sulphur oxidation is stimulated by the presence of sulphate ions. This sulphate effect is very specific. The only anion which can replace sulphate is selenate [21]. The oxidation of elemen-

tal sulphur by *T. ferrooxidans* cells can be inhibited by the addition of -SH reagents like *N*-ethylmaleimide, by freezing and thawing of cells and by the addition of uncouplers [2,21]. Published schemes for the oxidation of reduced sulphur compounds proposed in the literature involve sulphite as an essential intermediate. However, formation of sulphite from elemental sulphur by intact cells of acidophilic thiobacilli has not been reported in the literature. When *T. ferrooxidans* cells are incubated with elemental sulphur at pH values above 5.0, significant amounts of sulphite are formed (Fig. 4), demonstrating that indeed sulphite can be an intermediate of elemental sulphur oxidation. Oxidation of elemental sulphur at lower pH values does not result in the accumulation of sulphite. This observation can be explained by the low pH optimum for sulphite oxidation by *T. ferrooxidans* (Fig. 3).

Low rates of thiosulphate formation from elemental sulphur have been observed with cell-free extracts of *T. thiooxidans* prepared under a nitrogen atmosphere [31]. The enzyme activity, which required reduced glutathione (GSH) for activity, was partially purified and exhibited optimum activity at pH 7.5. Catalase stimulated the reaction after prolonged incubation times, whereas KCN was inhibitory [31]. The initial product of sulphur oxidation by cell-free extracts of *T. thiooxidans* was identified as sulphite [32]. The formation of

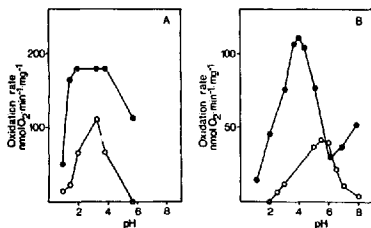


Fig. 3. pH optima of elemental sulphur (●) and sulphite (○) oxidation by *T. ferrooxidans* (A) and *T. acidophilus* (B). *T. acidophilus* was pregrown in a mixotrophic chemostat culture (5 mM glucose, 20 mM thiosulphate, $D = 0.05 \text{ h}^{-1}$, pH = 3.0, $T = 30^\circ\text{C}$). *T. ferrooxidans* was pregrown in a thiosulphate-limited chemostat culture ($D = 0.02 \text{ h}^{-1}$) [16].

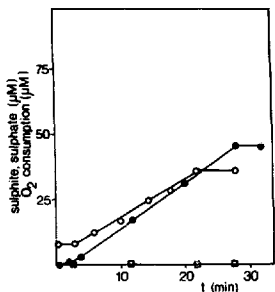


Fig. 4. Formation of sulphite during the oxidation of elemental sulphur by *T. ferrooxidans*. Prior to the addition of elemental sulphur ($3 \text{ mg} \cdot \text{l}^{-1}$ final concentration), the pH of the cell suspension was increased to pH 5.5. *T. ferrooxidans* was pregrown in a thiosulphate-limited chemostat culture [16]. ○, sulphite; ●, O₂ consumption; □, sulphate.

thiosulphate can be explained from a chemical reaction of sulphite with elemental sulphur. Based on experiments with ¹⁸O, Suzuki [33] concluded that an oxygenase was involved in the formation of thiosulphate. However, ¹⁸O incorporation in thiosulphate did not occur with all preparations tested and the overall incorporation of ¹⁸O was very low in all experiments. From a bio-energetic point of view, direct oxygenation of sulphur with molecular oxygen seems wasteful, since such a process does not involve transfer of electrons via an electron transport chain.

Using a crude cell-free extract of *T. thiooxidans*, containing a particulate fraction, Kodama and Mori [34] observed GSH-independent oxidation of sulphur. Anaerobic conditions during cell disruption were necessary to obtain active cell-free extracts. The active components could be separated in an oxygen-sensitive, particulate fraction and a soluble fraction, both of which were needed for catalytic activity. The soluble fraction contained both high molecular weight components and a low molecular weight component. The latter could be replaced by either NAD or NADP [35]. The high molecular weight soluble fraction contained two essential proteins, a 120 000-Da non-heme iron protein and a 23 000-Da non-heme iron

flavoprotein [36]. *T. thiooxidans* cell-free sulphur-oxidizing systems have been reported to be sensitive to metal-chelating compounds, CO and thiolbinding agents [34,36,37].

Oxidation of elemental sulphur by a cell-free preparation of *T. ferrooxidans* was first described by Silver and Lundgren [38]. Like the *T. thiooxidans* cell-free system described by Suzuki [31], this system required GSH for activity and oxidized elemental sulphur to sulphite. The partially purified enzyme had a pH optimum of 7.8 and contained non-heme iron and acid-labile sulphide.

With intact cells of both *T. ferrooxidans* and *T. thiooxidans*, ferric ions can act as electron acceptors for the oxidation of elemental sulphur [39,40]. Based on these observations, Sugio et al. [41] proposed a mechanism for elemental sulphur oxidation by *T. ferrooxidans*. In this Fe^{3+}/Fe^{2+} cycling model, Fe^{3+} is the initial electron acceptor for the oxidation of elemental sulphur. According to the model, ferrous iron formed during sulphur oxidation is then oxidized by the ferrous iron-oxidizing enzyme system present in *T. ferrooxidans*. An enzyme catalysing the oxidation of elemental sulphur to sulphite with ferric iron as an electron acceptor was detected in cell-free extracts and purified to homogeneity [42]. Since osmotic shock treatment resulted in the release of enzyme activity from the cells, a periplasmic localization was proposed, although the purified enzyme preparation exhibited a pH-optimum of 6.5. Enzyme activity was strictly dependent on the addition of GSH. The purified enzyme consisted of two 23000-Da subunits [42].

The physiological significance of this sulphur-ferric iron oxidoreductase has been questioned. In *T. ferrooxidans*, the oxidation of sulphur, but not the oxidation of ferrous iron, is inhibited by HOQNO (2-*n*-heptyl-4-hydroxyquinoline *N*-oxide). This suggests involvement of the *bcl* complex in electron transfer from elemental sulphur to oxygen. This respiratory chain component is apparently not involved in electron transfer from ferrous iron to oxygen, which as pointed out by Corbett and Ingledew [43] is in contradiction with the Fe^{3+}/Fe^{2+} cycling model. Secondly, these authors demonstrated that the transfer of electrons from elemental sulphur to ferric iron in-

volves participation of cytoplasmic components, which is not in agreement with the presence of a periplasmic elemental sulphur-ferric iron oxidoreductase as proposed by Sugio et al. [42]. Also the observation that the rates of elemental sulphur-dependent oxygen uptake by *T. ferrooxidans* are independent of the presence of Fe^{3+} -ions seems difficult to reconcile with the Fe^{3+}/Fe^{2+} cycling model [43]. When *T. ferrooxidans* is grown in tetrathionate-limited chemostat cultures, the ability to oxidize ferrous iron is completely repressed. However, cells grown under these conditions retain the ability to oxidize elemental sulphur [16]. Also this observation is in contradiction with an Fe^{3+}/Fe^{2+} cycling mechanism. As mentioned before, growth yields (expressed as g biomass per mol of electrons) of *T. ferrooxidans* grown on reduced sulphur compounds are higher than the molar growth yields on ferrous iron [8]. If oxidation of (intermediary) sulphur would be coupled to the electron transport chain via the reduction of ferric iron, lower growth yields on reduced sulphur compounds would be expected.

Recently, Sugio and co-workers have reported that sulphide, rather than elemental sulphur is the actual substrate for the ferric iron-reducing enzyme [44]. In this recent model, elemental sulphur is first reduced to sulphide with GSH. Subsequently, sulphide is oxidized directly to sulphite with ferric iron as an electron acceptor. If long-chain intermediary sulphur compounds are true intermediates during the oxidation of sulphide [21], it seems difficult to envisage sulphide as an intermediate during the oxidation of elemental sulphur.

4. OXIDATION OF THIOSULPHATE

Thiosulphate is relatively unstable in acidic environments. At pH values below pH 4, decomposition to sulphur and sulphite may occur [45]. For this reason, batch cultures are ill-suited to study growth of acidophilic thiobacilli on thiosulphate. The rate of chemical decomposition strongly depends on the thiosulphate concentration. In thiosulphate-limited chemostat cultures, the rate of chemical decomposition is negligible in compari-

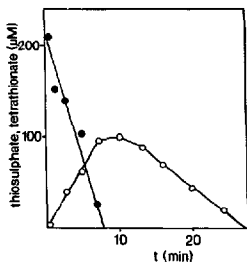


Fig. 5. Formation of tetrathionate (○) during the oxidation of thiosulphate (●) by a *T. ferrooxidans* cell suspension. *T. ferrooxidans* was pregrown in a thiosulphate-limited chemostat culture ($D = 0.02 \text{ h}^{-1}$) [16].

son with the biological oxidation rates, due to the low residual substrate concentrations.

Growth on thiosulphate as a sole source of energy has been demonstrated with *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus* [6]. Different groups have shown that in these three organisms the initial step in thiosulphate metabolism is its oxidation to tetrathionate [46,47]; Meulenber et al., unpublished). When low concentrations ($< 1 \text{ mmol l}^{-1}$) of thiosulphate are added to cell suspensions of *T. ferrooxidans*, transient accumulation of tetrathionate occurs (Fig. 5). Near-quantitative conversion of thiosulphate to tetrathionate can be observed during the first phase of thiosulphate oxidation. This strongly suggests that the oxidation of thiosulphate via tetrathionate is the major, if not the only route of thiosulphate metabolism in *T. ferrooxidans*.

Oxidation of thiosulphate with oxygen as an electron acceptor has been demonstrated in crude cell-free extracts of *T. thiooxidans* [47]. Tetrathionate was formed as an intermediate. Thiosulphate oxidation by these extracts exhibited a low pH optimum.

A thiosulphate-oxidizing enzyme system was purified from cell-free extracts of *T. ferrooxidans* by Silver and Lundgren [48]. The enzyme catalysed the oxidation of thiosulphate to tetrathionate with ferricyanide as an artificial electron acceptor. Enzyme activity was not measured at pH

values below 4.5 because of the instability of thiosulphate at lower pH values. However, enzyme activities were very low at near-neutral pH values, suggesting that the initial step in thiosulphate oxidation by *T. ferrooxidans* occurs extracytoplasmically. The K_m of the purified enzyme preparation, measured at pH 4.5, was 0.9 mM. Kinetic analysis of thiosulphate oxidation by intact cells of *T. ferrooxidans* suggests that the affinity for thiosulphate may be higher at low pH values [16]. The purified enzyme preparation described by Silver and Lundgren [48] showed three bands after acrylamide gel electrophoresis. Further research is needed to assess whether thiosulphate-acceptor oxido-reductase from *T. ferrooxidans* is a multi-subunit enzyme. Although similar enzymes in other thiobacilli have been demonstrated to use cytochrome *c* as an electron acceptor, this has not yet been demonstrated for the *T. ferrooxidans* enzyme. Also with *T. acidophilus* cell-free extracts, oxidation of thiosulphate with ferricyanide as an artificial electron acceptor has been demonstrated [9].

Cell-free extracts of *T. ferrooxidans* also contain rhodanese (thiosulphate-cyanide sulphur transferase, EC 2.8.1.1). *T. ferrooxidans* rhodanese has been purified [49]. Rhodanese activity is not restricted to sulphur-oxidizing bacteria. The enzyme has also been demonstrated in mammalian tissues and in some heterotrophic bacteria [50]. A significant role of rhodanese in thiosulphate oxidation by *T. ferrooxidans* seems unlikely in view of the near-quantitative formation of tetrathionate from thiosulphate observed with cell suspensions (Fig. 5).

5. OXIDATION OF TETRATHIONATE

Tetrathionate formed as an intermediate during the oxidation of thiosulphate is readily oxidized by *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus*. These three organisms are also capable of autotrophic growth with tetrathionate as a sole energy source [6].

In contrast to thiosulphate, tetrathionate is relatively stable in acidic environments. However, in the presence of other reduced sulphur compounds, formation of pentathionate may occur [46].

Cell suspensions of *T. ferrooxidans* and *T. acidophilus* exhibit very high affinities towards tetrathionate, with affinity constants of 4 μM and 0.6 μM , respectively ([16]; Meulenberg, unpublished).

When low concentrations of tetrathionate are added to cell suspensions of *T. ferrooxidans*, a transient increase of the turbidity can be observed [21]. Analysis of acetone extracts of cell suspensions revealed that the increase in turbidity is due to the accumulation of some form of intermediary sulphur. The formation of long-chain sulphur compounds from tetrathionate can be monitored as an increase of the optical density in the UV region. Electron microscopic analysis revealed that intermediary sulphur globules were deposited between the inner and outer membrane of *T. ferrooxidans* cells during the oxidation of tetrathionate.

A detailed chemical analysis of the intermediary sulphur formed from tetrathionate by *T. ferrooxidans* was performed by Steudel and co-workers in collaboration with our laboratory [23]. The intermediary sulphur was found to consist of long-chain (up to S_{13}) polythionates and some elemental sulphur, the latter mainly in the form of S_8^0 . The combination of these compounds might produce the form of hydrophilic sulphur shown in Fig. 6.

A set of reactions has been proposed to explain the formation of polythionates and elemental sulphur from tetrathionate (Fig. 7; [23]). Key intermediates in this pathway of intermediary sulphur formation are the sulphane-monosulphonic acids. These are highly reactive compounds, which may undergo spontaneous elongation reactions to eventually form elemental sulphur and sulphite. The formation of polythionates can be explained from an oxidative condensation of two sulphane-monosulphonic acids. Formation of polythionates from sulphane-monosulphonic acids is also possible under anaerobic conditions. In this case hydrogen sulphide is formed (Fig. 7; [23]).

This proposed scheme for the formation of elemental sulphur and polythionates from tetrathionate has a number of attractive properties:

- The scheme explains the formation and the observed composition of intermediary sulphur

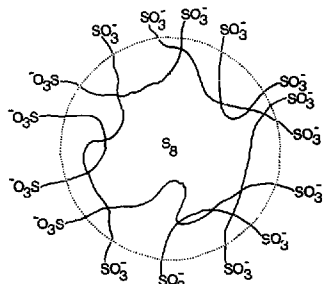
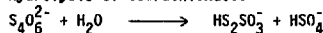


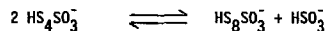
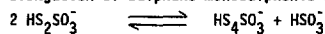
Fig. 6. Proposed structure of hydrophilic intermediary sulphur globules formed during the oxidation of tetrathionate by *T. ferrooxidans*. The intermediary sulphur has been demonstrated to consist of long-chain polythionates and elemental sulphur (mainly in the form of S_8). (Reproduced with permission from [23])

during tetrathionate oxidation by *T. ferrooxidans*. The scheme is in agreement with the observation that formation of intermediary sulphur from tetrathionate may also occur un-

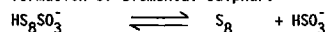
hydrolysis of tetrathionate:



elongation of sulphane-monosulphonic acids:



formation of elemental sulphur:



formation of polythionates:

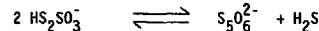
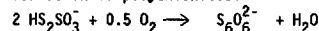


Fig. 7. Proposed pathway for the formation of polythionates and elemental sulphur during the oxidation of tetrathionate by *T. ferrooxidans* [23].

der anaerobic conditions (Hazeu, unpublished).

- Formation of trithionate as an intermediate during tetrathionate oxidation has been reported for *T. ferrooxidans* [46] and *T. thiooxidans* [51,52]. Trithionate can be formed chemically by a reaction of tetrathionate with sulphite [53], which is formed during the chain elongation reactions. Alternatively, trithionate might be formed by (biological) oxidation of S_3 -sulphane-monosulphonic acid.
- The reactions leading to the formation of polythionates and elemental sulphur from S_3 -sulphane-monosulphonic acid are reversible. Therefore, although this scheme explains the transient accumulation of intermediary sulphur after the pulse-wise addition of tetrathionate to *T. ferrooxidans*, it does not imply that long-chain polythionates or elemental sulphur are obligatory intermediates during tetrathionate oxidation. If trithionate can be formed by the biological oxidation of S_3 -sulphane-monosulphonic acid, a cyclic pathway similar to that proposed by Sinha and Walden [46] can be envisaged (Fig. 8). Hydrolysis of trithionate, yielding thiosulphate, has been reported for *T. thiooxidans* [54]. As discussed earlier, the biological oxidation of thiosulphate to tetrathionate is well established in the acidophilic thiobacilli.

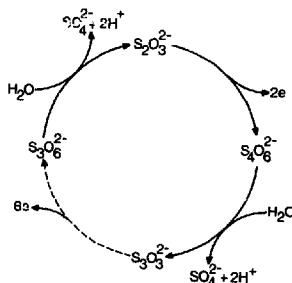


Fig. 8. Hypothetical cyclic pathway for the oxidation of sulphur oxy-anions by acidophilic thiobacilli. The reactions indicated by solid arrows have been demonstrated in *Thiobacillus* species.

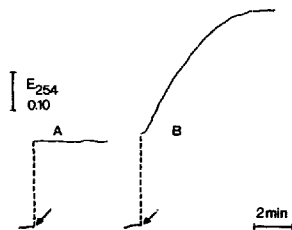


Fig. 9. Formation of long-chain intermediary sulphur compounds from tetrathionate by cell-free extracts of *T. ferrooxidans* in the absence (A) and presence (B) of 50 mM sodium sulphate. Tetrathionate (400 μ M) was added at the time indicated by an arrow. Formation of long-chain sulphur compounds was measured as increase in optical density at 254 nm.

The formation of intermediary sulphur from sulphane-monosulphonic acids according to the scheme proposed by Steudel et al. [23] is reversible. Therefore, these reactions could catalyse the entry of sulphur atoms from elemental sulphur into the cyclic pathway described above. However, such a pathway of elemental sulphur oxidation would require a net input of three sulphite molecules for each S_8 molecule entering the cycle. As yet, there is no experimental evidence to support such a mechanism of sulphur oxidation.

The key enzyme in the scheme suggested by Steudel et al. [23] catalyses the hydrolysis of tetrathionate to yield S_3 -sulphane-monosulphonic acid (Fig. 7). We recently observed that cell-free extracts of *T. ferrooxidans* catalyse the formation of long-chain sulphur compounds from tetrathionate (Hazeu, unpublished). The enzyme activity catalysing this reaction did not require oxygen and was strictly dependent on the presence of sulphate anions (Fig. 9). The only anion which could replace sulphate was selenate. We propose the name tetrathionate hydrolase for this enzyme activity. The product of the reaction, sulphate, has not been measured quantitatively due to the high background sulphate concentrations. The enzyme is active at low, but not at near-neutral pH values (Hazeu, unpublished). This is in agreement with the observation that intermediary sulphur forma-

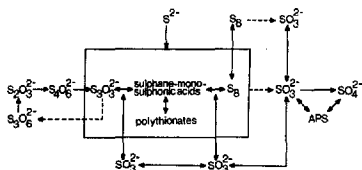


Fig. 10. Scheme for the oxidation of reduced sulphur compounds by the acidophilic thiobacilli. The boxed set of compounds and reactions represents a pool of intermediary sulphur compounds (sulphane-mono-sulphonic acids, polythionates, elemental sulphur and possibly polysulphides). The nature of the intermediary sulphur compounds formed from sulphide and elemental sulphur are unknown. Also the mechanism of sulphite formation from elemental sulphur remains to be elucidated. S_8 -sulphur may combine with polythionates to form hydrophilic sulphur complexes as shown in Fig. 6.

tion occurs between the inner and outer membranes of *T. ferrooxidans* cells [21].

6. OXIDATION OF SULPHITE

Sulphite solutions are readily auto-oxidized in air, a process catalysed by a number of metal ions. The catalytic effect of metal ions can be prevented by the addition of metal-chelating compounds [24].

Sulphite is an essential intermediate in proposed pathways for the oxidation of reduced sulphur compounds by thiobacilli [11,12]. However, autotrophic growth of the acidophilic thiobacilli on sulphite has not been demonstrated [6]. At pH 3, cell suspensions of *T. ferrooxidans* exhibit significant rates of sulphite oxidation. The K_m for sulphite of thiosulphate-grown *T. ferrooxidans* cells is approximately 50 μM [16]. Oxidation of sulphite by this organism can be coupled to the phosphorylation of ADP (Hazeu, unpublished). These observations suggest that sulphite-limited growth of *T. ferrooxidans* is possible when practical problems with sulphite auto-oxidation can be overcome.

Intact cells of *T. thiooxidans* [29] and *T. acidophilus* (Fig. 4) exhibit only very low rates of sulphite oxidation at pH 3. Only when the pH of cell suspensions is increased to approximately pH

6, significant rates of sulphite oxidation can be observed. Sulphite oxidation at these non-physiological pH values may be a (specific) periplasmic reaction. Alternatively, oxidation of exogenous sulphite in these organisms may be catalysed by a cytoplasmic enzyme. In the latter case, the increase in activity at elevated pH values might be explained from an increased accessibility of the enzyme for sulphite.

At the enzyme level, two mechanisms have been proposed for the biological oxidation of sulphite. First, sulphite may be oxidized directly to sulphate by a cytochrome *c*-linked oxidoreductase. Alternatively, sulphite may undergo an oxidative condensation with adenosine 5'-monophosphate (AMP) to form adenosine 5'-phosphosulphate (APS). The liberation of sulphate from APS is coupled to the formation of ADP. AMP can subsequently be regenerated by the action of adenylate kinase. This substrate-level phosphorylation mechanism yields half a molecule of ATP for each molecule of oxidized sulphite. Of course, it must be assumed that the electrons from the AMP-dependent pathway are also fed into the electron transport chain and thus may contribute to the formation of a proton-motive force.

Literature data on the enzymes involved in sulphite oxidation by the acidophilic thiobacilli are sparse and sometimes conflicting. The presence of an APS reductase system in *T. thiooxidans* has been reported by Peck [55]. However, AMP-independent oxidation of sulphite by cell-free membrane preparations of this organism has also been reported [29,37,20]. In the latter papers, sulphite-dependent oxygen uptake was measured at near-neutral pH values.

An AMP-independent, sulphite-oxidizing enzyme has been partially purified from cell-free extracts of *T. ferrooxidans* [56]. Oxidation of sulphite by this enzyme could be coupled to reduction of ferricyanide or horse heart cytochrome *c*. The K_m of the enzyme for sulphite was 0.58 mM. The affinity constant of intact *T. ferrooxidans* cells for sulphite is about ten-fold lower [16]. The enzyme was active over a broad range of pH values (pH 5.4–9.0). No data are available on the activity of this sulphite oxidoreductase at low pH values.

7. CONCLUSIONS

Literature data suggest that there is little unity in the catabolic reactions employed by different sulphur-oxidizing bacteria, even within the genus *Thiobacillus* [11,12]. Based on the data discussed above, it is not possible to construct a definite scheme for the oxidation of reduced inorganic sulphur compounds by the acidophilic thiobacilli. In Fig. 10, an attempt has been made to summarize our view on the current knowledge in this field. This model has been constructed from on one hand intact cell studies and on the other hand work with cell-free extracts and purified enzymes. In the following, we will discuss these two aspects separately.

7.1. Intact cells

Experiments with intact cells of the acidophilic thiobacilli demonstrate that tetrathionate is the first intermediate during the oxidation of thiosulphate.

There is now ample evidence demonstrating that some form of long-chain intermediary sulphur can be formed during the oxidation of sulphide and tetrathionate. In both cases, the intermediary sulphur is hydrophilic in nature [21]. Intermediary sulphur formed from tetrathionate by *T. ferrooxidans* consists mainly of long-chain polythionates and elemental sulphur [23]. The chemical composition of the intermediary sulphur formed from sulphide has not been investigated in detail. It seems likely that, after an initial activation step [28], elemental sulphur also enters an intermediary sulphur 'pool'. Further research is needed to study the composition of intermediary sulphur compounds formed during the oxidation of elemental sulphur and sulphide. Detailed analysis of the chemical structure of the intermediary sulphur may also provide more insight in the reactions involved in the oxidation of sulphide and elemental sulphur.

Chemical analysis of hydrophilic sulphur produced by other microorganisms is needed to see whether its composition is similar to the intermediary sulphur produced by *T. ferrooxidans*. It has been suggested that the amphipatic character of the long-chain polythionates would, in addition

to the structures shown in Fig. 6, also allow the formation of micelles or vesicle-like structures, which would be filled with water [57]. Such structures, or an analogous type of vesicles, might explain the low buoyant density of sulphur globules formed by *Chromatium* species [58].

An attractive model to explain accumulation of intermediary sulphur compounds during tetrathionate oxidation has been proposed by Steudel et al. [23]. In this model, sulphane-monosulphonic acids are key intermediates. Although formation of long-chain intermediary sulphur compounds can be explained by this model, it is not clear whether or not these are obligate intermediates. Alternatively, accumulation long-chain sulphur compounds may reflect a bottleneck, resulting in overflow, in the sulphur-metabolizing pathways. If they are not, accumulation of a reactive intermediate (for example S_2 -sulphane-monosulphonic acid in the model of Steudel et al. [23]) might then set off a series of chemical reactions leading to chain elongation and, eventually, the formation of elemental sulphur. At sub-optimal substrate concentrations, oxidation of substrates might then proceed via a cyclic pathway as shown in Fig. 8. Interestingly, this hypothetical cyclic pathway does not necessarily involve sulphite as an intermediate. It should be stressed that direct enzymatic evidence for the involvement of a cyclic pathway in sulphur compound oxidation by the acidophilic thiobacilli is lacking at present.

Oxidation of elemental sulphur via the cyclic pathway mentioned above would require a net input of sulphite. In contrast to this, production of sulphite can be observed during the oxidation of elemental sulphur by *T. ferrooxidans* cells at elevated pH values (Fig. 4). Although this observation suggests that sulphite is an intermediate during the oxidation of elemental sulphur, the molecular mechanism of sulphite formation remains unclear.

With respect to the oxidation of sulphite, there seems to be a significant difference between on one hand *T. ferrooxidans* and on the other hand *T. acidophilus* and *T. thiooxidans*. In the latter two species, added sulphite is only oxidized at significant rates at elevated, non-physiological pH values. This implies that sulphite generated extra-cy-

toplasmically can not be oxidized by growing cells of these bacteria. Since *T. acidophilus* does not accumulate sulphite during thiosulphate-limited growth (Pronk, unpublished), sulphite is either formed intracellularly or not involved as an intermediate in thiosulphate oxidation by this organism.

7.2. Cell-free systems

Although oxidation of all reduced sulphur compounds discussed here has been demonstrated in cell-free systems, the data obtained are often incomplete and deserve further attention. Experimental work with cell-free systems is complicated by the reactivity of many substrates and intermediates and by the lack of suitable (acid-resistant) artificial electron acceptors.

Sulphide oxidation has been demonstrated with cell-free membrane preparations of *T. thiooxidans* [18–20]. However, the enzyme involved has not been purified, nor has its cellular localization been studied.

Considerable effort has been put into the isolation and characterization of cell-free systems capable of oxidizing elemental sulphur. For various reasons discussed above, the oxygenase reaction reported by Suzuki [33] and the $\text{Fe}^{3+}/\text{Fe}^{2+}$ cycling mechanism proposed by Sugio and co-workers [41,42,44] are unlikely pathways to account for the bulk of elemental sulphur oxidation in any of the acidophilic thiobacilli. Further research is needed to assess the physiological significance of the enzyme activities described by these authors. A detailed study of elemental sulphur oxidation was performed with cell-free preparations of *T. thiooxidans* [34–36]. The results demonstrate that oxidation of elemental sulphur by *T. thiooxidans* requires various enzyme activities, both soluble and particulate. The exact catalytic function of the proteins involved and their cellular localization are still unknown.

In *T. ferrooxidans* and *T. acidophilus* cell-free extracts, the oxidation of thiosulphate to tetrathionate can be coupled to the reduction of ferricyanide [48,9]. The enzyme activity responsible for this reaction has been purified to a significant extent from *T. ferrooxidans* [48]. Although the available data suggest a periplasmic localization

and coupling to the electron transport chain at the level of cytochrome *c*, conclusive evidence is still lacking.

Formation of long-chain sulphur compounds from tetrathionate has been demonstrated with cell-free extracts of *T. ferrooxidans* (Hazeu, unpublished). A tetrathionate hydrolase, producing S_2 -sulphane-monosulphonic acid, has been implicated as the enzyme activity responsible for this reaction. Further characterization of this enzyme activity is currently in progress in our laboratory.

Data obtained with intact cells suggest that the mechanism of sulphite oxidation may differ significantly among the acidophilic thiobacilli. A detailed study of the enzyme activities involved in sulphite oxidation by the different species may provide more insight in a possible role of sulphite as an intermediate during the oxidation of the more reduced inorganic sulphur compounds. Sulphite is a very reactive compound. When studying the enzymology of sulphite oxidation, it is important to rule out aspecific reactions of sulphite (e.g. with electron transport chain components). To assess the physiological significance of sulphite oxidation in cell-free systems, the catalytic behaviour of such systems should always be compared to that of intact cells.

As discussed above, a cyclic pathway as shown in Fig. 8 might be involved in the oxidation of reduced sulphur compounds by the acidophilic thiobacilli. One of the possible key reactions in this process is the oxidative conversion of tetrathionate into trithionate. This conversion may involve S_2 -sulphane-monosulphonic acid as an intermediate. Additionally, a number of other reactive intermediates may be involved. Detailed studies of sulphur compound oxidation by purified, cell-free systems may either prove or disclaim the involvement of the set of reactions shown in Fig. 8.

The characterization of enzymes involved in inorganic sulphur metabolism by the acidophilic thiobacilli is an intriguing, but complicated field of research. An understanding of the reactions involved in the biological oxidation of simple inorganic sulphur species may eventually yield useful information for the application of these organisms for the leaching of metal ores and the desulphurization of coal.

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