OXIDATION OF THIOSULPHATE BY INTACT CELLS OF *THIOBACILLUS X*: EFFECTS OF SOME EXPERIMENTAL CONDITIONS

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Summary

Low cell concentrations of *Thiobacillus X*, grown with low aeration, failed to oxidize thiosulphate to completion in air, and tetrathionate accumulated. The extent to which thiosulphate was oxidized was decreased in 100% oxygen and depended upon the substrate concentration.

The rate of oxidation of thiosulphate in air by organisms grown with high aeration fell off progressively with time but little or no tetrathionate accumulated: 100% oxygen did not affect the extent to which thiosulphate was oxidized.

Sulphite inhibited thiosulphate oxidation and was formed to a small extent during thiosulphate oxidation.

During the oxidation of outer-labelled [^{35}S]thiosulphate by *Thiobacillus X*, ^{35}S became incorporated into the inner position of thiosulphate.

Under all conditions studied approximately two molecules of sulphate were formed from the inner sulphur of thiosulphate, for every one from the outer sulphur, in the early stages of thiosulphate oxidation.

Some quantitative relationships between the products of oxidation of outer-labelled [^{35}S]thiosulphate are described.

A hypothesis is presented to account for these results.

I. Introduction

Although many studies have been made to elucidate the mechanism of thiosulphate oxidation by thiobacilli, the autotrophic sulphur bacteria (see reviews by Vishniac and Santer 1957; Lees 1960; Vishniac and Trudinger 1962), there is still no general agreement over its essential features. Uncertainties in the interpretation of the results of experiments with growing cultures and washed intact cell preparations have arisen from differences in the results obtained by different workers carrying out apparently similar experiments.

A number of recent papers have emphasized the influence that the experimental conditions may have on the metabolism of sulphur compounds by thiobacilli. The amount and type of polythionate accumulated by growing cultures of *Thiobacillus thioparus* are influenced by the phosphate concentration and the ratio of sodium to potassium in the medium (Jones and Happold 1961) and differences in polythionate production by different species of thiobacilli have been reported (Wooley, Jones, and Happold 1962). The extent to which thiosulphate is oxidized by washed *T. novellus* is determined by the concentration of bacteria (De Ley and van Poucke 1961), while the oxidation of tetrathionate by washed cells of *Thiobacillus X* and

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T. thioparus is markedly affected by a number of experimental conditions (Trudinger 1964*a*).

The present paper describes experiments which show that the products of thiosulphate oxidation by washed cell preparations of *Thiobacillus X* and the fate of the individual sulphur groups are influenced quantitatively by changes in the oxygen tension during the growth of the organism and by the concentrations of substrate, organisms, and oxygen used for the experiments.

II. Materials and Methods

(*a*) *Organism and Growth Conditions*

Thiobacillus X (Parker and Prisk 1953) was grown at 30°C in the medium described by Vishniac and Santer (1957) for the growth of *T. thioparus*. Three conditions were used:

(1) The bacteria were grown at pH 6·0 in 2 litres of medium in a Quickfit culture vessel (No. FV2L) with vigorous stirring and automatic pH control (Trudinger 1964*b*). Air was forced through the medium at the rate of 120 litres/hr. The growth was exponential and was complete in about 12 hr with a 5% inoculum.

(2) This was the same as for (1) except that the aeration rate was 5 litres/hr. About 30 hr were required for complete growth: the growth was linear for about the last 24 hr, indicating that oxygen was limiting.

(3) The bacteria were grown on a rotary shaker in 2-litre Fernbach flasks containing 500 ml of medium. The culture was maintained at pH 6–7 by periodical addition of Na_2CO_3. In the experiments to be described bacteria grown under these conditions behaved in the same way as those grown under (2) above.

The bacteria were harvested by centrifugation when growth was approximately 80% complete and were washed twice with 0·2M potassium phosphate, pH 7. Between the first and second washes the bacteria were shaken at 30°C in buffer for 30 min to remove endogenous substrates and any elemental sulphur which may have precipitated with the bacteria.

(*b*) *Manometry*

The conventional Warburg manometric technique was used. Flasks of approximately 15-ml volume were shaken at 30°C at 120 oscillations/min and an amplitude of 4 cm. The limiting oxidation rates referred to in the text are those obtained when oxygen diffusion is limiting.

(*c*) *Experimental Procedure with Labelled Thiosulphate*

Washed bacteria were incubated at 30°C with [^{35}S]thiosulphate in Warburg flasks and the reactions were stopped by the addition of 2 ml cold 10% (v/v) acetic acid in 50% (v/v) ethanol. The mixtures were made to 10 ml with water and centrifuged. Aliquots of the supernatants were analysed by chromatography on Dowex

1X2 as described previously (Trudinger 1964*b*). The precipitates were washed three times with 0·1M phosphate, pH 7, and digested with a mixture of HCl, HNO_3, and Br_2 to oxidize any sulphur present to sulphate (Trudinger 1964*b*).

(*d*) *Labelled Thiosulphates*

Sodium thiosulphate ($Na_2S_2O_3$), labelled with ^{35}S in either the outer (–S) or inner ($–SO_3$) positions, was obtained from New England Nuclear Corporation, Boston, Mass. Some of the samples of inner-labelled thiosulphate contained appreciable amounts of radioactive sulphate. This was removed by adding a slight excess of $BaCl_2$ to the solution and removing the precipitated $BaSO_4$ by centrifuging. Barium thiosulphate was then precipitated by the addition of 50% ethanol and sodium thiosulphate regenerated by double decomposition with Na_2SO_4.

(*e*) *Estimation of Sulphate*

Sulphate was eluted from Dowex 1X2 by 1M ammonium acetate, pH 5, and the total ^{35}S in the eluate measured. The eluate was then evaporated to dryness at approximately 180°C: most of the ammonium acetate volatilized during the drying. The dry material was taken up in 10% (v/v) acetic acid and a small amount of insoluble material (probably from degradation of the resin) removed by centrifugation. Sulphate was then precipitated by adding an excess of 1% (w/v) benzidine in acetone. The benzidine sulphate precipitate was washed four times with 100% acetone and dissolved completely in 1% (w/v) sodium borate in 0·1N NaOH. The benzidine content of this solution was determined colorimetrically using sodium β-naphthoquinone-4-sulphonate (Lange and Tarver 1957), and the ^{35}S with a Geiger–Müller counter. The specific activity of the sulphate was determined and this value was used for calculating the total sulphate in the original eluate.

(*f*) *Estimation of Thiosulphate and Tetrathionate*

In experiments using outer-labelled [^{35}S]$Na_2S_2O_3$, thiosulphate was estimated, after elution from Dowex 1X2, from the radioactivity present in the outer (–S) sulphur after it had been established that no reduction of inner to outer sulphur occurred (Table 4). Similarly tetrathionate was estimated from the ^{35}S in its inner (–S–) position. In aqueous solutions there is an almost instantaneous exchange of sulphur between thiosulphate and tetrathionate (Fava 1953) and, since analysis of the distribution of sulphur in tetrathionate in 6N HCl (the eluting solution) is tedious and unreliable, the value of the ratio ^{35}S (outer)/^{35}S (inner) found for thiosulphate, was also used in the estimation of tetrathionate. When sufficient material was available, thiosulphate was also estimated by titration with standard iodine using starch as an indicator, and tetrathionate by titration with standard KIO_4 in 6N HCl using chloroform as an indicator (Kurtenacher, Mutschin, and Stasny 1935).

In some experiments, in which unlabelled thiosulphate was used as substrate, tetrathionate in reaction media was determined directly by titration with standard iodine after reaction with excess sulphite (Starkey 1935).

(*g*) *Other Analytical Methods*

Methods for the analysis of the distribution of ^{35}S in thiosulphate, the paper electrophoresis of sulphur compounds, and the determination of ^{35}S have been described in previous papers (Trudinger 1961*b*, 1964*b*).

III. Results

(*a*) *Effect of Oxygen and Bacterial Concentration on Thiosulphate Oxidation by Organisms Grown with Low Aeration*

The extent of thiosulphate oxidation by *Thiobacillus X* grown with low aeration depended upon the bacterial concentration and the oxygen concentration in the gas phase (Fig. 1). Complete oxidation occurred only with relatively high bacterial

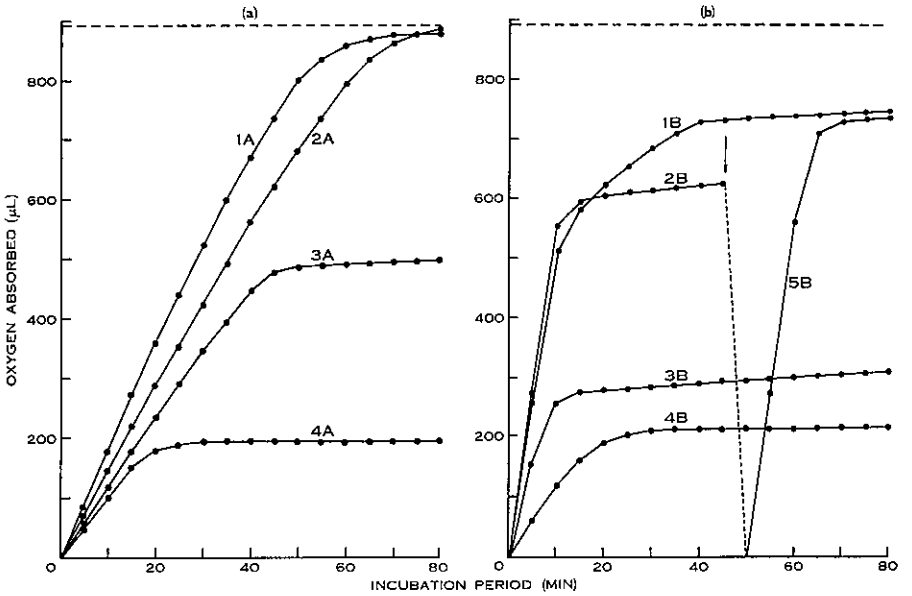


Fig. 1.—(*a*) and (*b*) Effect of gas phase [(*a*) air, (*b*) 100% oxygen] and bacterial concentration on the oxidation of thiosulphate by *Thiobacillus X* grown with low aeration. Various concentrations of *Thiobacillus X* grown under condition (2) were incubated with 200 μmoles of potassium phosphate, pH 7, and 20 μmoles of $Na_2S_2O_3$ in a volume of 1·8 ml. Dry weights of organisms (mg) were: curves 1*A*, 1*B*, 6·0; curves 2*A*, 2*B*, 3·0; curves 3*A*, 3*B*, 1·2; curves 4*A*, 4*B*, 0·6; curve 5*B* as for 2*B* except that a second addition of 20 μmoles of $Na_2S_2O_3$ was made at the time indicated by the arrow; the second part of the curve shows the *additional* oxygen consumed. No oxygen was absorbed in the absence of substrate. The horizontal dotted lines represent the theoretical oxygen consumption (896 μl) for the complete oxidation of 40 μmoles thiosulphate to sulphate.

populations in air (Fig. 1(*a*), curves 1*A* and 2*A*); under these conditions the rate of oxygen uptake approached the limiting oxygen uptake rate of the system. At lower concentrations of bacteria there was a sharp break in the rate of oxygen uptake, to 5% or less of the initial rate at an intermediate stage of oxidation. The stage at which the break occurred depended on the actual bacterial concentration used.

Except at the lowest bacterial concentration (Figs. 1(*a*), 1(*b*), curves 4*A*, 4*B*), oxidation was less complete in 100% oxygen than in air. That oxygen did not cause an irreversible inactivation of one of the components of the metabolic system is shown by Figure 1(*b*), curve 5*B*, where a second sample of thiosulphate was added after oxidation of the original substrate in 100% oxygen had ceased: immediate and rapid oxidation of the second sample of substrate ensued.

The reaction mixtures from the experiment described in Figure 1 were examined qualitatively by paper electrophoresis after an incubation time of 80 min. They contained no thiosulphate but considerable amounts of tetrathionate, which accounted for the difference between the observed oxygen uptake and that required for complete oxidation of thiosulphate to sulphate (Table 1).

TABLE 1

PRODUCTS OF THIOSULPHATE OXIDATION BY THIOBACILLUS X GROWN WITH LOW AERATION

Flask contents at 80 min from the experiment described in Figure 1 were titrated with iodine and tetrathionate determined from the iodine titre after treatment with excess sulphite

Flask	Final pH	Iodine Titre before Treatment with $-SO_3^{2-}$ (μ-equiv.)	Oxygen Unconsumed (μl)*	Tetrathionate Formed (μmoles)	Oxygen Equivalent of Tetrathionate† (μl)
1A	6·40	0	2	0	—
2A	6·45	0	10	0	—
3A	6·80	0·1	398	5·0	392
4A	7·05	0	706	9·1	714
1B	6·65	0	249	2·9	227
2B	6·25	0·1	433	5·6	438
3B	7·00	0	582	7·1	557
4B	7·05	0	693	9·2	772

* Measured from curves at 80 min.

† Calculated from the equation $Na_2S_4O_6 + 3{\cdot}5\ O_2 + 3H_2O \rightarrow Na_2SO_4 + 3H_2SO_4$.

(*b*) *Effect of Substrate Concentration on the Extent of Thiosulphate Oxidation by Bacteria Grown with Low Aeration*

In 100% oxygen, with a constant bacterial population, oxidation became less complete as the substrate concentration was raised (Table 2). Similar results were obtained in air and in each case the accumulated tetrathionate accounted for the deficiency in oxygen absorbed. A similar dependence of the extent of thiosulphate oxidation upon substrate concentration has been reported by Vishniac (1952) but, in that case, a complicating factor was a marked fall in pH associated with oxidation at the higher substrate levels. Moreover, incomplete oxidation was assumed to be due to the precipitation of elementary sulphur; no measurements of any tetrathionate accumulation were reported.

(*c*) *Oxidation of Thiosulphate by Bacteria Grown with High Aeration*

The effects of oxygen and bacterial concentrations on the oxidation of thiosulphate by organisms grown with high aeration are shown in Figure 2. With the

lower bacterial concentrations (Fig. 2 (*a*), curves 2*A* and 3*A*) there was a progressive falling off of the rate of oxidation in air with time. The rate of oxidation reverted

TABLE 2

EFFECT OF SUBSTRATE CONCENTRATION ON THE EXTENT OF THIOSULPHATE OXIDATION IN OXYGEN

Washed cells of *Thiobacillus X* (1·8 mg dry wt.) grown under condition (3) (p. 739) were incubated with 200 μmoles of potassium phosphate, pH 7, and $Na_2S_2O_3$ in a volume of 2·5 ml under 100% oxygen

$Na_2S_2O_3$ (μmoles/flask)	Oxygen Used (μl)				Percentage of Theoretical*
	5 Min	10 Min	15 Min	20 Min	
1	28	32	33	33	71·4
2	46	49	50	50	55·8
5	85	90	92	92	41·0
10	123	139	143	143	31·9
20	213	283	289	289	32·3

* For complete oxidation of thiosulphate to sulphate.

to the initial rate on the addition of more substrate (curve 5*A*). In contrast to the oxidation by organisms grown at low aeration, 100% oxygen had little effect on

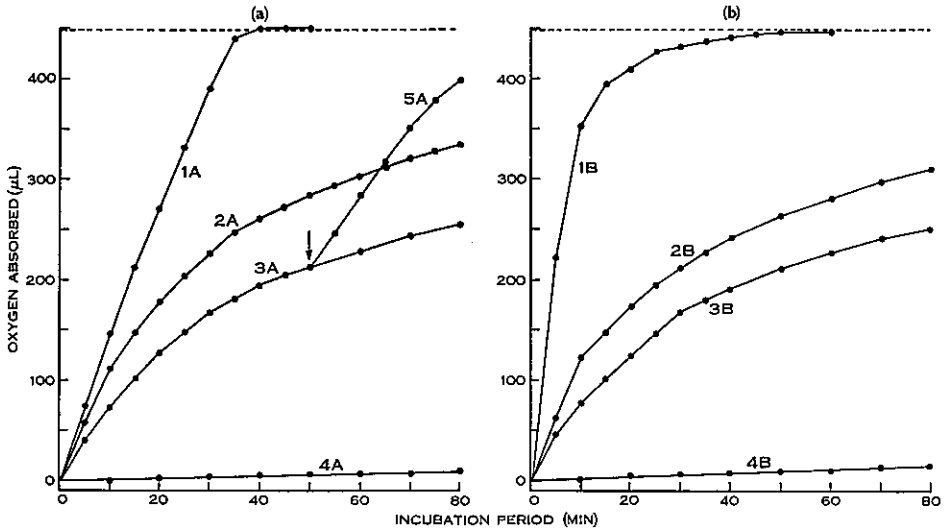


Fig. 2.—(*a*) and (*b*) Effect of gas phase and bacterial concentration on thiosulphate oxidation by *Thiobacillus X* grown with high aeration under condition (1). 10 μmoles $Na_2S_2O_3$ added; other conditions and gas phases were as for Figures 1(*a*) and 1(*b*) respectively. Dry weights of organisms (mg) were: curves 1*A*, 1*B*, 5·0; curves 2*A*, 2*B*, 1·0; curves 3*A*, 3*B*, 0·5; curves 4*A* and 4*B*, 5·0 (no $Na_2S_2O_3$). Curve 5*A*: as for 3*A* except that a second addition of 10 μmoles of $Na_2S_2O_3$ was made at the time indicated by the arrow. The horizontal dotted lines represent the oxygen uptake (448 μl) for complete oxidation of 10 μmoles of $Na_2S_2O_3$.

thiosulphate oxidation except at the highest bacterial concentration where oxygen was limiting in air (Figure 2, curves 1*A* and 1*B*). Only traces of polythionates

accumulated during the oxidation of thiosulphate by bacteria grown with high aeration (e.g. Table 5): the thiosulphate remaining accounted almost entirely for the unused oxygen.

(*d*) *Effect of Aeration Rate during Growth on the Thiosulphate-oxidizing Enzyme Content of* Thiobacillus X

Thiobacillus X contains a soluble enzyme catalysing the oxidation of thiosulphate to tetrathionate in the presence of ferricyanide (Trudinger 1961*b*). The levels of this enzyme in extracts of *Thiobacillus X* were determined using the assay described by Trudinger (1961*a*), and were of the order of 4 and 2 units per milligram dry weight of organisms for bacteria grown with low and high aeration respectively.

TABLE 3

INHIBITION OF THIOSULPHATE OXIDATION BY SULPHITE

Reaction mixtures contained washed cells of *Thiobacillus X*, 10 μmoles of $Na_2S_2O_3$, and 200 μmoles of potassium phosphate, pH 7, in a volume of 1·8 ml. The results are expressed as percentage inhibition during the first 15 min

Sulphite Concn. (mM)	Inhibition (%)		
	Expt. 1*	Expt. 2*	Expt. 3†
5·5	79	95	91
2·2	42	89	78
1·1	15	77	54
0·55	12	52	38

* Organisms (expt. 1, 4·2 mg dry wt.; expt. 2, 0·8 mg dry wt.) grown with high aeration [condition (1), p. 739].

† Organisms (2·1 mg dry wt.) grown with low aeration [condition (2), p. 739].

(*e*) *Inhibition of Thiosulphate Oxidation by Sulphite*

De Ley and van Poucke (1961) have attributed the incomplete oxidation of thiosulphate by *T. novellus* to inhibition by sulphite which accumulated in their experiments. In the present work traces of a material with the properties of sulphite have been detected in reaction mixtures both by direct iodine titration and by chromatography on Dowex 1X2. However, in no case has the level of sulphite exceeded about 0·5 mM while in many instances it was undetectable (e.g. Table 1). Table 3 shows the inhibition by sulphite of thiosulphate oxidation by *Thiobacillus X*. Oxidation of thiosulphate was inhibited to a greater extent at the lower bacterial concentrations, but over the range of bacterial concentrations examined at least 1 mM sulphite was necessary for 80% inhibition.

Thus, while with very low bacterial concentrations the accumulation of sulphite may have contributed to the reduction in the rate of thiosulphate oxidation by

Thiobacillus X during the later stages of metabolism, this explanation does not appear to account for the results with higher bacterial concentrations described in Figures 1 and 2.

(*f*) *Formation of Doubly Labelled* [^{35}S]*Thiosulphate from Outer-labelled* [^{35}S]*Thiosulphate*

During the oxidation of outer-labelled [^{35}S]thiosulphate by bacteria grown either with high or low aeration the inner sulphur of thiosulphate became labelled (Table 4). No significant amount of ^{35}S appeared in the outer group during the oxidation of inner-labelled [^{35}S]thiosulphate.

TABLE 4

DISTRIBUTION OF ^{35}S IN THIOSULPHATE DURING THE OXIDATION OF SINGLY LABELLED [^{35}S]THIOSULPHATE

Bacteria were incubated in air in a volume of 2·5 ml with 200 μmoles of potassium phosphate and 32 μmoles of [^{35}S]$Na_2S_2O_3$ labelled in either the outer or inner position. After incubation the remaining thiosulphate was isolated and the distribution of ^{35}S determined

Expt. No.	^{35}S in Outer Position (counts/min)		^{35}S in Inner Position (counts/min)	
	Initial	Final	Initial	Final
1A*	2770	891	0	222
1B*	0	12	6000	1746
2A†	4661	3426	0	254
2B†	0	2	4475	3226

* Organisms (7·2 mg dry wt.) grown with low aeration [condition (3), p. 739]; incubation time 25 min, oxygen consumed 37 μg-atoms.

† Organisms (7·4 mg dry wt.) grown with high aeration [condition (1), p. 739); incubation time 10 min, oxygen consumed 12·9 μg-atoms.

(*g*) *Effect of Growth Conditions and Gas Phase on the Products of Thiosulphate Oxidation*

The amounts of sulphate, elemental sulphur, and polythionates formed during the early stages of thiosulphate oxidation by dense bacterial populations are shown in Table 5. These products accounted for over 95% of the metabolized thiosulphate: traces of ^{35}S were found in the sulphide + sulphite fraction in some instances. At equivalent stages of oxidation (as measured by oxygen uptake, e.g. expts. 1 and 2) much larger amounts of polythionates were produced by bacteria grown with low aeration than by those grown with high aeration. Oxygen (100%) stimulated polythionate production by organisms grown with low aeration (expt. 3) but not by organisms grown at high aeration (expt. 1).

Elemental sulphur was produced only from the outer sulphur of thiosulphate (cf. Skarszynski, Ostrowski, and Krawczyk 1957) and only by bacteria grown with low aeration. Even with the latter the amounts of elemental sulphur produced under a standard set of conditions varied considerably between batches of organisms.

TABLE 5

EFFECT OF GROWTH CONDITIONS AND GAS PHASE ON THE PRODUCTS OF THIOSULPHATE OXIDATION BY THIOBACILLUS X

Organisms were incubated with 200 μmoles of potassium phosphate, pH 7, in a final volume of 2·3 ml. Duplicate flasks were used for each incubation, one containing outer-labelled $[^{35}S]Na_2S_2O_3$ and the other inner-labelled $[^{35}S]Na_2S_2O_3$. The results are expressed as μg-atoms of oxygen or sulphur

Expt. No.	Growth Conditions	Gas Phase during Incubation	Dry Weight of Organisms (mg)	Initial Thiosulphate	Incubation Period (min)	Sulphate Formed		S^0 Formed	Polythionates Formed*	Oxygen Consumed (μl)
						From Inner S	From Outer S			
1	High aeration [condition (1)]†	Air	7·4	64	6	2·8	1·5	0	3·2	8·9
		Air	7·4	64	10	6·0	2·8	0	0·5	12·9
		100% O_2	7·4	64	2·5	7·3	3·5	0	0	12·2
2	Low aeration [condition (2)]†	Air	10·9	64	10	6·2	3·3	2·1	20·8	14·1
3	Low aeration [condition (3)]†	Air	6·0	44	22	12·7	6·8	1·0	4·8	29·4
		100% O_2	6·0	44	6	13·6	7·4	0·5	25·6	29·6

* Over 95% of this fraction was tetrathionate; traces of trithionate and pentathionate were also present.

† See text, p. 739.

Approximately two molecules of sulphate were formed from the inner sulphur of thiosulphate for every one derived from the outer sulphur. This is in agreement with the results reported earlier for one experimental condition (bacteria grown under condition (3), gas phase air—cf. Trudinger 1964*c*).

(h) Effect of Substrate Concentration on the Oxidation of Outer-labelled [^{35}S]Thiosulphate

Thiobacillus X, grown with low aeration, was incubated with various concentrations of outer-labelled [^{35}S]thiosulphate and the reaction mixtures analysed when the oxygen consumption in each case was approximately 45% of the amount required for complete oxidation of the thiosulphate to sulphate. The results are shown in Table 6 and have been expressed on the basis of 100 μmoles of thiosulphate of specific activity 100 counts per minute per μmole.

TABLE 6

EFFECT OF SUBSTRATE CONCENTRATION ON THE OXIDATION OF OUTER-LABELLED [^{35}S]THIOSULPHATE

Washed cells of *Thiobacillus X* (4·2 mg dry wt.) grown under condition (3) were incubated with 200 μmoles of potassium phosphate, pH 7, and outer-labelled [^{35}S]$Na_2S_2O_3$. The gas phase was air and the volume 2·4 ml. To facilitate comparison of the results they have been expressed relative to 100 μmoles of thiosulphate with a specific activity of 100 counts per minute per μmole.

Oxygen required for complete oxidation of substrate = 400 μg-atoms

Flask	1	2	3	4
Thiosulphate concentration (mM)	2·9	5·0	9·2	13·3
Incubation period (min)	9	16	31	37
Sulphate formed (μmoles)	136	135	133	133
Specific activity of sulphate (counts/min)	35·5	34·0	36·5	34·5
Thiosulphate remaining (μmoles)	10·7	9·2	11·0	11·5
Specific activity of inner S (counts/min)	61·0	38·5	26·5	14·5
Polythionate formed (μmoles)*	7·5	8·8	8·0	9·1
Sulphur formed (μg-atoms)	4·9	8·8	11·7	13·7
Total [$^{35}S^0 + {}^{35}SO_3$]†	20·6	19·1	18·8	18·0
Recovery of ^{35}S (%)	94	98	98·5	93·5
Oxygen consumed (μg-atoms)	172	172	182	184
Theoretical oxygen (μg-atoms)‡	268	243	293	225
Oxygen deficit (μg-atoms)	96	71	111	41

* Expressed as tetrathionate, see footnote to Table 5.

† See Section III(*h*).

‡ This is the oxygen consumption calculated for the formation of sulphate, elemental sulphur, and the labelled ($-SO_3$) groups of thiosulphate and polythionates.

Changes in substrate concentration had little influence on the amounts of thiosulphate remaining, the amounts of polythionates and sulphate formed, or the specific activity of the sulphate. The latter value was close to 33% of the initial specific activity of thiosulphate which is the theoretical specific activity for the sulphate produced if one molecule arose from the outer sulphur and two from the inner sulphur of thiosulphate.

With increasing thiosulphate concentration there was a considerable increase in the amount of sulphur precipitated and a marked decrease in the specific activity

of the inner sulphur of thiosulphate. The total amount of ^{35}S in the elemental sulphur and in the ($-SO_3$) groups of thiosulphate and polythionates ($^{35}S^0 + {}^{35}SO_3$), however, remained fairly constant indicating that the labelled ($-SO_3$) groups and elemental sulphur were derived from the same source.

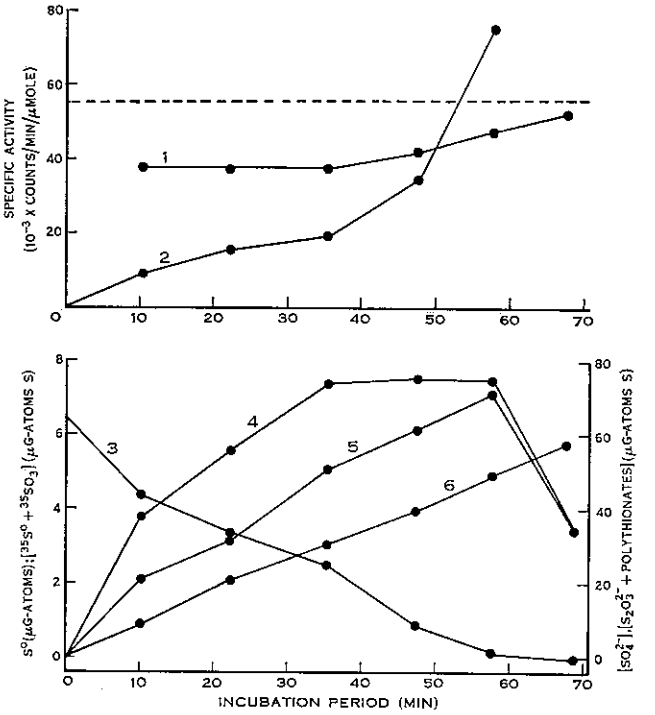


Fig. 3.—Oxidation of thiosulphate by *Thiobacillus X*: course of reaction with time. *Thiobacillus X* (10·9 mg dry wt.) grown with low aeration [condition (3)] was incubated with 200 μmoles of potassium phosphate, pH 7, and 32 μmoles of outer-labelled [^{35}S]$Na_2S_2O_3$ (specific activity = 110×10^3 counts per minute per mole) in a volume of 2·4 ml. Curves: **1**, specific activity of sulphate; **2**, specific activity of inner sulphur of thiosulphate; **3**, thiosulphate+polythionates; **4**, total [$^{35}S^0 + {}^{35}SO_3$] (see Section III(*h*)); **5**, elemental sulphur; **6**, sulphate. The horizontal dotted line represents the theoretical specific activity of sulphate (55) at complete oxidation.

(*i*) *Course of Oxidation of Outer-Labelled* [^{35}S]*Thiosulphate*

Figure 3 compares the specific activities of sulphate and of the inner sulphur of thiosulphate with the production of sulphur and sulphate during the course of oxidation of outer-labelled [^{35}S]thiosulphate by *Thiobacillus X* grown with low aeration. For the first 35 min, during which time the specific activity of sulphate remained fairly constant, there was a slow increase in the specific activity of the inner sulphate of thiosulphate. For the next 20 min, although elemental sulphur was still precipitated, the total ($^{35}S^0 + {}^{35}SO_3$) remained constant. At the same time the specific activity of sulphate rose to approach the value expected at complete oxidation, and there was a dramatic rise in the specific activity of the inner sulphur

of thiosulphate. The latter reached about 70% of the specific activity of the outer sulphur of thiosulphate showing that by this time the major part of the inner sulphur was derived from the outer group.

(*j*) *A Possible Reduced Intermediate in Thiosulphate Oxidation*

It was found consistently that the oxygen required for the formation of the products isolated at intermediate stages of thiosulphate oxidation was in excess of the oxygen actually consumed. An example is shown in Table 6 (cf. oxygen consumed, theoretical oxygen, and oxygen deficit). The values for theoretical oxygen are subject to considerable error since any errors in the values for sulphate and ($-SO_3$) are magnified four times. Nevertheless the possibility remains that some of the products isolated were in a more reduced form and became oxidized during analysis. No direct evidence for such a reduced intermediate has been obtained.

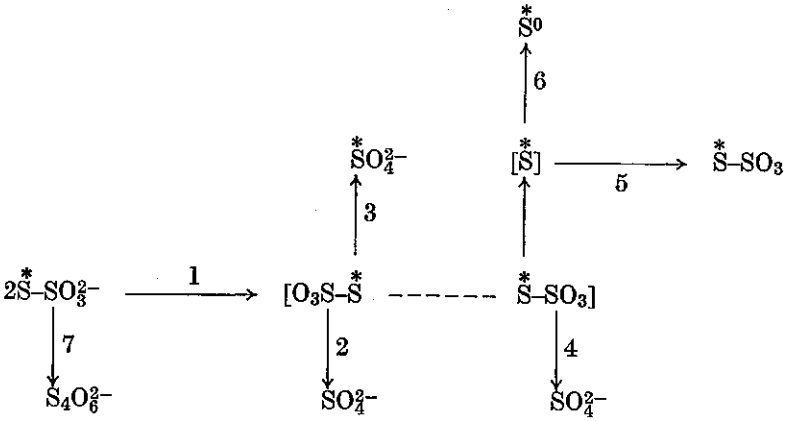
IV. Discussion

The results reported in this paper show that the amounts of polythionates and elemental sulphur accumulating during thiosulphate oxidation by *Thiobacillus X* depend very greatly on the growth history of the bacteria and upon parameters such as the gas phase and the concentrations of substrate and bacteria used in the experiments. These observations, together with those of other workers mentioned in the Introduction, emphasize the need for cautious interpretation of the results of experiments with growing cultures and washed bacteria.

Nevertheless, under all the experimental conditions employed, a consistent result has been the formation of two molecules of sulphate from the inner sulphur of thiosulphate, for every one derived from the outer group, during the early stages of oxidation. This ratio is indicative of a four-sulphur intermediate in the conversion of thiosulphate to sulphate (Trudinger 1964*c*). The conclusion may be drawn, therefore, that formation of sulphate through this unknown four-sulphur intermediate is the main pathway of thiosulphate metabolism and that elemental sulphur and polythionates arise by secondary reactions the rates of which are modified by experimental conditions.

The inverse relationship between the appearance of ^{35}S in elemental sulphur and the ($-SO_3$) groups of thiosulphate and polythionates during the oxidation of outer-labelled [^{35}S]thiosulphate (Table 6) suggests a common origin for elemental sulphur and the labelled ($-SO_3$) groups. That elemental sulphur is oxidized by way of thiosulphate is indicated by the results described in Figure 3. The specific activity of the inner sulphur of thiosulphate reached a very high value, when most of the oxidizable sulphur remaining in the medium was in the form of elemental sulphur. Earlier work has already indicated that thiosulphate is an intermediate in sulphur oxidation by thiobacilli (Vishniac and Santer 1957; Suzuki and Werkman 1959). However, since free elemental sulphur was not precipitated during the oxidation of thiosulphate by bacteria grown with high aeration (Table 5) it may not be an obligatory intermediate in thiosulphate oxidation but may arise from the breakdown of

a precursor of the inner sulphur of thiosulphate. These ideas are outlined in the following scheme:



Reactions 1–5 represent the main pathway of thiosulphate oxidation and reactions 6 and 7 secondary reactions which are suppressed in bacteria grown with high aeration.

The results reported in this paper provide no explanation for the differences between bacteria grown with low and high aeration. While there appeared to be less thiosulphate-oxidizing enzyme in bacteria grown with high aeration it is doubtful whether the difference was sufficiently great to account for the difference in polythionate production by organisms grown under the two conditions.

The oxidation of thiosulphate by extracts of *T. thioparus* studied by Peck and his colleagues (see review by Peck 1962) appeared to involve an initial reduction of the thiosulphate molecule to sulphide and sulphite. Although polythionates were formed under some conditions (Peck and Fisher 1962) no evidence for the direct participation of a four-sulphur intermediate in the oxidation of thiosulphate to sulphate was obtained. Trudinger (1964c), however, has pointed out that a partial resolution of the reaction sequence could result during extraction of the bacteria and that reactions may be modified by the presence of excess reduced glutathione, which is required for thiosulphate oxidation by cell-free extracts. The necessity for considering the possible effects of non-specific chemical interactions between inorganic sulphur compounds and thiols has recently been emphasized by Postgate (1963). There are, moreover, some obvious metabolic differences between *Thiobacillus X* and the strain of *T. thioparus* used by Peck. For example, growing cultures and washed cells of the latter carry out only a partial oxidation of thiosulphate and large amounts of elementary sulphur accumulate (Peck 1960; Trudinger, unpublished results). On the other hand, formation of free sulphur by *Thiobacillus X* is variable and, when produced, it is later rapidly and completely oxidized to sulphate.

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