THE METABOLISM OF THIOBACILLUS THIOPARUS

I. The Oxidation of Thiosulfate

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There is evidence that the metabolism of inorganic sulfur compounds follows one general pathway between sulfide and sulfate. Thiosulfate, polythionates, and sulfite arise as intermediate products in this reaction chain. Guittonneau (1926) and Guittonneau and Keilling (1932) showed that sulfur was oxidized rapidly in the soil by common heterotrophic organisms, and that thiosulfate and polythionates arose transiently. The reduction of sulfite by mutants of *Escherichia coli* unable to use sulfate was described by Lampen *et al.* (1947). Hockenhull (1949) discussed the reduction of sulfate via sulfite and thiosulfate as observed with mutant strains of *Aspergillus nidulans*. The reduction of tetrathionate to thiosulfate by coliform bacteria has been found by Pollock and Knox (1943). Fromageot (1947) discussed the oxidation of sulfide to thiosulfate in animal tissue. The most extensive turnover of inorganic sulfur compounds takes place in thiobacilli which obligately oxidize a variety of inorganic sulfur compounds and, therefore, are suitable organisms for the study of inorganic sulfur metabolism. The pathway of thiosulfate oxidation by *Thiobacillus thioparus* has been variously formulated: Starkey (1934*b*, 1935) believed that thiosulfate was oxidized directly to sulfate and sulfur without the intermediate formation of polythionates; other workers, notably Nathansohn (1902) and Tamiya *et al.* (1941) observed the transitory formation of polythionates. The data presented in this paper support the view that polythionates arise in the course of thiosulfate oxidation.

MATERIALS AND METHODS

Cultures. A motile strain of *T. thioparus* was isolated from a thiosulfate enrichment medium inoculated with marine mud and cultivated on Starkey's medium no. 2 (1934*a*): KH_2PO_4, 4.0 g; K_2HPO_4, 4.0 g; $Na_2S_2O_3 \cdot 5H_2O$, 10.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 0.1 g; NH_4Cl, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.02 g; $MnSO_4 \cdot 4H_2O$, 0.02 g; H_2O, 1,000 ml. Dr. Starkey kindly supplied an authentic culture of *T. thioparus* for comparison; it differed only in being nonmotile and growing more slowly. The course of thiosulfate oxidation appeared identical in both strains.

Cultivation of bacteria. The organisms were grown in flasks containing 100 ml of medium and incubated at 28 C with forced aeration (air + 2 to 5 per

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cent CO_2). To provide material for manometric work bacteria were grown in Fernbach flasks containing 1 L of medium with forced aeration and continuous agitation on a rotary shaker. Such cultures attained a density of 10 mg of bacterial N per liter in 3 to 4 days. Suspensions of freshly harvested and washed cells carried out such active autorespiration (R.Q. $\sim$ 1) that the bacteria frequently had to be starved for several hours prior to experimentation. These suspensions oxidized thiosulfate rapidly and completely, i.e., 2.0 μM of O_2 were consumed per μM of thiosulfate. The cultures used by Tamiya *et al.* (1941) yielded only 0.08 to 0.25 mg bacterial N per liter, perhaps because much less iron and manganese were supplied than in Starkey's medium. It was noted in the present experiments that growth decreased if the iron and manganese levels were lowered below those employed by Starkey. Tamiya and coworkers reported that harvested cells oxidized thiosulfate slowly and incompletely with a maximum uptake of 18.0 μl O_2 per μM of thiosulfate; furthermore, the cells did not oxidize tetrathionate nor produce measurable amounts of CO_2 in endogenous respiration.

Dry cellular preparations. T. thioparus was grown with forced aeration in 50 L of medium, harvested with a Sharples supercentrifuge, and washed twice with tap water in a Sorvall angle head centrifuge. The sediment consisted of two distinct layers: a heavy, granular, bright yellow layer of sulfur; and a lighter, slimy, bright orange layer of bacteria. The bacteria were separated from the sulfur, washed again with tap water, and then dried for 10 hours in a desiccator over "drierite" at 0.03 mm Hg pressure. Such a preparation contained no viable cells but rapidly oxidized appropriate sulfur compounds.

Sulfur compounds. Tetrathionate ($S_4O_6^-$) was prepared by the oxidation of thiosulfate with iodine; trithionate ($S_3O_6^-$) by the oxidation of thiosulfate with sulfur dioxide; and dithionate ($S_2O_6^-$) by the oxidation of sulfur dioxide with manganic dioxide; all as described in Abegg's *Handbuch* (Auerbach and Koppel, 1927).

RESULTS AND DISCUSSION

Formation and utilization of tetrathionate. According to Starkey (1935) the oxidation of thiosulfate proceeds directly to sulfate without formation of intermediate compounds; it follows that oxygen uptake and growth should cease as soon as all thiosulfate is consumed. However, 60 per cent of total growth occurred after the disappearance of all iodine titrable thiosulfate (table 1), suggesting that at least part of the thiosulfate was converted into a utilizable intermediate product which did not react with iodine. Although suspensions of freshly harvested bacteria and dry cellular preparations were capable of complete thiosulfate oxidation, suspensions stored overnight in the refrigerator or suspensions of dried cells which had not been previously washed and freed from sulfur carried out only a limited oxidation of thiosulfate. Per μM of thiosulfate only 0.25 μM of O_2 was taken up, and one microequivalent of alkali was formed (table 2). In the oxidation of thiosulfate by freshly harvested bacteria, in which the theoretical maximum uptake of O_2 was approached, distinct changes in the rate

of oxidation occurred after 0.25 and 0.68 μM of O_2 had been consumed per μM of thiosulfate (figure 1, curve 1). In the course of this oxidation the initial formation of alkali was soon counterbalanced by acidification (figure 2). These observations can be accounted for by the oxidation of thiosulfate to tetrathionate as the first step in thiosulfate degradation (reaction 1).

TABLE 1

Growth of Thiobacillus thioparus and consumption of thiosulfate

TIME	BACTERIAL NITROGEN	RESIDUAL $Na_2S_2O_3 \cdot 5H_2O$
days	*mg*	*g*
0.75		0.95
1.5		0.80
2.0		0.61
2.5		0.30
3.0	0.53	0
4.0	1.05	
5.0	1.40	
6.0	1.40	

The figures represent amounts in 100 ml of culture medium. Cultures grown as described under Materials and Methods.

TABLE 2

Incomplete oxidation of thiosulfate by Thiobacillus thioparus

	EXPERIMENT NO.		
	1		2
μM of thiosulfate added	0	9.6	9.4
Initial bicarbonate in μl CO_2	211	211	159
Final bicarbonate in μl CO_2	209	429	372
Change in bicarbonate in μl CO_2	−2	+218	+213
Oxygen consumed in μl	1	51	52

Each vessel contained bacterial matter equivalent to 1.0 mg of nitrogen and 20 μM of $NaHCO_3$ in a total volume of 2.0 ml. Experiment no. 1 was conducted with unwashed dried cells, experiment no. 2 with a stored suspension of intact cells. Bicarbonate was determined as CO_2 by adding 200 μM of H_2SO_4 from the side arm. Atmosphere, 10 per cent CO_2 in air. Temperature, 30 C.

(1) $$S_2O_3^{=} + \tfrac{1}{4}O_2 + \tfrac{1}{2}H_2O \rightarrow \tfrac{1}{2}S_4O_6^{=} + OH^-$$

The complete disappearance of thiosulfate and the formation of tetrathionate were shown in three series of qualitative determinations (table 3). Each series consisted of 6 replicate Warburg vessels in which bacterial suspensions were oxidizing 20 μM of thiosulfate. Every 5 minutes a vessel was withdrawn for analysis. The vessel contents of the first series was filtered through asbestos pulp, mixed with 4 volumes of 95 per cent ethanol, and examined microscopically for crystals of sodium thiosulfate. Vessels of the second series contained in one side

arm starch and an amount of iodine barely sufficient for blue coloration. At intervals the starch-iodine mixture was tipped into the main compartment; failure to discharge the color indicated absence of thiosulfate. In the third series mercurous nitrate was tipped from the side arm into the main compartment. Mercurous nitrate forms a black precipitate with thiosulfate and trithionate and a brilliant yellow precipitate with tetrathionate (Kurtenacker, 1938). The results indicated that no detectable amount of thiosulfate remained when 0.25 μM of O_2 had been consumed per μM of thiosulfate and that tetrathionate was formed.

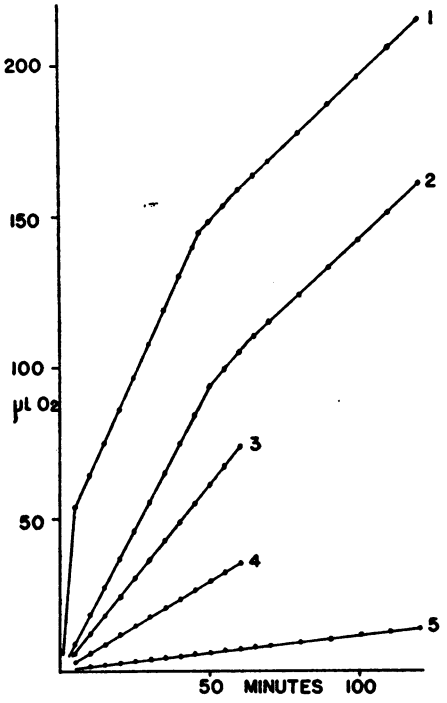


Figure 1. Consumption of oxygen by *Thiobacillus thioparus* during the oxidation of sulfur compounds.

Each vessel contained bacterial cells equivalent to 1.0 mg of nitrogen, 135 μM of phosphate buffer, pH 7.0, and the following substrates: curve 1, 10 μM of thiosulfate; curve 2, 5 μM of tetrathionate; curve 3, 5 μM of trithionate; curve 4, 10 μM of dithionate; curve 5, none. The center well contained 100 μM of KOH. Volume, 2.2 ml. Atmosphere, air. Temperature, 30 C.

The first inflection in curve 1 (figure 1) occurs, therefore, when all thiosulfate has been consumed and tetrathionate oxidation begins.

If tetrathionate is an intermediate product of thiosulfate oxidation and is excreted into the medium, *T. thioparus* should be able to oxidize added tetrathionate. Sodium tetrathionate was recrystallized 8 times from water and ethanol to remove inhibitors present in the crude preparation. Then adequately buffered (M/15 phosphate, pH 7.0) resting cells of *T. thioparus* oxidized tetrathionate at a rate equal to that of thiosulfate oxidation after the first inflection

in curve 1 (figure 1, curve 2). Tetrathionate likewise supported good growth of *T. thioparus* and *T. thiooxidans* and was oxidized readily by dried cells of *T. thioparus*. Tamiya *et al.* (1941) claim that tetrathionate cannot be utilized by *T. thioparus* and believe that although thiosulfate is first oxidized to tetrathionate, further oxidation depends on the spontaneous conversion of tetrathionate to other biologically oxidizable, sulfur compounds. However, tetrathionate does

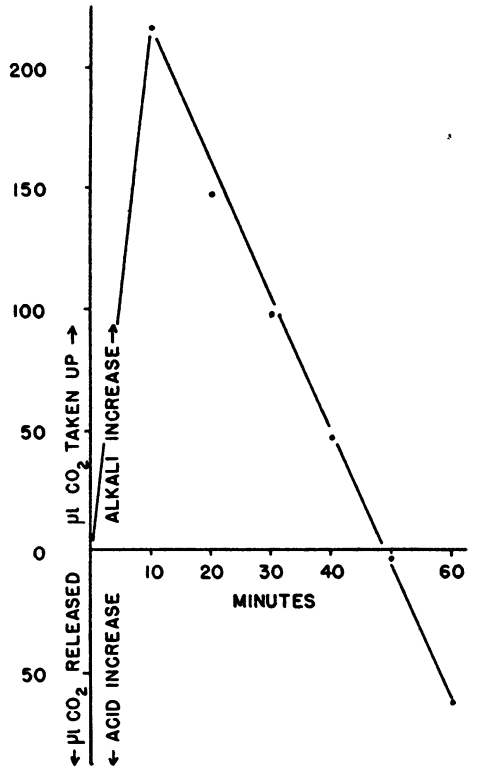


Figure 2. Changes in acidity during the oxidation of thiosulfate by *Thiobacillus thioparus*.

Each vessel contained bacterial cells equivalent to 1.0 mg of nitrogen, 25 μM of sodium bicarbonate, 5 μM of potassium phosphate, and 10 μM of thiosulfate. Volume, 2.2 ml. Atmosphere, 3 per cent CO_2 in air. Temperature, 30 C. The reaction was terminated after 10, 20, 30, 40, 50, and 60 minutes by tipping 200 μM of H_2SO_4 from the side arm. Changes in bicarbonate level are referred to the initial level (established by a zero time control) and expressed in μl of CO_2.

not decompose spontaneously fast enough to permit rapid growth of *T. thioparus*. According to Yost and Russell (1944) a 0.1 M tetrathionate solution decomposes to the extent of 25 per cent in 40 days.

Formation and utilization of trithionate. Of the two changes in the rate of thiosulfate oxidation (figure 1, curve 1) the first has been shown to be associated with the formation of tetrathionate. The second change occurs when 150 μl of

O_2 have been consumed per 10 μM of thiosulfate. A corresponding inflection occurs when 92 μl of O_2 are consumed in the oxidation of 5 μM of tetrathionate (figure 1, curve 2), in agreement with the difference between the two inflections in curve 1. These inflections were not observed in every single experiment. Their occurrence depends in part on low endogenous activity of the bacteria and on a moderate rate of substrate oxidation. But whenever they occurred the inflections indicated points at which 58 and 150 μl of O_2 had been consumed per 10 μM of thiosulfate or 92 μl of O_2 per 5 μM of tetrathionate. On two occasions injured cellular suspensions of the type used for the experiment in table 2 did not terminate thiosulfate oxidation when 58 μl of O_2 had been taken up but consumed 150 μl. The available evidence points, therefore, to a change in oxidative reaction when 150 μl of O_2 (6.67 μM) have been taken up per 10 μM of thiosulfate, or 92 μl of O_2 (4.13 μM) per 5 μM of tetrathionate. The oxidation of a mole of thio-

TABLE 3

Formation of tetrathionate during the oxidation of thiosulfate by Thiobacillus thioparus

TIME	μL OXYGEN CONSUMED	THIOSULFATE CRYSTALS ON ADDITION OF ETHANOL	COLOR ON ADDITION OF IODINE-STARCH	COLOR ON ADDITION OF $HgNO_3$
min				
5	19	many	colorless	black
10	46	many	colorless	black
15	79	few	colorless	black
20	100	none	colorless	brown
25	134	none	blue	bright yellow
30	144	none	blue	light brown

Each vessel contained bacterial cells equivalent to 1.0 mg of nitrogen, 135 μM of potassium phosphate buffer, pH 7.0, and 20 μM of sodium thiosulfate. The center well contained 100 μM of KOH. Volume, 2.0 ml. Atmosphere, air. Temperature, 30 C.

sulfate with 0.67 moles of O_2, or of a mole of tetrathionate with 0.83 moles of O_2, results in a product on the oxidation level of trithionate.

The formation of trithionate was demonstrated in an experiment in which intact bacteria were allowed to oxidize 10 μM of thiosulfate in each of eight Warburg flasks (table 4). At intervals flasks were withdrawn, the contents treated with excess $BaCO_3$ and $BaCl_2$ to remove sulfate and phosphate, and filtered. The clear filtrate was boiled and the immediate precipitation of $BaSO_4$ indicated specifically the presence of trithionate (Kurtenacker, 1938). Trithionate was most abundant when 150 μl of O_2 had been consumed. Synthetic trithionate was readily oxidized by bacterial suspensions (figure 1, curve 3) and by dry cellular preparations. The instability of trithionate made growth experiments impractical. The present data indicate that tetrathionate, formed by the oxidation of thiosulfate, is oxidized to trithionate (reaction 2).

$$(2) \qquad 3S_4O_6^{=} + 5/2O_2 + H_2O \rightarrow 4S_3O_6^{=} + 2H^+$$

Possible intermediates in trithionate oxidation. Of the sulfur compounds that

are intermediate in oxidation level between trithionate and sulfate, sulfite and dithionate seem to be intermediates in the further oxidation of trithionate. Sulfite cannot readily be tested as a substrate for growth and oxidation because of its rapid spontaneous oxidation. Dithionate, a possible intermediate in the oxidation of sulfite to sulfate, has repeatedly been reported as a product of or substrate for various strains of thiobacilli (Lieske, 1912; Trautwein, 1921; Starkey, 1934*a*). Added dithionate was oxidized only slowly by the strain of *T. thioparus* used here (figure 1, curve 4), similar to Starkey's observation (1934*a*). Beijerinck (1904) found no effect of dithionate on his culture of *T. thioparus*.

Formation of elemental sulfur. The reactions considered so far do not account for the precipitates of sulfur that are so conspicuous in cultures of *T. thioparus*. Starkey (1935) observed a constant ratio between sulfate formed and sulfur deposited: in cultures which initially contained 1 per cent of $Na_2S_2O_3 \cdot 5H_2O$,

TABLE 4

Formation of trithionate during the oxidation of thiosulfate by Thiobacillus thioparus

TIME	μL OXYGEN CONSUMED	TRITHIONATE, ESTIMATED AS BARIUM PRECIPITATE
min		
5	26	−
10	63	−
15	86	+
20	99	++
30	130	+++
40	155	+++
50	194	++
60	210	+

Each vessel contained bacterial cells equivalent to 1.0 mg of nitrogen, 135 μM of potassium phosphate buffer, pH 7.0, and 10 μM of sodium thiosulfate. The center well contained 100 μM of KOH. Volume, 2.1 ml. Atmosphere, air. Temperature, 30 C. The trithionate was carried out as described in the text. No precipitate, −; turbidity, +; distinct precipitate, ++; heavy precipitate, +++.

60 per cent of the total sulfur consumed at any one time was recovered as sulfate and 40 per cent was precipitated as elemental sulfur. Starkey concluded that elemental sulfur arose by a specific biological mechanism and formulated the oxidation of thiosulfate by *T. thioparus* as in reaction 3.

$$(3) \qquad 5S_2O_3^{=} + 4O_2 + H_2O \rightarrow 6SO_4^{=} + 4S + 2H^+$$

Such a reaction would permit a maximum O_2 uptake of 17.9 μl per μM of thiosulfate consumed. It was found, however, that both resting suspensions and dried cells of *T. thioparus* completely oxidized 10 μM of thiosulfate to sulfate with the uptake of 448 μl of O_2 in one to several hours. Since resting cells of *T. thioparus* oxidize sulfur at a barely measurable rate, it cannot be assumed that thiosulfate was utilized as in reaction 3 and that the observed oxygen uptake was the result of slower sulfur oxidation. Dry cellular preparations which were entirely unable

to oxidize elemental sulfur oxidized 10 μM of thiosulfate completely in one hour. Obviously a complete oxidation of thiosulfate cannot give rise to free sulfur. The discrepancy between the behavior of growing cultures and resting suspensions appeared to depend on the relative concentrations of bacteria and thiosulfate. Sulfur was precipitated when high initial thiosulfate concentrations (0.04 M in culture media, as compared to 0.005 M in manometric experiments) were removed only slowly by the growth of bacteria from small inocula.

The effect of the concentration of thiosulfate on the amount of sulfur precipitated was investigated by using resting cellular suspensions oxidizing various amounts of thiosulfate (table 5). Only oxygen changes were recorded, and it was assumed that no sulfur was formed in vessels where the theoretically required amount of oxygen was consumed. Conversely, a low oxygen uptake was taken to indicate a high proportion of precipitated sulfur. No changes above the control vessel were observed after 48 hours, and no titrable thiosulfate remained at that time. Only at the two lowest concentrations had thiosulfate been oxidized

TABLE 5

Effect of thiosulfate concentration on the extent of its oxidation by Thiobacillus thioparus

INITIAL AMOUNT OF THIOSULFATE IN μM	μL OXYGEN CONSUMED	μL OXYGEN CONSUMED CORRECTED FOR BLANK	PER CENT OF OXYGEN REQUIRED FOR COMPLETE OXIDATION
0	1,245		
10	1,763	518	115
50	3,711	2,466	109
200	6,969	5,724	64
1,000	3,435	2,190	5

Each vessel contained bacterial cells equivalent to 2.0 mg of nitrogen, 675 μM of potassium phosphate buffer, pH 7.0, and amounts of thiosulfate as indicated. The center well contained 500 μM of KOH. Volume, 11.5 ml. Atmosphere, O_2. Temperature, 30 C. Time, 48 hours.

to completion. Similar observations have been made by Lange-Posdeeva (1930), who found the characteristic sulfur pellicle on a culture of *T. thioparus* at high concentrations of thiosulfate but not when the initial concentration was "below 1 per cent", and by Saslavsky (1927), whose halophilic *Thiobacillus* strain did not form elemental sulfur when the initial thiosulfate concentration was only 0.25 per cent. These findings indicate that sulfur precipitation is dependent on a high initial concentration of thiosulfate. Since the precipitation of sulfur occurs over a wide pH range (table 6), it cannot be caused by acid decomposition of thiosulfate.

Tamiya *et al.*, (1941) suggested the spontaneous decomposition of tetrathionate as the source of the precipitated sulfur and as the mechanism by which tetrathionate is converted to trithionate (reactions 4 and 5).

(4) $$2S_4O_6^- \rightarrow S_5O_6^- + S_3O_6^-$$

(5) $$S_5O_6^- \rightarrow S_4O_6^- + S$$

But *T. thioparus* can oxidize tetrathionate, whereas reactions 4 and 5 do not ordinarily proceed with sufficient rapidity to account for thiosulfate oxidation (Yost and Russell, 1944). However, reactions 4 and 5 are greatly accelerated by thiosulfate which induces a highly polar configuration in the higher polythionates (Foss, 1947). The effect of a high initial concentration of thiosulfate on sulfur formation can be interpreted as follows. It takes 2 to 3 days for thiosulfate to disappear from a culture which initially contained 1 per cent. Therefore, both thiosulfate and tetrathionate are present sufficiently long for thiosulfate to catalyze the formation of pentathionate (reaction 4) and subsequently of sulfur (reaction 5). In manometric experiments reactions 4 and 5 cannot take place because all thiosulfate is converted into tetrathionate in a matter of minutes, except when the reaction time is prolonged by the addition of large amounts of thiosulfate (table 5). Reactions 4 and 5 are entirely nonbiological, as suggested by Tamiya and co-workers, but instead of forming an important link in the biological utilization of thiosulfate they are mere side reactions dependent on catalysis by thiosulfate. Their data show a rapid formation of pentathionate and sulfur as long as thiosulfate is present, and little change thereafter.

TABLE 6

Formation of elemental sulfur by Thiobacillus thioparus at different pH levels

INITIAL pH	FINAL pH	RESIDUAL $Na_2S_2O_3 \cdot 5H_2O$, G	SULFUR PRECIPITATE	BACTERIAL YIELD IN MG N
6.0	4.6	0	heavy	1.15
7.0	5.5	0	heavy	1.16
8.0	6.0	0	heavy	1.10
9.0	7.6	0	heavy	0.89
10.0	8.8	0.61	light	0.24

The figures represent amounts in 100 ml of culture medium. Cultures were grown as described under Materials and Methods.

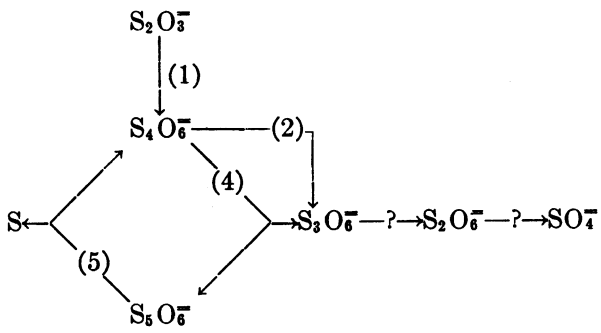
The precipitation of sulfur in filtered culture fluid and the inhibition of such precipitation by KCN (Nathansohn, 1902) at times have been interpreted as evidence for an extracellular cyanide sensitive enzyme which decomposes thiosulfate in the medium, although Nathansohn never held that view. Tamiya was unable to inhibit sulfur precipitation in culture filtrates by the addition of KCN or urethane or by O_2 deprivation. If Tamiya used concentrations of KCN predicated on the idea of enzyme inhibition, this is not surprising since the effect of KCN is strictly stoichiometric (reactions 6 and 7).

$$(6) \qquad S_4O_6^- + CN^- + OH^- \rightarrow S_2O_3^- + SCN^- + HSO_4^-$$

$$(7) \qquad S_5O_6^- + 2CN^- + OH^- \rightarrow S_2O_3^- + 2SCN^- + HSO_4^-$$

Thus KCN removes the polythionates from which elemental sulfur is formed.

Pathway of thiosulfate oxidation. The oxidation of thiosulfate by *T. thioparus* may be summarized as follows:



The numbers refer to the reactions discussed in the text. Reactions 1 and 2 are enzymatic, whereas reactions 4 and 5 are nonbiological and depend on catalysis by excess thiosulfate. In short term manometric experiments reactions 4 and 5 do not take place, but in cultures they account for the formation of sulfur. The pathway of oxidation for trithionate has not been sufficiently investigated yet. The work of Foss (1947) has clarified considerably the chemistry of polythionates. His formulation makes it possible to resolve seemingly complex conversions, such as the oxidation of tetrathionate to trithionate, into a series of single step reactions. Whether Foss's ideas can be applied to the study of biological polythionate oxidation must await the outcome of detailed enzymatic studies.

SUMMARY

Thiobacillus thioparus oxidizes thiosulfate with the intermediate formation of tetrathionate and trithionate; dithionate also may be involved. Elemental sulfur which is precipitated in cultures of *T. thioparus* arises by a purely nonbiological mechanism; excess thiosulfate catalyzes the dismutation of tetrathionate to trithionate and pentathionate and the subsequent decomposition of pentathionate to tetrathionate and sulfur.

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