The Role of Tetrathionate in the Oxidation of Thiosulphate by *Chromatium* sp. strain D

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SUMMARY

Sulphate was progressively replaced by tetrathionate as the end product of thiosulphate oxidation by suspensions of *Chromatium* D when the pH value was decreased from 7·3 to 6·25; tetrathionate was not itself oxidized to sulphate within this range. The effect of pH value on the oxidation of endogenous sulphur was less marked than on the production of sulphate from thiosulphate. Extracts of *Chromatium* D catalysed the oxidation of thiosulphate to tetrathionate in the presence of ferricyanide; pH 5·0 was the optimum of the purified enzyme. Tetrathionate inhibited growth and the complete oxidation of thiosulphate by suspensions. At pH 6·75 where sulphur accumulated during thiosulphate utilization and sulphate and tetrathionate were formed, added tetrathionate inhibited the accumulation of endogenous sulphur but not the production of tetrathionate from thiosulphate. Tetrathionate had no effect on the oxidation of endogenous sulphur by suspensions. It is concluded that in *Chromatium* D thiosulphate is metabolized by two alternative pathways depending on the conditions: (1) the cleavage of one molecule of thiosulphate in a reaction similar to that catalysed by thiosulphate sulphur-transferase E.C.2.8.1.1. (rhodanese); (2) the oxidative coupling of two molecules of thiosulphate to give tetrathionate, catalysed by the thiosulphate-oxidizing enzyme which only operates at low pH values.

INTRODUCTION

Tetrathionate has been suggested as an intermediate in the light-dependent oxidation of thiosulphate to sulphate by purple sulphur bacteria (van Niel, 1936; Eymers & Wassink, 1937; Wassink, 1941). The evidence, largely indirect, was based on the kinetics of carbon dioxide uptake by suspensions of purple sulphur bacteria in the presence of thiosulphate. In similar studies with *Chlorobium thiosulphatophilum* which grows on sulphide and thiosulphate but not on tetrathionate, Larsen (1952, 1953) reported that tetrathionate was oxidized by suspensions with the concomitant fixation of carbon dioxide. He suggested that tetrathionate might be an intermediate in the oxidation of thiosulphate by this organism. Manometric studies, however, gave no indication of an accumulation of tetrathionate during thiosulphate oxidation.

Tetrathionate not only supports the chemo-autotrophic growth of several species of *Thiobacillus*, but also accumulates during their growth on other inorganic sulphur compounds (Parker & Prisk, 1953; Pratt, 1958; Pankhurst, 1964). Several

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workers have reported the accumulation of tetrathionate during the oxidation of sulphur compounds by suspensions of thiobacilli (Vishniac, 1952; Baalsrud & Baalsrud, 1954; Vishniac & Santer, 1957; Trudinger, 1959; Jones & Happold, 1961).

Extracts of *Thiobacillus thioparus* and *T. thio-oxidans* have recently been shown to catalyse the oxidation of sulphide, thiosulphate, tetrathionate, trithionate and sulphite to sulphate (Rittenberg & London, 1964). Tetrathionate was identified as a likely intermediate in the oxidation of sulphide and thiosulphate. Extracts of *Thiobacillus* X contain an enzyme which catalyses the oxidation of thiosulphate to tetrathionate (Trudinger, 1958); by using soluble and particulate cytochrome preparations isolated from crude extracts, thiosulphate oxidation has been coupled to the uptake of oxygen. This enzyme is thought to catalyse the initial step in the oxidation of thiosulphate by *Thiobacillus*.

In the present work, the production of tetrathionate from thiosulphate and the effect of tetrathionate on the complete oxidation of thiosulphate and endogenous sulphur by *Chromatium* strain D were investigated to establish the role, if any, of polythionate in the oxidation of thiosulphate to sulphate. Extracts were used to provide an enzymological basis for the observation made with intact organisms.

METHODS

Organisms, media and growth of cultures. The culture of *Chromatium* sp. strain D was grown as described by Smith & Lascelles, (1966). This organism was used throughout these studies except in some experiments with extracts of the Athiorhodaceae *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. These organisms were grown as described previously (Smith & Lascelles, 1966).

Preparation and incubation of suspensions. Organisms for suspensions were prepared and incubated as described previously (Smith & Lascelles, 1966). In the investigation of the effect of pH value on the oxidation of sulphur compounds, the concentration of sodium bicarbonate was decreased to 3 mM and the pH value of the potassium phosphate buffer chosen so as to give the required final pH value when gassed with N₂ 95% (v/v) + CO₂ 5% (v/v).

Assay of the thiosulphate-oxidizing enzyme. Extracts were prepared as described by Smith & Lascelles (1966) except that the washed organisms were finally suspended in 0.04 M-potassium phosphate buffer (pH 7.5). A modification of the Trudinger (1961) assay was used. The reaction mixture contained, in a volume of 3 ml.: acetate buffer (pH 5.5), 350 μ moles; sodium thiosulphate, 25 μ moles; potassium ferricyanide, 7.5 μ moles; enzyme preparation equivalent to 1.5 mg. protein of crude extract. The reaction was started by the adding of enzyme. The reference cuvette contained the complete reaction mixture without thiosulphate. Ferricyanide reduction was followed at room temperature (18 to 20°) by using a recording double-beam grating spectrophotometer (Optica CF4DR, Optica U.K. Ltd., Gateshead-on-Tyne, England). The millimolar extinction coefficient of ferricyanide at 418 m μ is 1.0 (Ibers & Davidson, 1951), and a unit of enzyme activity was defined as the amount which catalysed the reduction of 1 μ mole ferricyanide/min.

Purification of the thiosulphate-oxidizing enzyme. Particle-bound pigment was removed from the extract prepared from 12 l. fully grown culture by centrifugation at 100,000 g for 60 min. in a Spinco model L ultracentrifuge. The supernatant

fluid fraction of the extract was treated with 5% (w/v) streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) in 0.04 M-potassium phosphate buffer (pH 7.5) to give a final concentration of 1 mg. streptomycin sulphate/mg. extract protein. Precipitated nucleic acid was removed after 15 min. by centrifugation at 18,000 *g* for 20 min. The supernatant fluid was adjusted to pH 4.6 with N-hydrochloric acid and precipitated protein removed after 5 min. by centrifugation at 18,000 *g* for 10 min. The supernatant fluid was adjusted to pH 7.0 with N-sodium hydroxide. The enzyme was precipitated by 35–55% of saturation (by volume) with ammonium sulphate. Precipitated protein was removed after 30 min. by centrifugation at 18,000 *g* for 20 min. and dissolved in a minimum amount of 0.04 M-potassium phosphate buffer (pH 7.0). After dialysis for 12 hr against mM-potassium phosphate buffer (pH 7.0), the concentrated enzyme preparation was applied to a column (15 × 1.5 cm.) of DEAE-DE 50 cellulose (Whatman, Colnbrook, England). Protein was eluted with potassium phosphate buffer (pH 7.0) by using a concentration gradient (1–200 mM); the flow rate was 1 ml./min. and the eluate was collected in 5 ml. fractions.

Analytical methods. Protein, phosphate, thiosulphate, sulphate and endogenous sulphur were determined as described previously (Smith & Lascelles, 1966). The amount of tetrathionate in suspensions was determined by the method of Sorbo (1957) in samples of the supernatant medium after the organisms had been removed by centrifugation.

Chromatography of inorganic sulphur compounds. Thiosulphate and polythionates were separated by descending chromatography on Whatman no. 1 paper with the solvent systems described by Skarzynski & Szczepkowski (1959) and Trudinger (1959). The chromatograms were dried at room temperature and the sulphur compounds located by the method of Trevelyan (1950).

Inorganic sulphur compounds. Sodium salts were used throughout these studies.

RESULTS

Effect of pH value on growth and the oxidation of inorganic sulphur compounds

Chromatium D grew well on the thiosulphate + salts medium at pH 7.3 whereas the organism did not grow on the same medium at pH 6.5. Growth on pyruvate showed a similar dependence on the pH value of the medium.

Effect of pH value on the oxidation of thiosulphate. At pH 7.3, suspensions of organisms oxidized thiosulphate completely to sulphate under anaerobic conditions in the light in the presence of bicarbonate; there was a transient accumulation of sulphur within the organisms (Fig. 1). At pH 6.25, thiosulphate disappearance was more rapid than at pH 7.3 but sulphate production was less than 25% of the theoretical (Fig. 1) and an accumulation of sulphur within the organisms was not observed. Tetrathionate was found chromatographically in suspensions oxidizing thiosulphate at pH 6.25; there was no trace of polythionate in suspensions oxidizing thiosulphate at pH 7.3. The pattern of tetrathionate production in suspensions oxidizing thiosulphate at various pH values was established by using a quantitative method for determining polythionate (Fig. 1). As the pH value was decreased the amount of tetrathionate produced

from thiosulphate increased until, at pH 6.25, it was the principal product of thiosulphate utilization. At each pH value, the sum of tetrathionate + sulphate produced accounted quantitatively for the thiosulphate used by the suspensions.

Tetrathionate, whether produced from thiosulphate by suspensions under acidic conditions (Fig. 1) or added to suspensions in the absence of thiosulphate, was not oxidized to sulphate in the range pH 7.3–6.25.

There was no disappearance of thiosulphate or production of polythionates in suspensions of heat-killed organisms or in the absence of organisms between pH 7.3 and pH 6.25. The lowering of the pH of suspensions was therefore accompanied by a fundamental change in the pattern of thiosulphate oxidation; sulphur accumulation and sulphate production were progressively replaced by the conversion of thiosulphate to tetrathionate.

Effect of pH on the oxidation of endogenous sulphur. Suspensions of organisms containing considerable amounts of intracellular sulphur oxidized this sulphur to

Fig. 1. Effect of pH value on the oxidation of thiosulphate by suspensions. Suspensions of thiosulphate-grown organisms containing 0.25 mg. protein/ml. were incubated at various pH values under anaerobic conditions with a light intensity of 500 ft.c. in the presence of thiosulphate (2 mM). Thiosulphate (—△—) disappearance, sulphur (---■---) and tetrathionate (—●—) accumulation and sulphate (—○—) production were determined.

Table 1. *Effect of pH value on the oxidation of endogenous sulphur*

Suspensions of thiosulphate-grown *Chromatium* D containing intracellular sulphur (equiv. 0.25 mg. protein/ml.) were incubated at various pH values under anaerobic conditions at a light intensity of 500 ft.c. The sulphate produced after incubation for 1 hr and 8 hr was determined. The figures in parentheses refer to sulphate production by suspensions of sulphur-free organisms oxidizing thiosulphate (2 mM) under similar conditions.

Time (hr)	pH		
	7.3	6.75	6.25
	Sulphate production (μmole/hr/ml.)		
1	1.2 (1.5)	1.2 (1.2)	1.2 (0.1)
8	3.1 (4.0)	3.1 (3.2)	2.3 (0.4)

sulphate under anaerobic conditions in the light (Smith & Lascelles, 1966). The effect of pH value on the oxidation of endogenous sulphur was less marked than its effect on the production of sulphate from thiosulphate (Table 1). Sulphate appeared to be the only product of endogenous sulphur oxidation by suspensions in the range pH 7.8–6.25.

Tetrathionate production by extracts

Extracts of Chromatium D catalysed the rapid and complete oxidation of thiosulphate to tetrathionate in the presence of potassium ferricyanide according to the equation

The reaction did not occur with heated extracts.

Properties of the thiosulphate-oxidizing enzyme. The optimum pH value of the reaction catalysed by crude extracts was below 5.5. The enzyme in crude extracts was insensitive to EDTA (10 mM), fluoride (10 mM), iodoacetate (10 mM) and arsenite (1 mM); azide (10 mM) decreased the reaction rate by 50 %. The Michaelis constant of the enzyme for thiosulphate was 1.5 mM. Extracts did not catalyse the reduction of ferricyanide when thiosulphate was replaced by tetrathionate.

Extracts of Chromatium D grown on organic carbon had about half the activity of extracts from thiosulphate-grown organisms; the enzyme was not found in extracts of *Rhodospirillum rubrum* or *Rhodopseudomonas spheroides*.

Table 2. *Purification of the thiosulphate-oxidizing enzyme of Chromatium D*

The details of the individual steps are given in Methods. Five ml. of the dialysed protein fraction were applied to the column of DEAE cellulose.

Stage	Treatment	Product volume (ml.)	Total protein (mg.)	Total enzyme (units)	Specific activity (units/mg. protein)
I	French press	27	1350	1000	0.7
II	High-speed centrifugation	22	572	853	1.5
III	Streptomycin sulphate	24	480	804	1.7
IV	Acid precipitation	24	209	670	3.2
V	Ammonium sulphate (35–55 % saturation)	5.2	85	624	7.3
VI	Dialysis	6.6	85	521	6.1
(Fractions 15–19)					
VII	Chromatography on DEAE cellulose	25	3.2	253	79

Purification of thiosulphate-oxidizing enzyme. The enzyme from extracts of thiosulphate-grown Chromatium D was partially purified in a seven stage process (Table 2) which involved column chromatography on DEAE cellulose. The thio-sulphate-cleaving and thiosulphate-oxidizing enzymes were completely separated on the column of substituted cellulose (Fig. 2). The reaction catalysed by the 100-fold purified enzyme was optimal at pH 5.0 (Fig. 3).

Effect of tetrathionate on the oxidation of inorganic sulphur compounds

Tetrathionate did not support the growth of *Chromatium D* under autotrophic conditions and it inhibited the growth of the organism on the thiosulphate-salts medium as well as on organic media (Smith & Lascelles, 1966). Thiosulphate utilization and sulphur accumulation by suspensions at pH 7.3 were inhibited by

Fig. 2

Fig. 3

Fig. 2. Chromatography of the thiosulphate-oxidizing enzyme on DEAE cellulose. The protein fraction from stage VI was applied to a column of DEAE-DE 50 cellulose. The thiosulphate-oxidizing enzyme was eluted with 75–90 mM buffer (column fractions 15–19) whilst rhodanese was eluted from the column with 10–35 mM buffer (column fractions 5–9). Protein (---), enzyme activity (—), and buffer concentration (---) were determined.

Fig. 3. Optimum pH value of the thiosulphate-oxidizing enzyme. Phosphate (full symbols) and acetate (open symbols) buffers of the appropriate pH were used in the determination of the activity of the enzyme in a crude extract (---) and in a purified preparation (—) from the DEAE cellulose column. Protein was precipitated from reaction mixtures containing crude extract below pH 5.5.

Table 3. *Effect of tetrathionate on thiosulphate utilization and sulphur accumulation by Chromatium D at pH 7.3 and 6.25*

Suspensions of thiosulphate-grown *Chromatium D* (equiv. 0.25 mg. protein/ml.) at pH 7.3 or pH 6.25 were incubated at a light intensity of 500 ft.c. under anaerobic conditions with thiosulphate (5 mM) and various concentrations of tetrathionate.

pH	Tetrathionate concentration (μmoles/ml.)	Thiosulphate utilization (μmoles/hr/ml.)	Sulphur accumulation (maximum turbidity change)
7.3	0	2.7	12.8
	2.5	0.6	2.8
	5	0.3	0.9
6.25	0	4.8	0
	2.5	4.6	0
	5	4.2	0

tetrathionate (Table 3); the inhibition was non-competitive and reversed by washing. The effect of tetrathionate on thiosulphate disappearance was much less at pH 6.25 than at pH 7.3 (Table 3). At the intermediate value pH 6.75, where sulphur accumulated transiently and sulphate and tetrathionate were the products of thiosulphate oxidation, sulphur accumulation was markedly inhibited by added tetrathionate, whereas tetrathionate production from thiosulphate was, if anything, increased (Fig. 4). Added tetrathionate had no effect on the oxidation of endogenous sulphur by suspensions. Tetrathionate reacts spontaneously with sulphide and sulphite and this prevented a study of its effect on the oxidation of these compounds.

Fig. 4. Differential effect of added tetrathionate on the accumulation of sulphur (a) and tetrathionate (b) at pH 6.75. Suspensions of thiosulphate-grown organisms containing 0.2 mg. protein/ml. were incubated anaerobically in the light at pH 6.75 in the presence of thiosulphate (6 mM) and various concentrations of tetrathionate: 10 mM (—■—), 1 mM (—△—) and none (—○—).

DISCUSSION

Chromatium D unlike *Thiobacillus thio-oxidans* did not grow on inorganic media at pH 6.5. Nevertheless, suspensions of Chromatium D utilized thiosulphate at pH values below 6.5. Sulphur accumulation and sulphate production were, however, greatly decreased at pH 6.25; the yield of sulphate under these conditions was less than 25% of the theoretical. Tetrathionate was the principal product of thiosulphate utilization at pH 6.25 whereas at pH 7.3 there was no detectable accumulation of tetrathionate during thiosulphate oxidation. Two possible explanations of the accumulation of tetrathionate at acid pH values were: (1) the accumulation of tetrathionate, a possible intermediate in thiosulphate oxidation, was due to the inactivity at acid pH of the enzymes for its further oxidation; (2) tetrathionate was the product of an enzyme reaction which was only functional at low pH values. The fact that tetrathionate was not oxidized in the range 7.3–6.25 favoured the second explanation.

Extracts of *Chromatium D* contained a soluble enzyme distinct from rhodanese which catalysed the oxidation of thiosulphate to tetrathionate. Such an enzyme was described by Trudinger (1958) in extracts from *Thiobacillus* strain x. The enzyme in crude extracts of *Chromatium D* was only slightly active at pH 7.0; the purified enzyme had an optimum at pH 5.0. In view of its properties it is likely that this enzyme is involved in the production of tetrathionate from thiosulphate by intact organisms at low pH values.

The inhibition by tetrathionate of thiosulphate utilization and of sulphur accumulation at pH 7.3 was an additional complicating factor. At the intermediate value pH 6.75, where both sulphate and tetrathionate were products of thiosulphate oxidation, added tetrathionate inhibited the intracellular accumulation of sulphur but did not affect the production of tetrathionate from thiosulphate.

Fig. 5. The oxidation of thiosulphate by *Chromatium D*.

In contrast, tetrathionate had no effect on the oxidation of endogenous sulphur. The site of the inhibition of the conversion of thiosulphate to sulphate was therefore likely to be at a stage before the oxidation of endogenous sulphur. The results of this and other work (Smith & Lascelles, 1966) suggested that thiosulphate was metabolized by the two mechanisms outlined in the scheme shown in Fig. 5, one catalysed by a rhodanese-like enzyme and the other by the thiosulphate-oxidizing enzyme.

Wassink, Katz & Dorrestein (1941) investigated the fixation of carbon dioxide by suspensions of a species of *Chromatium* and found it to be most rapid at pH 6.3 in the presence of excess thiosulphate. At this pH value carbon dioxide fixation was accompanied by the production of alkali (measured by chemical binding of carbon dioxide) which was attributed to the conversion of thiosulphate to tetrathionate. On this basis, tetrathionate was proposed as an intermediate in thiosulphate oxidation. In the present work direct analysis for thiosulphate and its oxidation products has shown that lowering from pH 7.3 to 6.25 resulted in a fundamental change in the reactions for the oxidation of thiosulphate in which sulphate was replaced by tetrathionate as the end-product of oxidation. Wassink (1941) correctly interpreted the results of his experiments at pH 6.3 in terms of the oxidation of thiosulphate to tetrathionate; his investigation, based only on measurement of carbon dioxide fixation, did not however reveal the change in the pattern of thiosulphate oxidation which accompanied the change in pH. Since

fixation of carbon dioxide can apparently be coupled to the conversion of thiosulphate to tetrathionate at a pH value where growth is not possible, this oxidation process might be of some importance to the organism. The change from sulphate to tetrathionate production at acid pH values would be beneficial to an organism oxidizing thiosulphate in that acidic products would be replaced by the production of alkali: (1) complete oxidation: $\text{Na}_2\text{S}_2\text{O}_3 \rightarrow \text{Na}_2\text{SO}_4 + \text{H}_2\text{SO}_4$; (2) incomplete oxidation: $2\text{Na}_2\text{S}_2\text{O}_3 \rightarrow \text{Na}_2\text{S}_4\text{O}_6 + 2\text{NaOH}$.

The inhibition of the complete oxidation of thiosulphate by tetrathionate would also prevent the further production of acidic oxidation products, whilst permitting the assimilation of carbon dioxide coupled to the oxidation of thiosulphate to tetrathionate.

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