Studies on Biochemistry of the Thiobacilli

Part VII. Metabolism of Tetrathionate by *T. thiooxidans*

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Quantitative oxidation of tetrathionate to sulfate was achieved with freshly harvested cells of *T. thiooxidans*; pH optimum for the oxidation of tetrathionate by the washed cells was 2~3, and the activity fell markedly at pH above 3.5.

Tetrathionate might be enzymatically dismutated to pentathionate and trithionate under anaerobic conditions with crude extracts of *T. thiooxidans*; pH optimum for the reaction was about 2.7 and the activity fell strikingly at pH 4.7. The formed trithionate might be further hydrolyzed to thiosulfate and sulfate.

Gleen and Quastel⁶⁾ noted that soil enriched with sulfur-oxidizing bacteria by perfusion with thiosulfate will oxidize tetrathionate without a lag phase and at the same rate as thiosulfate.

Tetrathionate is oxidized rapidly by *T. thioparus*,¹⁾ *T. denitrificans*,²⁾ and by the green sulfur bacterium *Chlorobium thiosulfatophilum*.³⁾ Parker and Prisk⁴⁾ reported that *Thiobacillus X* (apparently identical with *T. thioparus*) oxidized tetrathionate, but failed to find tetrathionate oxidation with *T. thioparus* and *T. thiooxidans*. Vishniac et al.,⁵⁾ however, believed that Parker et al. might have worked with virtually inactive suspensions, and reported that *T. thioparus* grown without Fe²⁺ and Mn²⁺, was unable to oxidize thiosulfate to completion.

The fate of tetrathionate is obscure, but there are some evidences that trithionate or

a closely related compound is formed during tetrathionate oxidation.^{13,14)}

Tamiya et al.^{7,12)} reported that tetrathionate was nonbiologically dismutated to trithionate and pentathionate (equation 1).

On the other hand, according to Vishniac's investigations,^{1,8)} tetrathionate seems to undergo a sort of oxidation which, at least in part, give rise to trithionate, and his manometric data were in agreement with a quantitative conversion of tetrathionate to trithionate (equation 2).

The further innestigation on the biological conversion of tetrathionate are reported in this paper.

MATERIALS AND METHODS

Organism and crude extracts

The organism used throughout this studies was *Thiobacillus thiooxidans* which was isolated in our laboratory. Cells were cultured and harvested by the same procedures as described in the previous paper.¹⁰⁾

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The washed cells were suspended in 0.1 M phosphate buffer (pH 7.0) at a final concentration of 14~16 mg dry weight per ml. The suspension was exposed to a Raytheon sonic oscillator (20 kc/sec., 200W) for fifteen minutes and centrifuged in the cold for thirty minutes at 12,000 r.p.m. to remove intact cells and debris. Solid ammonium sulfate was added to the supernatant with stirring up to 80% saturation. pH of the solution was kept at 7.0 with ammonium hydroxide during the ammonium sulfate fractionation. The precipitate was collected and dissolved in 0.1 M phosphate buffer (pH 7.0). The pH of the crude extracts obtained in this way was carefully adjusted to about pH 3.5 with 1 N H₂SO₄.

Experiments were conducted with the crude extracts except the special cases, in which the conditions were particularly noted somewhere else.

Substrates

Sodium tetrathionate was synthesized according to Auerbach's method,⁹⁾ by the oxidation of sodium thiosulfate with iodine, and the product obtained was recrystallized three times from 50% ethanol.

Measurement of Tetrathionate Oxidation

The tetrathionate oxidation was followed by the oxygen uptake using Warburg manometers. After cessation of the reactions, the residual tetrathionate and formed other compounds were determined.

General Procedure of the Experiments of Tetrathionate Dismutation

Tetrathionate dismutation was carried out under anaerobic conditions in usual Thunberg tubes (1.8 cm external diameter). The crude extracts and some other reagents were placed in the main compartment and tetrathionate was added from the side arm. The reactions were carried out at 29~30°C, and after a certain period of incubation, the residual tetrathionate and formed trithionate, pentathionate and sulfate were immediately determined.

Determinations and paperchromatographys

Determinations and paperchromatographys of inorganic sulfur compounds were by the methods as described in the previous paper.¹⁰⁾

RESULTS AND DISCUSSION

Effect of pH on the Oxidation of Tetrathionate by Washed Cells

Iwatsuka and Mori¹¹⁾ described that a slight uptake of oxygen by washed cells of *T. thiooxidans* was observed at pH 3.0~5.0 when sodium tetrathionate was used as the substrate.

The author also studied manometrically the effect of pH on the oxidation of tetrathionate by washed cells.

FIG. 1. Effect of pH on Oxidation of Tetrathionate by Washed Cells of *T. thiooxidans*.

Each reaction mixture contained 1.0 ml of cell suspension (cells: 8 mg dry weight), and 15 μmoles of sodium tetrathionate to total volume of 2.3 ml. pH was adjusted to desired value by 1 N H₂SO₄. Incubation was durated for 45 minutes at 30°C.

As shown in Fig. 1, the pH optimum for the oxidation by the washed cells lay at about 2~3 and the activity fell markedly at about 3.7. HgNO₃ test and the paper-chromatography at the end of the experiments indicated that a most part of tetrathionate remained without being utilized in the reaction mixture of pH 3.7 and a slight amount of both thio-sulfate and trithionate was formed. Although Parker and Prisk⁴⁾ failed to find tetrathionate oxidation with *T. thiooxidans*, this might be caused either by incubation at pH above 5 or by use of virtually inactive cells.

According to Vishniac's data,¹⁾ tetrathionate was remarkably utilized even at pH 7.0 washed cells of *T. thioparus*, but in our experiments, the freshly harvested cells of *T. thiooxidans* were unable to utilize tetrathionate at pH 7.0. It is interesting that the pH curve obtained with the washed cells for tetrathionate oxidation was nearly similar to that obtained from

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FIG. 2. Oxidation of Tetrathionate by Washed Cells of *T. thiooxidans*.

The reaction mixture (pH 3.0) contained 2.0 ml of the same suspension as employed in the above experiment, and 10 μ moles of tetrathionate to total volume of 2.3 ml. The reaction was carried out at 30°C with Warburg manometer.

thiosulfate oxidation.

Since tetrathionate oxidation took place most readily at pH 3 with the washed cells, manometric experiments of complete oxidation of tetrathionate to sulfate was, further, carried out at the pH value as mentioned above (Fig. 2).

As shown in Fig. 2, *T. thiooxidans* oxidized tetrathionate at a single linear rate until the oxidation was complete. When 10 μ moles of tetrathionate was supplied, the oxygen uptake commenced after about 5 to 10 minutes of induction period, and it proceeded thereafter almost linearly with time for about 4 hours to attain finally (in almost six hours) a stationary level. About 780 μ l of oxygen uptake per 10 μ moles of the substrate was observed, and the value was equivalent to about 35 μ moles. Further, the presence of thiosulfate, trithionate, tetrathionate, and sulfite in the reaction mixture after cessation of the reaction was not indicated by addition of I_2 and $HgNO_3$ reagents. These substances were not identified by the paper chromatography. These results indicated that the quantitative oxidation of tetrathionate to sulfate was achieved with washed cells of *T. thiooxidans* (equation 3).

FIG. 3. Effect of pH on Oxidation of Tetrathionate by Crude Extracts of *T. thiooxidans*.

- 1) pH effect: Each reaction mixture contained 2.0 ml of crude extracts, and 20 μ moles of tetrathionate to total volume of 2.3 ml.
 - 2) Relationship between time course and oxidation of tetrathionate: The reaction mixture had the same composition as described above, except that the pH was 3.0.
- Both the reactions were carried out at 30°C with Warburg manometer; and incubation period in the case of (1) was 120 minutes.

Effect of pH on the Oxidation of Tetrathionate by Crude Extracts

As shown in Fig. 3, the pH optimum for the oxidation lay at about 3, and the activity fell markedly at pH above 3.3; consequently, the result was similar to that obtained with the washed cells.

Further manometric experiment of the oxidation of tetrathionate was carried out at pH 3.0 with the same extract as employed in the above experiment.

The oxygen uptake commenced after about five minutes of induction period, thereafter proceeded almost linearly with time for about 210 minutes to attain finally a stationary level.

Oxygen consumption was about 200 μ l (equivalent to 8.9 μ moles) after 4 hours, and no residue of tetrathionate in the reaction mixture at the end of the incubation was indicated by the addition of $HgNO_3$ reagent. Therefore, 8.9 μ moles of oxygen consumed are equivalent to about a half of 20 μ moles of tetrathionate added initially, and this is in good agreement to the result of the previous paper.¹⁰⁾

Decomposition of Tetrathionate under Anaerobic Condition

According to Tamiya's report,⁷⁾ tetrathionate was nonenzymatically dismutated to trithionate and pentathionate (equation 1).

In our manometric experiments on the tetrathionate oxidation, the lag phase was observed for first ten minutes. Concerning the occurrence of lag phase, it is expected that tetrathionate may be dismutated to tri- and pentathionate, which may be further oxidized.

Thus, experiments on the dismutation of tetrathionate were conducted with the crude extracts under anaerobic condition. The result is shown in Table I.

TABLE I. ANAEROBIC REACTION OF TETRATHIONATE BY CRUDE EXTRACTS OF *T. thiooxidans*

	$S_2O_3^{2-}$ formed (μ moles)	$S_3O_6^{2-}$ formed (μ moles)	$S_5O_6^{2-}$ formed (μ moles)	$S_4O_6^{2-}$ formed (μ moles)
Crude extracts	4.4	7.2	6.3	81.9
Heated extracts	0.0	0.0	0.0	99.2

Each 11.0 ml of the reaction mixtures (pH 3.0) contained 5.0 ml of crude extracts, 600 μ moles of phosphate and 100 μ moles of tetrathionate. Incubation was continued for 60 minutes at 30°C under anaerobic condition.

Heated extract: The extract was boiled at 100°C for 5 minutes.

Firstly, since equimolar trithionate and pentathionate were formed, the dismutation of tetrathionate may take place, as indicated in equation 1. Secondly, it is considered that the heated crude extracts do not attack tetrathionate, since consumption of tetrathionate was little after one hour incubation. Therefore, the tetrathionate dismutation observed with the crude extracts must be an enzymatic reaction. Thirdly, the formation of thiosulfate was observed, and it is considered that the thiosulfate formation may be attributed to reduction of tetrathionate, or to decomposition or either the formed trithionate or pentathionate. Consequently, the oxygen uptake observed in manometric experiments of the tetrathionate oxidation may not be attributed to the oxidation of tetrathionate itself. It is postulated that, under aerobic conditions tetra-

thionate may be first dismutated enzymatically to yield trithionate and pentathionate, which may be further oxidized to consume oxygen secondarily.

Effect of pH on the Decomposition of Tetrathionate

In order to determine optimal pH for the tetrathionate decomposition under anaerobic condition, the experiment was conducted with the crude extracts at such pH values as indicated in Table II.

TABLE II. EFFECT OF pH ON THE DECOMPOSITION OF TETRATHIONATE BY CRUDE EXTRACTS

pH	$S_2O_3^{2-}$ formed (μ moles)	$S_3O_6^{2-}$ formed (μ moles)	$S_4O_6^{2-}$ residue (μ moles)
1.7	32.4	38.1	11.6
2.8	36.8	43.2	2.9
4.7	0	0	94.8
6.4	0	0	95.3

Each reaction mixture contained 5.0 ml of crude extracts, 500 μ moles of phosphate and 100 μ moles of tetrathionate to total volume of 6.5 ml. pH was adjusted to desired value by 1 N H_2SO_4 . All reaction mixtures were incubated at 30°C for 180 minutes under anaerobic condition.

The pH optimum for the decomposition lay at about 2.7, and activity fell strikingly at pH 4.7. This result was similar to the result obtained from the manometric experiment on the effect of pH for the tetrathionate oxidation (Fig. 2). These results suggest that, tetrathionate itself is not oxidized, but is dismutated enzymatically to trithionate and pentathionate, which may be secondarily oxidized to some things.

Quantitative Analysis on Products Obtained in Anaerobic Reaction of Tetrathionate

In above experiments, thiosulfate was formed in anaerobic reactions of tetrathionate, but whether the formation of thiosulfate was attributed to the reduction of tetrathionate itself or to secondary conversion of the products formed by dismutation of tetrathionate remained unknown. Thus, experiments were carried out in order to investigate relations between time course and thiosulfate formation under anaerobic conditions with crude ex-

FIG. 4. Decomposition of Tetrathionate by Crude Extracts.

The reaction mixture contained 5 ml of crude extracts, 500 μ moles of phosphate and 100 μ moles of tetrathionate to total volume of 7.0 ml. The reaction was carried out at 30°C under anaerobic condition.

tracts. The result is illustrated in Fig. 4.

As shown in the figure, the formation of pentathionate proceeded linearly from the beginning. The formation of thiosulfate commenced after a while, and it proceeded thereafter almost linearly with time. Equimolar formation of thiosulfate and pentathionate was observed during about three hours' incubation. In this connection, trithionate was roughly equimolar to pentathionate for first one hour of incubation and it diminished thereafter slowly with time. On the other hand, when the heated extracts were employed instead of the intact extracts, tetrathionate as substrate was little consumed all the time, and the percentage of tetrathionate recovered was 99.4%. In case in which the substrate was not added

to the reaction mixture, the formation of thiosulfate, sulfite, tri-, tetra-, and pentathionate was not found throughout the incubation period.

At the early stage, the equimolar formation of trithionate and pentathionate was observed, while a little thiosulfate was formed, and thereafter, the formation of thiosulfate as well as pentathionate increased with time, whereas the trithionate diminished. Huzisige and Haga¹²⁾ expressed that trithionate was biologically hydrolyzed to yield thiosulfate and sulfate with washed cells of *T. thioparus* (equation 4).

If the hydrolysis of trithionate had not taken place, the formation of trithionate would proceed almost linearly with time, and would be equimolar to the pentathionate.

Accordingly, these facts suggest that tetrathionate may firstly be dismutated to yield trithionate and pentathionate, and the formed trithionate may be secondarily hydrolyzed to form thiosulfate and sulfate (equations 1 and 4).

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