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# Anaerobic Oxidation of Thiosulfate and Elemental Sulfur in *Thiobacillus denitrificans*

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**Abstract.** *Thiobacillus denitrificans* strain RT could be grown anaerobically in batch culture on thiosulfate but not on other reduced sulfur compounds like sulfide, elemental sulfur, thiocyanate, polythionates or sulfite. During growth on thiosulfate the assimilated cell sulfur was derived totally from the outer or sulfane sulfur. Thiosulfate oxidation started with a rhodanese type cleavage between sulfane and sulfone sulfur leading to elemental sulfur and sulfite. As long as thiosulfate was present elemental sulfur was transiently accumulated within the cells in a form that could be shown to be more reactive than elemental sulfur present in a hydrophilic sulfur sol, however, less reactive than sulfane sulfur of polythionates or organic and inorganic polysulfides. When thiosulfate had been completely consumed, intracellular elemental sulfur was rapidly oxidized to sulfate with a specific rate of 45 natom S°/min·mg protein. Extracellularly offered elemental sulfur was not oxidized under anaerobic conditions.

**Key words:** *Thiobacillus denitrificans* – Sulfur metabolism – Thiosulfate – Elemental sulfur – Siroheme sulfite reductase – Rhodanese.

Bacteria of the genus *Thiobacillus* derive energy from the oxidation of reduced sulfur compounds (sulfide, elemental sulfur, thiosulfate) to sulfate. In aerobic thiobacilli the electrons liberated are transferred to oxygen either directly as in the oxidation of elemental sulfur to sulfite – this reaction is catalyzed by an oxygenase which utilizes molecular oxygen as substrate – or via an electron transport chain as in the further oxidation of sulfite to sulfate (Suzuki, 1974).

The facultative *Thiobacillus denitrificans* utilizes both oxygen and nitrate as electron acceptors. During growth on nitrate reduced sulfur compounds are oxidized in the absence of molecular oxygen. The mechanism by which anaerobic cells of *T. denitrificans* form sulfite from elemental sulfur and/or sulfide, therefore, must be different from the oxygenase-type reaction in aerobic thiobacilli.

In a preceding publication we reported that cells of *T. denitrificans* contain large i.e. "dissimilatory" amounts of a siroheme-containing sulfite reductase (Schedel and Trüper, 1979). This enzyme in its molecular and catalytic properties was very similar to desulfoviridin, the sulfite reductase isolated from *Desulfovibrio* species. It seems likely that in *T. denitrificans* its in vivo function is not to reduce but rather to form sulfite from either sulfide or elemental sulfur and, therefore, that in its function its corresponds to the elemental sulfur oxygenase of aerobic thiobacilli.

The present paper summarizes experiments on the anaerobic oxidation of elemental sulfur and thiosulfate in whole cells of *T. denitrificans*. They were carried out to elucidate the pathway of sulfite formation from more reduced sulfur compounds in the absence of oxygen and to substantiate the proposed role of siroheme sulfite reductase isolated from *T. denitrificans*.

## Material and Methods

*Culture Medium, Preparation of Concentrated Cell Suspensions*

*Thiobacillus denitrificans* strain RT (Baldensperger and Garcia, 1975) was grown in a medium which contained in 1 l: *A*: 7.5 g $Na_2S_2O_3 \times 5\ H_2O$, 5.0 g $KNO_3$, 0.5 g $MgCl_2 \times 6\ H_2O$, 2 ml of the tenfold concentrated trace element solution of Pfennig and Lippert (1966); *B*: 2.7 g $KH_2PO_4$; *C*: 3.9 g $NaHCO_3$. *A* was dissolved in 930 ml, *B* in 20 ml and *C* in 50 ml distilled water, respectively; all three solutions were autoclaved at 121°C for 20 min and combined after cooling. The pH was 7.0. Cultures were inoculated to 10% (v/v) with a preculture in the same medium taken from the exponential growth phase and incubated at 30°C in almost completely filled 5 l carboys closed with a cotton plug. The final optical density (450 nm) in this medium was 0.9.

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For experiments with concentrated cell suspensions cells were harvested at an optical density at 450 nm between 0.5 and 0.7, centrifuged at room temperature, washed once in an oxygen-free medium that contained in 1 l 3.0 g $KNO_3$, 0.5 g $MgCl_2 \times 6\ H_2O$, 2.7 g $KH_2PO_4$ and 3.9 g $NaHCO_3$ (pH 7.0), resuspended in the same medium to 10 mg protein/ml and used either immediately or stored at room temperature under 75% $N_2$ + 25% $CO_2$ for up to 6 h.

*Experiments with Concentrated Cell Suspensions*

Experiments with concentrated cell suspensions were carried out in a 10 ml or 100 ml cylindrical glass vessel with a water jacket. The vessel was magnetically stirred and closed with a rubber plug that possessed a gas inlet and an outlet and that allowed a pH electrode to be inserted and aliquots of the reaction mixture to be removed. The temperature was 30° C, the gas atmosphere 75% $N_2$ + 25% $CO_2$. Cell suspensions were preincubated for 15 min before the reaction was started by adding a concentrated solution of thiosulfate or an elemental sulfur sol. After different intervals of time aliquots were removed, diluted with 4 volumes of cold (0° C) distilled water and filtered through membrane filters of pore size 0.1 μm. Elemental sulfur was determined in the material retained on the filter, the soluble sulfur compounds in the filtrate.

*Experiments with Growing Cultures*

Experiments with growing cultures were performed in the medium described above in 2 l carboys which were closed with a rubber plug, placed in a water bath at 30° C and stirred magnetically. Cells used for inoculation were taken from a logarithmic preculture, centrifuged under steril conditions and resuspended in distilled water. To achieve anaerobic conditions the gas phase was flushed with sterile nitrogen. Aliquots were removed after different intervals of time with nitrogen pressure.

*Determination of Enzyme Activities*

Rhodanese activity was determined in a modified assay system according to Bowen et al. (1965): 0.9 ml appropriately diluted enzyme solution, 0.1 ml 1 M Tris-acetate (pH 8.7) and 0.5 ml 0.2 M NaCN were preincubated at 30° C for 5 min. The reaction was started with 0.5 ml 0.1 M $Na_2S_2O_3$ and stopped after 10 min with 0.5 ml 35% formaldehyde. The thiocyanate formed was determined by adding 7.0 ml distilled water and 0.5 ml 0.75 M Fe $(NO_3)_3$ in 20% $HNO_3$. The optical density was measured at 460 nm against a blank to which formaldehyde was added before $Na_2S_2O_3$. Thiosulfate reductase activity was measured in a Warburg manometer according to Hashwa (1972); as reductant lipoic acid freshly reduced with borohydride was used. Thiosulfate oxidase was photometrically determined according to Lyric and Suzuki (1970).

1 enzyme unit (U) was defined as that amount of enzyme which utilized 1 μmol $S_2O_3^{2-}$ per min at 30° C.

*Preparative Methods*

A hydrophilic sulfur sol was prepared according to Janek (1933), trithionate according to Stamm and Goehring (1942), pentathionate according to Stamm et al. (1941). Inorganic polysulfides ($S_3^{2-}$) were prepared following the method given by Fehér and Laue (1956), organic polysulfides (Cys-$S_3$-Cys) according to Fletcher and Robson (1963).

*Analytical Methods*

Elemental sulfur was determined in the following assay system: An aliquot containing 0–60 μg elemental sulfur was filtered through membrane filters of pore size 0.1 μm. The filter which retained intra- and extracellular elemental sulfur was incubated in 3 ml 0.1 M NaCN at 90° C for 10 min. After cooling 6.5 ml distilled water and 0.5 ml 0.75 M Fe$(NO_3)_3$ in 20% $HNO_3$ were added. The optical density was measured at 460 nm against a reagent blank. Other sulfur compounds were determined according to the following methods described in the literature: Sulfide (Trüper and Schlegel, 1964), thiosulfate, tri- tetra- and pentathionate (Kelly et al., 1969), sulfite (Grant, 1947), sulfate (Dodgson, 1961). The total sulfur content of biological material was determined as sulfate after complete digestion in $HNO_3/HClO_3$ according to Evans and St. John (1944).

Protein was determined according to Stickland (1951). For dry weight determination *T. denitrificans* cells were centrifuged, washed twice with distilled water and dried over night at 95° C.

*Chromatography of Sulfur Compounds*

Sulfur compounds ($S_2O_3^{2-}$, $S_4O_6^{2-}$, $SO_3^{2-}$, $SO_4^{2-}$) were separated on Dowex ion exchange resin (Cl-form, 1 × 2, 200–400 mesh) according to Trudinger (1964). If sulfide was present, it was removed at pH 5.0 by adding ammonium acetate buffer, adsorbed in 5 N NaOH and determined separately. If sulfite was present, formaldehyde was added to a concentration of 0.5 M. Sulfite was chromatographed as sulfite-formaldehyde adduct.

*Determination of the Activation Energy of Cyanolysis*

0.5 ml 1 M Tris-HCl (pH 9.0) were added to 2.0 ml of a sample containing 3–6 μmol sulfane sulfur (soluble sulfane sulfur containing compounds were used as appropriately diluted solutions in distilled water; from insoluble forms of elemental sulfur an aliquot was filtered through a membrane filter of pore size 0.1 μm, the filter was then placed into 2.0 ml distilled water) and the mixture preincubated at various temperatures between 30 and 90° C. Cyanolysis was started by adding 0.5 ml 0.2 M NaCN pre-equilibrated at the same temperature. To measure the increase in $SCN^-$ 0.5 ml aliquots were removed, mixed immediately with 0.1 ml 0.75 M Fe$(NO_3)_3$ in 20% $HNO_3$ and made up to 2.0 ml with distilled water. The optical density was measured at 460 nm against a reagent blank. The activation energy (dimension: kJ/mol $SCN^-$) was determined from the Arrhenius plot.

*Radioactivity Measurements*

Radioactive samples (0.2 ml, 0.5 ml or 1.0 ml) were mixed with 10 ml of the scintillation cocktail "PCS" obtained from Amersham/Searle, USA, and left in darkness for at least 12 h. Radioactivity was measured in the liquid scintillation counter "Betascint" (Beckman, USA).

*Chemicals*

Tetrathionate was obtained from Merck, Darmstadt, $^{35}S$-thiosulfate from Amersham/Buchler, Braunschweig, cellulose nitrate membrane filters from Sartorius, Göttingen.

## Results

### *Growth of Thiobacillus denitrificans on Various Sulfur Compounds*

Thiosulfate was the only sulfur source which *T. denitrificans* strain RT could use as electron donor for anaerobic growth in batch culture. No measurable increase in protein or sulfate could be found when *T. denitrificans* was grown in the medium described in "Methods" when thiosulfate was replaced by sulfide

(initial concentration: 5 mM), elemental sulfur added as hydrophilic sulfur sol (3 mM), thiocyanate (10 mM), tetrathionate (10 mM), or sulfite (5 mM). Sulfide did not support growth but was oxidized to elemental sulfur, which was deposited outside the cells and not further oxidized.

### *Assimilation of Cell Sulfur During Growth on Thiosulfate*

In order to answer the question which of the two sulfur moieties of thiosulfate – the outer (sulfane) sulfur which has the oxidation state of elemental sulfur or the inner (sulfone) sulfur which has the oxidation state of sulfite – was assimilated during growth on thiosulfate, the following experiment was carried out: *T. denitrificans* was grown on $^{35}S$-thiosulfate labelled either in the sulfane or the sulfone moiety. After different intervals of time cells from appropriate culture volumes were sedimented by centrifugation, washed three times with distilled water, subjected to complete digestion in $HNO_3/HClO_3$, and the radioactivity determined in the remaining solution. Figure 1 shows that the assimilated cell sulfur was radioactively labelled only after growth on sulfane-labelled thiosulfate, but not when sulfone-labelled thiosulfate was used as substrate. During growth on thiosulfate cell sulfur therefore was exclusively assimilated from the outer or sulfane sulfur. From the fact that no radioactive lable appeared in the cell sulfur during growth on sulfone-labelled thiosulfate it further follows that under physiological conditions sulfite is not reduced in *T. denitrificans* in measurable amounts. Otherwise one could expect an exchange of label between sulfone and sulfane sulfur.

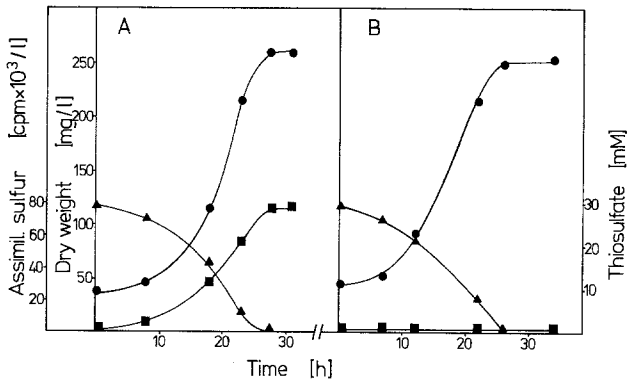


**Fig. 1 A and B.** Assimilation of cell sulfur by *Thiobacillus denitrificans* during growth on $^{35}S$-labelled thiosulfate. From a growing culture samples were removed at different intervals of time and centrifuged. Thiosulfate was determined in the supernatant. The sediment was washed twice with distilled water; after determination of its dry weight it was completely oxidized with $HNO_3/HClO_3$ and made up to a standard volume with distilled water. In an aliquot hereof the radioactivity was measured. **A** Growth of *T. denitrificans* on sulfane labelled thiosulfate (0.85 μCi/mmol $S_2O_3^{2-}$). **B** Growth of *T. denitrificans* on sulfone-labelled thiosulfate (2.0 μCi/mmol $S_2O_3^{2-}$). Thiosulfate (▲); dry weight (●); assimilated cell sulfur (■)

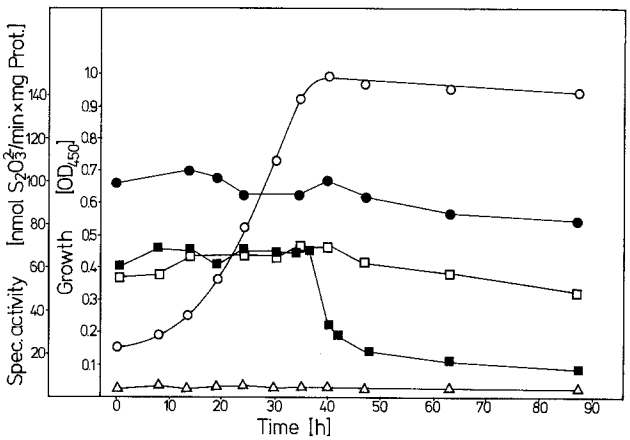


**Fig. 2.** Comparison of thiosulfate uptake rate of whole cells of *Thiobacillus denitrificans* with the specific activities of rhodanese, thiosulfate reductase and thiosulfate oxidase in crude extracts. Experimental details are described in the text and in "*Methods*". Optical density (OD) of growing culture at 450 nm (○); rate of thiosulfate uptake of whole cell (●); rhodanese (□); thiosulfate reductase (■); thiosulfate oxidase (△)

### *The Thiosulfate-Utilizing Enzyme*

The next experiment was carried out to decide which of the three enzymes, rhodanese, thiosulfate reductase or thiosulfate oxidase was operative in *T. denitrificans*.

Aliquots from a *T. denitrificans* culture growing anaerobically on thiosulfate were removed after different intervals of time, centrifuged, washed once with 50 mM K-phosphate (pH 7.0) and resuspended in the same buffer to 10 mg protein/ml (all steps carried out as anaerobically as possible). One half was supplemented with $KNO_3$ to 30 mM and with $NaHCO_3$ to 30 mM (pH adjusted to 7.0). The rate of thiosulfate utilization shown by whole cells was measured with this concentrated cell suspension. The other half was passed twice through a French pressure cell at about 138 MPa. After separating debris and unbroken cells by centrifugation the specific activity of the three enzymes was determined in the supernatant. The result is shown in Fig. 2. The thiosulfate uptake rate of whole cells was about 100 nmol $S_2O_3^{2-}$/min·mg protein and almost independent of the growth phase. Likewise the specific rhodanese activity did not depend on the growth phase, however, amounted only to about 2/3 of the thiosulfate uptake rate of whole cells (70 nmol $S_2O_3^{2-}$/min · mg protein). The specific thiosulfate reductase activity equaled the specific rhodanese activity in the exponential phase, but dropped to about 1/4 of its value (20 nmol $S_2O_3^{2-}$/min · mg protein) in less than half a generation time when thiosulfate in the medium was

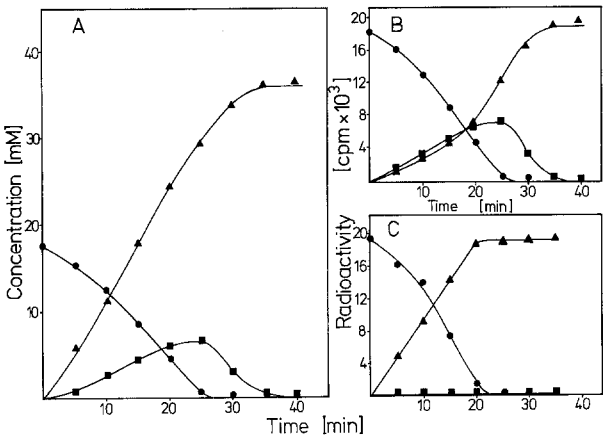


**Fig. 3 A–C.** Thiosulfate oxidation by concentrated cell suspensions of *Thiobacillus denitrificans*. **A** Oxidation of non-labelled thiosulfate. **B** Oxidation of $^{35}$S-sulfane thiosulfate (18 μCi/mmol $S_2O_3^{2-}$). **C** Oxidation of $^{35}$S-sulfone thiosulfate (22 μCi/mmol $S_2O_3^{2-}$). In experiments *B* and *C* the reaction volume was 30 ml. At the time given, 2.0 ml samples were removed. To stop the reaction the temperature was rapidly lowered to 0° C and 0.2 ml 4 M $NH_4$-acetate (pH 5.0) and 0.2 ml 6 M $CH_2O$ were added. Cells were separated by centrifugation. A standard volume of the supernatant was subjected to chromatography on Dowex ion-exchange resin (column: 0.7 cm × 6 cm). Radioactivity was determined in aliquots of the pooled thiosulfate and sulfate fractions of the eluate. To measure the radioactivity of intracellular elemental sulfur the sedimented cells were resuspended in 2.0 ml distilled water. 0.2 ml of this suspension was filtered through a 0.1 μm pore size membrane filter. The filter was incubated in 2 ml 0.1 M NaCN for 10 min at 90° C. After centrifugation radioactivity was measured in an aliquot of the supernatant. Thiosulfate (●); elemental sulfur (■); sulfate (▲)

used up. The specific thiosulfate oxidase activity was low (4 nmol $S_2O_3^{2-}$/min · mg protein). Based on the assumption that the rate of thiosulfate utilization by whole cells is limited by the specific activity of the first enzyme of the pathway of thiosulfate oxidation these results can be taken to indicate that rhodanese is the thiosulfate-splitting enzyme. If thiosulfate was reductively cleaved the rate of thiosulfate uptake of whole cells should also drop at the transition to the stationary phase. The specific thiosulfate oxidase activity was too low to explain the rate of thiosulfate utilization of whole cells.

### *The Anaerobic Oxidation of Thiosulfate*

The kinetic of anaerobic oxidation of thiosulfate to sulfate was studied with concentrated cell suspensions. Figure 3A shows that a substantial amount of elemental sulfur (about 50% of the thiosulfate sulfane sulfur) was transiently accumulated and then rapidly oxidized further to sulfate after all thiosulfate had been taken up. No other intermediary products ($S^{2-}$, $SO_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$, $S_5O_6^{2-}$) could be detected. The elemental sulfur was macroscopically visible by the milky appearance of the cell suspension. However, under the microscope no elemental sulfur droplets could be seen within the cells. To demonstrate that the elemental sulfur was exclusively formed from the sulfane sulfur the experiment was repeated with $^{35}$S-thiosulfate. The intermediary formed elemental sulfur carried radioactivity only when sulfane- (Fig. 3 B) but not when sulfone-labelled thiosulfate was used (Fig. 3C).

### *The Anaerobic Oxidation of Elemental Sulfur*

As shown above, *T. denitrificans* strain RT did not grow on elemental sulfur added to the medium. However, elemental sulfur that was formed from thiosulfate intracellularly could be rapidly oxidized to sulfate (Fig. 3). The obvious lack of a transport system for elemental sulfur functioning in *T. denitrificans* strain RT under anaerobic conditions (under aerobic conditions elemental sulfur was readily oxidized) could be demonstrated in the following experiment (Fig. 4). With one half of a cell suspension prepared as described in "Methods" the experiment of Fig. 3A was repeated. When the maximal amount of elemental sulfur had been formed the reaction was stopped by rapidly cooling the suspension. The cells were washed at 0° C in an oxygen free medium containing 20 mM $KH_2PO_4$, 40 mM $NaHCO_3$, 2.5 mM $MgCl_2$ and 30 mM $KNO_3$ (pH 7.0), resuspended in the original volume of the same medium and incubated under 75% $N_2$ + 25% $CO_2$. When the temperature was raised to 30° C the intracellular elemental sulfur was oxidized to sulfate at a specific rate of 45 natom S°/min · mg protein. When the second half of the original cell suspension was supplied with elemental sulfur in form of a reactive hydrophilic sulfur sol under otherwise identical conditions no oxidation occurred.

### *Characterization of Intracellular Elemental Sulfur*

Aminuddin and Nicholas (1973) showed that membrane bound elemental sulfur, formed during sulfide oxidation by *T. denitrificans*, was present in form of reactive polysulfide sulfane sulfur. In order to demonstrate that the intracellular elemental sulfur formed from the thisosulfate sulfane moiety was deposited in a reactive form, too, we tried to characterize it by comparison of its activation energy in the cyanolytic reaction with that of various other forms of elemental sulfur. Figure 5 shows that the intracellular elemental sulfur had an activation energy of 51 kJ/mol (12.2 kcal/mol) $SCN^-$. It was less reactive than sulfane sulfur present in completely soluble sulfur compounds (organic and inorganic polysulfides, polythionates), however, clearly more reactive than elemental sulfur present as hydrophilic sulfur sol, a form of elemental sulfur, that is generally considered as very reactive.

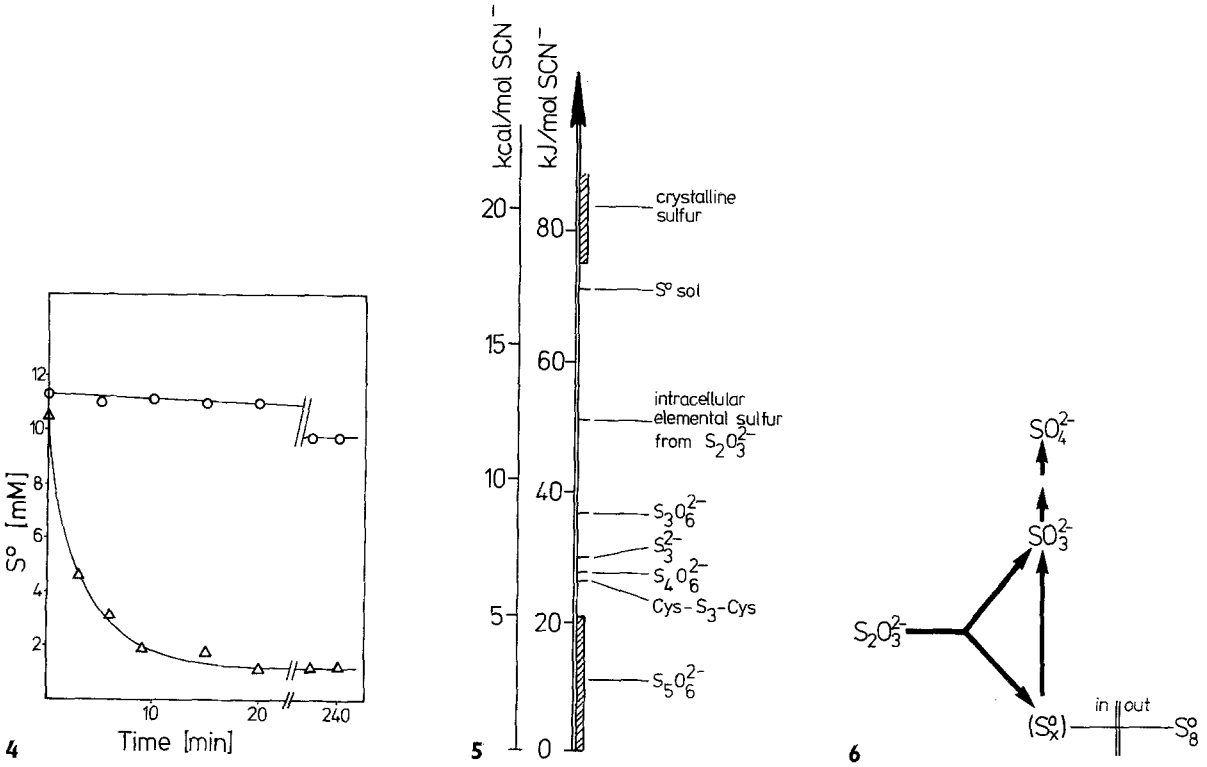


**Fig. 4.** Oxidation of elemental sulfur by concentrated cell suspensions of *Thiobacillus denitrificans*. Experimental details are described in the text. Extracellular elemental sulfur (○); intracellular elemental sulfur (△)

**Fig. 5.** Activation energy of cyanolysis of various forms of elemental sulfur. The activation energy of cyanolysis of intracellular elemental sulfur formed from thiosulfate was determined with whole cells of *T. denitrificans* which were prepared as described for the experiment of Fig. 4. With the method applied the activation energy in the hatched areas could not be determined exactly

**Fig. 6.** Pathway of thiosulfate metabolism in *Thiobacillus denitrificans* strain RT

## Discussion

The anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans* strain RT may be summarized as shown in Fig. 6. Thiosulfate is split by rhodanese to sulfite and elemental sulfur. Elemental sulfur is transiently deposited within the cell in a reactive form. Its oxidation to sulfite and further to sulfate is delayed as long as thiosulfate is present. The rate of elemental sulfur oxidation becomes maximal when all thiosulfate is consumed. *T. denitrificans* strain RT is unable to oxidize extracellular elemental sulfur under anaerobic conditions. Polythionates do not seem to play a role in the anaerobic oxidation of thiosulfate or elemental sulfur.

No results were obtained that support a physiological role of sulfide, neither as substrate for growth in batch culture nor as possible intermediate in thiosulfate utilization. The thiosulfate reductase activity measured in crude extracts of *T. denitrificans* may indeed be a second activity of rhodanese depending on the assay conditions as suggested by Westley (1973). In agreement with this assumption is the fact that the specific thiosulfate reductase activity equaled the specific rhodanese activity during logarithmic growth. When thiosulfate was used up the specific thiosulfate reductase activity dropped, however, to about 1/4 of its original value whereas the specific rhodanese activity remained constant. This result may be explained as follows: When Bowen et al. (1965) studied rhodanese of *T. denitrificans*, they found that the enzyme exists as an enzymatically active polymer, the monomers (which are also catalytically active) being linked by disulfide bridges. Consumption of the electron donor thiosulfate leads to a state of electron deficiency, i.e., to oxidative conditions which induce polymerisation of rhodanese monomers by oxidation of SH-groups. In the enzyme polymer only the small $CN^-$ – but not the bulky lipoate molecule has free access to the active center to accept the split-off sulfane sulfur. Polymerisation leads to a drop of specific thiosulfate but not of specific rhodanese activity.

*T. denitrificans* assimilated its cell sulfur totally from thiosulfate sulfane sulfur, i.e., from sulfur in the oxidation state of elemental sulfur. No sulfite was reduced under physiological conditions. This excludes an assimilatory function of *T. denitrificans* siroheme

sulfite reductase (Schedel and Trüper, 1979) as sulfite-reducing enzyme. Its in vivo function is, therefore, the formation of sulfite from elemental sulfur. Sulfide may be an additional substrate in vitro. Cysteine is synthesized in *T. denitrificans* from sulfide by O-acetylserine sulfhydrylase or from thiosulfate via S-sulfocysteine by S-sulfocysteine synthase (Hensel and Trüper, 1976).

The enzymatic reactions of thiosulfate splitting (rhodanese) and sulfite oxidation to sulfate (adenylylsulfate reductase/ADP – sulfurylase or sulfite oxidase) are very similar in both, aerobic and anaerobic bacteria able to oxidize reduced sulfur compounds (Suzuki, 1974; Trüper, 1978). The principle difference exists in the oxidation mechanism by which elemental sulfur is oxidized to sulfite. Aerobic sulfur oxidizers contain an oxygenase that catalyzes the direct reaction between elemental sulfur and molecular oxygen. Two of the four oxygen atoms of sulfate which is ultimately formed are derived from atmospheric oxygen. Bacteria able to anaerobically oxidize reduced sulfur compounds form sulfite from elemental sulfur in a reaction which is catalyzed by a siroheme-containing enzyme. In this case molecular oxygen is not involved; all four sulfate oxygen atoms are derived from water, if the sulfite oxidase pathway is functioning; three of the sulfate oxygen atoms are derived from water, one from phosphate, however, when the adenylylsulfate reductase/ADP-sulfurylase pathway is functioning.

Siroheme has been described as essential prosthetic group of enzymes catalyzing multiple electron transfer reactions like sulfite and nitrite reductases which transfer six electrons to the substrate (Murphy et al., 1974). Very similar siroheme-containing enzymes have now been shown to play a central role in the anaerobic oxidation of elemental sulfur to sulfite, likewise a multiple electron transfer reaction. In this case four electrons (if sulfide is the substrate: six electrons) are removed from the substrate. Besides from *T. denitrificans* (Schedel and Trüper, 1979) this enzyme has so far been purified and characterized from the phototrophic sulfur bacterium *Chromatium vinosum* (Schedel et al., 1979).

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