[³⁵S]Thiosulphate Oxidation by *Thiobacillus* Strain C

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1. *Thiobacillus* strain C oxidized [³⁵S]thiosulphate completely to sulphate.
2. During thiosulphate oxidation [³⁵S]sulphate was formed more rapidly from (S.³⁵SO₃)²⁻ than from (³⁵S·SO₃)²⁻. ³⁵S disappeared less rapidly from thiosulphate with (S.³⁵SO₃)²⁻ as substrate than with (S.³⁵SO₃)²⁻.
3. Thiosulphate labelled in both atoms was produced during (³⁵S·SO₃)²⁻ oxidation, but not during (S.³⁵SO₃)²⁻ oxidation.
4. No ³⁵S was precipitated as elementary sulphur either in the presence or absence of exogenous unlabelled sulphur.
5. During [³⁵S]thiosulphate oxidation, appreciable quantities of [³⁵S]trithionate accumulated and later disappeared. Other polythionates did not accumulate consistently.
6. [³⁵S]Trithionate was formed initially at a greater rate from (S.³⁵SO₃)²⁻ than from (³⁵S·SO₃)²⁻, but subsequently at a similar rate from each.
7. Trithionate formed from (S.³⁵SO₃)²⁻ was labelled only in the oxidized sulphur atoms, but that formed from (³⁵S·SO₃)²⁻ was labelled in both oxidized and reduced atoms. The proportion of ³⁵S in the oxidized atoms increased as more trithionate accumulated.
8. The results eliminate some mechanisms of trithionate formation but are consistent both with a mechanism of thiosulphate oxidation based on an initial reductive cleavage of the molecule and with a mechanism in which thiosulphate undergoes an initial oxidative reaction.

Chemoautotrophic bacteria of the genus *Thiobacillus* derive energy for growth from the oxidation of reduced inorganic sulphur compounds, the principal oxidation product being sulphate. Studies of these organisms have led to the postulation of two apparently conflicting mechanisms for the oxidation of thiosulphate to sulphate (Vishniac & Santer, 1957; Vishniac & Trudinger, 1962; Peck, 1962; Kelly, 1965). The first of these mechanisms, a polythionate pathway, involves the initial oxidation of thiosulphate to tetrathionate, and the subsequent oxidation of this to sulphate with the probable intermediate formation of other polythionates and possibly of organic sulphur compounds. London & Rittenberg (1964) and Pankhurst (1964) have presented evidence for such a mechanism, but Trudinger (1964b) has suggested the occurrence of an S₄ intermediate, other than tetrathionate, but derived from two thiosulphate ions. Such a compound might be the product *in vivo* of an enzyme that has been shown to form tetrathionate from thiosulphate *in vitro* (Trudinger, 1961; Vishniac & Trudinger, 1962). The second mechanism is based on work with cell-free extracts by Peck (1960) and involves the initial reduction of thiosulphate to

sulphide and sulphite. These products are subsequently oxidized to sulphate with the intermediate formation of adenosine 5'-sulphatophosphate but not of polythionates; the sulphide moiety may first be oxidized to thiosulphate.

Clearly, the mechanism of thiosulphate oxidation is complex, and is not yet explained satisfactorily. By using [³⁵S]thiosulphate we have sought to determine (1) whether there is discriminative oxidation of the two sulphur atoms of thiosulphate as expected from the Peck hypothesis (cf. Ostrowski & Krawczyk, 1957; Peck & Fisher, 1962; Trudinger, 1964b,c), and (2) the origin and function of polythionates in the oxidation. Our results are consistent with an oxidation mechanism based on Peck's hypothesis, but may also be consistent with a modified polythionate oxidation pathway.

MATERIALS AND METHODS

Organism. *Thiobacillus* strain C was used in all experiments. This organism has been described elsewhere as *T. thioparus* (Kelly & Syrett, 1964a,b; Kelly, 1965), but may more closely resemble *T. neapolitanus* (D. White & M. Hutchinson, personal communication). It was cultured as

described elsewhere (Kelly & Syrett, 1964a). Organisms centrifuged from batch cultures were washed in sodium phosphate buffer, pH 7.0, and resuspended in 0.1M- $\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$, pH 7.0, to give known cell densities between 0.2 and 2.0 mg. dry wt./ml.

Radioactive thiosulphate. Anhydrous $\text{Na}_2(\text{S}\cdot\text{SO}_3)$, labelled in either the 'outer' (S-) or 'inner' ($-\text{SO}_3$) position with ^{35}S , was obtained from The Radiochemical Centre, Amersham, Bucks. The nomenclature adopted in this paper to describe the position of the ^{35}S label is as follows: $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ is described as 'reduced-atom-labelled', and $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$ as 'oxidized-atom-labelled'. Similarly, the central sulphur atom of trithionate ($\text{O}_3\text{S}\cdot\text{S}\cdot\text{SO}_3$) $^{2-}$ is referred to as the 'reduced atom' and the outer two as the 'oxidized atoms'.

Experiments with [^{35}S]thiosulphate. The ^{35}S -labelled $\text{Na}_2\text{S}_2\text{O}_3$ was added to bacteria shaken vigorously in Warburg flasks or conical flasks at constant temperature. In kinetic experiments, bacteria were separated rapidly from the suspending medium by filtration through Oxoid or Millipore membranes, or were killed by pipetting samples of suspensions into equal volumes of ethanol, and subsequently separated by centrifuging. The filtrates and ethanolic supernatants were analysed by paper chromatography.

Oxygen consumption was followed by Warburg manometry at 30°. Analysis of Warburg flask contents was made after separating the solution from the bacteria by filtration or by centrifuging. In some experiments elementary sulphur added to the flasks was largely freed of bacteria by low-speed centrifugation, washed with 50% (v/v) ethanol and acetone, and dried at 60° before assay for ^{35}S .

Chromatography and radioautography. Paper chromatograms were run on Whatman no. 1 paper developed in one dimension with descending solvents at $22 \pm 1^\circ$. The solvent commonly used was butan-1-ol-acetone-water (2:2:1, by vol.) (Skarzynski & Szczepkowski, 1959), run for 16–20 hr. Other solvents used were pyridine-propan-1-ol-water (3:5:5, by vol.) (Trudinger, 1959) and butan-1-ol-methanol-water (1:1:1, by vol.) (The Radiochemical Centre Data Sheet DS3552). Marker compounds, $\text{Na}_2\text{S}_2\text{O}_3$, $\text{K}_2\text{S}_3\text{O}_6$ and $\text{K}_2\text{S}_4\text{O}_6$, were detected as Ag_2S on the papers by spraying with 0.5% (w/v) AgNO_3 in aq. NH_3 solution [5 vol. of aq. NH_3 (sp. gr. 0.88) + 95 vol. of water] and heating at 100°.

[^{35}S]Sulphate and [^{35}S]thiosulphate were also used as marker materials. [^{35}S]Polythionate markers were produced by incubating [^{35}S]thiosulphate with unlabelled $\text{K}_2\text{S}_3\text{O}_6$ and $\text{K}_2\text{S}_4\text{O}_6$, when distribution of ^{35}S among all three compounds occurred (Fava, 1953).

Radioactive compounds on chromatograms were detected by placing the papers on Kodak Kodirex X-ray film for 2–4 weeks. Radioactive areas were outlined by tracing over the developed film.

To distinguish between [^{35}S]thiosulphate and [^{35}S]sulphate (which have very similar R_f values in the solvents used) samples were treated with excess of iodine; this completely converted thiosulphate into tetrathionate, which was easily separated chromatographically from sulphate. The quantities of sulphate and thiosulphate were calculated from the difference between samples with and without iodine treatment. Sulphate and thiosulphate were also separated by running chromatograms in butan-1-ol-acetone-water for 24–36 hr.

Degradation of radioactive trithionate. [^{35}S]Trithionate

was eluted from preparative paper chromatograms, after location by radioautography, and degraded by reaction with HgCl_2 . Samples (1 or 2 ml.) of aqueous solutions of [^{35}S]trithionate in micro-centrifuge tubes were mixed with 0.5 ml. of 36% (w/v) formaldehyde solution and 0.5 ml. of 5% (w/v) HgCl_2 in 2% (w/v) sodium acetate. After the mixture had stood at 22° for 15 min., 2 mg. of unlabelled $\text{K}_2\text{S}_3\text{O}_6$ in 0.1 ml. of water was added, and the mixture was left for a further 1½–2 hr. Like tetrathionate (van der Heijde & Aten, 1952; Trudinger, 1961), trithionate broke down to sulphate and an insoluble mercury complex (Abegg, Auerbach & Koppel, 1927; Starkey, 1935) as follows:

Thus the reduced atom of trithionate was precipitated and the oxidized ones remained in solution as sulphate. The precipitate was removed by centrifuging and washed once with a 1:20 dilution of the $\text{H}\cdot\text{CHO}\text{--HgCl}_2$ -sodium acetate reagent. The supernatant and washings were made up to a known volume for assay of ^{35}S . The precipitate was oxidized with 1 ml. of conc. HNO_3 saturated with bromine and boiled gently for 30 min. on a sand bath. The solution was made up to standard volume with water.

Degradation of radioactive thiosulphate. Two methods were used.

(a) The method used for trithionate (with 4 mg. of $\text{Na}_2\text{S}_2\text{O}_3\cdot 5\text{H}_2\text{O}$ instead of $\text{K}_2\text{S}_3\text{O}_6$ as carrier) converted the oxidized atom of thiosulphate into sulphate, and precipitated the reduced atom as $\text{HgCl}_2\cdot 2\text{HgS}$ (van der Heijde & Aten, 1952), which was oxidized as above.

(b) The thiosulphate solution was heated at 95° with excess of AgNO_3 for 45 min. The oxidized atom was left in solution as sulphate and the reduced atom precipitated as Ag_2S (Brodsikii & Eremenko, 1954). The precipitate was removed by centrifuging and washed with water. The supernatant and washings were made up to standard volume, and the precipitate was either suspended in a standard volume of water or dissolved in 2% (w/v) KCN for assay of ^{35}S .

Assay of ^{35}S . (a) Radioactive spots on chromatograms were counted on the paper by using a thin end-window Geiger-Müller tube linked to a decade scaler (Ekco Electronics Ltd.). Counts were corrected for background radiation and, when necessary, for the 'dead time' of the counter. The count obtained from a radioactive spot was proportional to the amount of ^{35}S present (with [^{35}S]sulphate standards), at least over the range 1–200 mμC of ^{35}S /spot. Also, the count given by a radioactive spot was essentially the same from each side of the paper, after the chromatogram had been run and allowed to dry evenly (Kelly, 1965). Normally only one side of the paper was counted.

(b) Solutions of ^{35}S compounds were dried on 2.5 cm. diam. ground-glass disks, usually to give infinitesimally thin samples for counting with a thin end-window Geiger-Müller tube. When thick samples were used, corrections for self-absorption were applied by reference to samples of infinitesimal thinness. Samples were counted to give a counting error not greater than $\pm 3\%$.

(c) In some experiments with elementary sulphur, weighed quantities of dried sulphur were dissolved in CS_2 and dried on disks as infinitesimally thin samples for count-

ing as in (b). In one experiment, sulphur was oxidized with HNO₃-Br₂-HCl (Trudinger, 1961).

Sulphate estimation. Sulphate was determined turbidimetrically as BaSO₄ (Gleen & Quastel, 1953). Samples containing up to about 10 μmoles of sulphate were mixed with 2 ml. of 50% (v/v) glycerol and water to a total volume of about 7 ml. Then 1 ml. of 10% (w/v) BaCl₂ in 4.8% (v/v, conc. acid in water) HCl was added and the volume immediately made up to 10 ml. with water before mixing very thoroughly. Standards and suitable blanks were prepared and turbidities measured by using a Hilger Spekker absorptiometer with dark-blue filters and 1 cm. cuvettes.

Reagents. AnalaR chemicals were used when available. Other reagents were of the highest purity available commercially. K₂S₂O₈ and K₂S₄O₆ were a generous gift from Dr F. H. Pollard of Bristol University. Finely divided, easily wettable elementary sulphur was obtained from old cultures of *T. thio-oxidans* and repeatedly washed before drying at 108°.

RESULTS

Oxygen uptake during thiosulphate oxidation. Both dilute bacterial suspensions (0.1–0.5 mg. dry wt./Warburg flask) and dense ones (3–7 mg./flask) consumed oxygen at a high and often constant rate, giving a total uptake usually very close to that calculated for complete thiosulphate oxidation to sulphate (Starkey, 1935):

Dense suspensions did not visibly precipitate elementary sulphur in such experiments (contrast Vishniac & Trudinger, 1962).

Oxidation of Na₂(³⁵S·SO₃) (i.e. radioisotope in reduced sulphur atom). After the addition of [³⁵S]-thiosulphate to dilute suspensions, the disappear-

Fig. 1. Oxidation of Na₂(³⁵S·SO₃) by *Thiobacillus* strain C. Experimental details are given in Table 1. [³⁵S]Thiosulphate was added at zero time. Curve 1, thiosulphate; curve 2, sulphate formation; curve 3, trithionate; curve 4, tetrathionate.

ance of radioactivity from the thiosulphate fraction was accompanied by [³⁵S]sulphate production at an almost equal rate (Fig. 1). The identity of the

Table 1. Specific activity of [³⁵S]sulphate produced during the oxidation of Na₂(³⁵S·SO₃)

Thiobacillus strain C (10 mg. dry wt.) was shaken at 30° in a final volume of 48 ml. of 0.1 M-sodium phosphate buffer, pH 7.0, in a 500 ml. conical flask; 196 μmoles of Na₂(³⁵S·SO₃) (144 μCi) were added and 5 ml. samples filtered through membranes at intervals. Filtrates were analysed by paper chromatography (Fig. 1) and the total sulphate content was determined. No loss of ³⁵S accompanied filtration, indicating that no elementary [³⁵S]sulphur was precipitated. The added thiosulphate contained 331.8 counts/sec./μmole. If the two sulphur atoms of thiosulphate were converted into sulphate at equal rates, the specific activity of sulphate at any sampling time would be 165.9 counts/sec./μmole.

Sample time (min.)	Sulphate/ml. of filtrate (μmoles)	[³⁵ S]Sulphate/ml. (counts/sec.)	Specific activity (counts/sec./ μmole)	Specific activity (% of theoretical)
0	0	0	—	—
4	1.4	94.6	67.7	41
10	3.5	338.0	96.8	58
16	5.5	541.0	98.6	59
23	7.0	960.0	137.2	83
29	7.8	1122.0	144.0	87
36	8.0	1228.0	154.0	93
45	8.0	1271.0	159.0	96
60	8.2	1282.0	156.7	94
Calc. maximum	8.17	1355.4	165.9	100

sulphate was confirmed by chromatography in three solvents, and by its precipitation with 97–100% efficiency by acid barium chloride. A lag of 0.5–2 min. preceded the establishment of a high constant rate of [35]sulphate production (Figs. 1 and 4). A substance chromatographically indistinguishable from trithionate increased during thiosulphate disappearance, and decreased as thiosulphate became exhausted. With a dense cell suspension, similar but more rapid kinetics were obtained. Comparison of total sulphate and [35]sulphate production (Table 1) showed that the specific activity of the sulphate was initially less than half the expected value, but increased as more sulphate was formed. This result would occur if the unlabelled oxidized sulphur atom of thiosulphate were converted into sulphate faster than the

labelled reduced one. The final discrepancy in specific activity would be explained if the trithionate remaining after thiosulphate exhaustion (and accounting for about 5% of the total ^{35}S) were derived largely from the reduced atom of thiosulphate.

Accumulation of trithionate. [^{35}S]Trithionate accumulated consistently in all experiments (Figs. 1 and 2). Its persistence after thiosulphate exhaustion is doubtless related to its lower rate of oxidation by the bacteria, the Q_0 for thiosulphate oxidation being $1130 \pm 280 \mu\text{l. of oxygen/hr./mg. dry wt.}$ (mean $\pm$ S.D. of 27 values) compared with 430 ± 200 (mean $\pm$ S.D. of 5 values) for trithionate. When a dense cell suspension was used thiosulphate disappeared completely within 5 min. After its disappearance the trithionate that had accumulated was oxidized completely to sulphate at a rate significantly lower than the original rate of sulphate production from thiosulphate.

Accumulation of tetrathionate. Usually the accumulation of [^{35}S]tetrathionate and [^{35}S]penta-thionate during $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ oxidation accounted for less than 3% of the total ^{35}S . However, in one exceptional experiment, a transitory accumulation of a large amount of [^{35}S]tetrathionate was observed. Its formation was accompanied by a sudden fall of the amount of thiosulphate.

Comparison of oxidation of $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ and $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$. Fig. 2 shows that [^{35}S]sulphate production and [^{35}S]thiosulphate disappearance were more rapid from $(\text{S}\cdot^{35}\text{SO}_3)^{2-}$ than from $(^{35}\text{S}\cdot\text{SO}_3)^{2-}$. Degradation of thiosulphate in the 20 sec. and 60 sec. samples from the $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ series showed that 1–2% and 5–7% respectively of the ^{35}S in the thiosulphate was in the oxidized atom. [^{35}S]Trithionate was produced at a similar rate from both substrates, although it was produced faster initially from $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$. These results were supported by another experiment, in which doubly-labelled thiosulphate, $\text{Na}_2(^{35}\text{S}\cdot^{35}\text{SO}_3)$, was also supplied (Table 2).

Degradation of labelled trithionate. Table 3 shows the distribution of ^{35}S in the trithionate formed. Trithionate formed from $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$ was labelled only in the oxidized atoms, whereas that from $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ contained a progressively larger proportion of ^{35}S in the oxidized atoms, reaching a maximum of about 50% of the total ^{35}S in trithionate. The proportion in the oxidized atoms may have continued to increase slightly after exhaustion of the thiosulphate substrate. Similarly, trithionate formed from $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$ in the experiment of Table 2 showed no significant labelling of the reduced atom, whereas that derived from $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ after 300 and 420 sec. contained 28 and 31% respectively of its total ^{35}S in the oxidized atoms. The addition of unlabelled $\text{K}_2\text{S}_2\text{O}_8$ to

Fig. 2. Oxidation of $\text{Na}_2(^{35}\text{S}\cdot\text{SO}_3)$ or $\text{Na}_2(\text{S}\cdot^{35}\text{SO}_3)$ by *Thiobacillus* strain C. The 250 ml. flasks shaken at 23° contained (in a final volume of 9 ml.) 700 μmoles of sodium phosphate buffer, pH 7.0, and 14 mg. dry wt. of bacteria; 42 μmoles of $\text{Na}_2\text{S}_2\text{O}_3$ (containing 15 μC of ^{35}S in the reduced atom or 7 μC of ^{35}S in the oxidized atom) were added at zero time. Samples (1 ml.) were pipetted into 1 ml. of ethanol. Curves 1, 3 and 5: thiosulphate, sulphate and trithionate respectively from $(^{35}\text{S}\cdot\text{SO}_3)^{2-}$; curves 2, 4 and 6: thiosulphate, sulphate and trithionate respectively from $(\text{S}\cdot^{35}\text{SO}_3)^{2-}$.

Table 2. Oxidation of thiosulphate labelled in either or both positions with ³⁵S: the formation of [³⁵S]sulphate and [³⁵S]trithionate

Thiobacillus strain C (1.85 mg./ml.) in 14 ml. of 0.1 M-sodium phosphate buffer, pH 7.0, shaken at 23° in 250 ml. conical flasks, received 2 ml. of [³⁵S]thiosulphate (100 μmoles) containing 30 μC of ³⁵S in the oxidized or reduced atom or 30 μC in each atom. Samples (2 ml.) were pipetted into 2 ml. of ethanol at 0° at intervals, centrifuged and the supernatants analysed chromatographically. In all experiments, 95–103% of the added ³⁵S was recovered in S₂O₃²⁻ + SO₄²⁻ + S₃O₆²⁻ + S₄O₆²⁻ + S₅O₆²⁻.

Substrate ...	³⁵ S in each compound (% of ³⁵ S added)								
	Na ₂ (³⁵ S·SO ₃)			Na ₂ (S· ³⁵ SO ₃)			Na ₂ (³⁵ S· ³⁵ SO ₃)		
	S ₂ O ₃ ²⁻	SO ₄ ²⁻	S ₃ O ₆ ²⁻	S ₂ O ₃ ²⁻	SO ₄ ²⁻	S ₃ O ₆ ²⁻	S ₂ O ₃ ²⁻	SO ₄ ²⁻	S ₃ O ₆ ²⁻
Time (sec.)									
0	100	—	—	100	—	—	100	—	—
30	92	5.8	0.4	91	6.0	2.0	94	6.6	1.0
55	90	7.0	1.3	85	9.5	1.7	89	8.8	1.1
90	77	8.0	4.0	74	13.0	4.2	80	11.7	2.7
125	75	9.0	4.7	69	17.0	4.7	70	15.0	5.1
180	69	14.0	5.4	59	22.0	8.8	64	21.9	7.1
300	47	29.0	15.1	29	47.5	17.0	42	40.9	17.7
420	9	45.0	32.0	7	68.5	22.0	11	56.0	28.1

Table 3. Distribution of ³⁵S in trithionate formed from thiosulphate labelled in the reduced or oxidized atom

Expt. I. Details were as given for Fig. 2. Samples (1 ml.) were pipetted into an equal volume of ethanol and centrifuged, and trithionate in the supernatants was separated chromatographically for degradation. Expt. II. A 250 ml. conical flask shaken at 26° contained (in a final volume of 12 ml.) 33 mg. dry wt. of *Thiobacillus* strain C and 1 m-mole of sodium phosphate buffer, pH 7.1; 47 μmoles (23 μC) of Na₂(³⁵S·SO₃) were added at zero time.

Substrate ...	Sample time (sec.)	Na ₂ (³⁵ S·SO ₃)			Na ₂ (S· ³⁵ SO ₃)		
		Distribution (% of recovered ³⁵ S)		³⁵ S recovery in assay (%)	Distribution (% of recovered ³⁵ S)		³⁵ S recovery in assay (%)
		—S—	—SO ₃ —		—S—	—SO ₃ —	
Expt. I	20	80.0	20.0	94.1	0	100.0	104.2
	60	77.9	22.1	96.1	0	100.0	95.5
	90	70.9	29.1	93.0	—	—	—
	120	68.8	31.2	98.7	1.0	99.0	98.6
	180	59.3	40.7	98.2	—	—	—
	300	48.2	51.8	104.0	1.6	98.4	95.5
Expt. II	480	44.3	55.7	105.0	1.3	98.7	103.2
	420	46.7	53.3	112.0	—	—	—
	600	52.0	48.0	98.5	—	—	—

oxidized-atom-labelled trithionate before degradation did not decrease the proportion of ³⁵S in the oxidized sulphur atoms. Spontaneous [³⁵S]trithionate formation occurs in mixtures of [³⁵S]thiosulphate and unlabelled trithionate but at a rate that is much too low to account for its formation in our experiments (Fava, 1953; D. P. Kelly, unpublished work).

Use of a 'trap' of elementary sulphur to detect free sulphur formation from the reduced atom of thiosulphate. In the absence of added elementary sulphur no significant loss of ³⁵S from solution was

ever demonstrated when the reaction mixtures were either membrane-filtered or centrifuged (contrast Trudinger, 1964*b,c*). Fig. 3 shows the oxygen consumption of dilute cell suspensions of *Thiobacillus* strain C oxidizing Na₂(³⁵S·SO₃) in the absence or presence of a large 'pool' of finely divided elementary sulphur, which also served as a substrate for oxidation. Table 4 demonstrates that, after the cessation of rapid oxidation, no ³⁵S had been retained in the sulphur 'trap'. This result contrasts with that of Peck & Fisher (1962). Chromatography showed 96–100% of the added ³⁵S to be present as

Fig. 3. Simultaneous oxidation of $\text{Na}_2(^{35}\text{S} \cdot \text{SO}_3)$ and elementary sulphur by *Thiobacillus* strain C. Experimental details were as given in Table 4. Thiosulphate was added at 40 min. Curve 1, with sulphur; curve 2, without sulphur; curve 3, boiled bacteria, with or without sulphur.

Table 4. $[^{35}\text{S}]$ Thiosulphate oxidation in the presence of a 'trap' of elementary sulphur

Warburg flasks contained (in a final volume of 2.4 ml.) 0.5 mg. dry wt. of living or dead (boiled for 2 min.) *Thiobacillus* strain C and 200 μmoles of sodium phosphate buffer, pH 7.0, with or without 100 mg. of fine elementary sulphur, and shaken at 30° . After preincubation for 40–50 min. 9.8 μmoles (7.2 μC) of $\text{Na}_2(^{35}\text{S} \cdot \text{SO}_3)$ were added. Oxidation is shown in Fig. 3. After the final manometric reading, the flask contents were filtered through membranes and the ^{35}S content of filtrates and residues was determined. Washed membranes + sulphur were first oxidized with $\text{Br}_2\text{--HNO}_3\text{--HCl}$. Average values for duplicates are given.

Treatment	$10^{-4} \times ^{35}\text{S}$ (counts/ 100 sec./flask)		Recovery of added ^{35}S in filtrate (%)
	Filtrate	Sulphur residue	
Initial thiosulphate	5.437	0	100.0
Live cells—sulphur	5.374	0	98.5
Live cells+ sulphur	5.500	0	101.0
Dead cells—sulphur	5.160	0	95.0
Dead cells+ sulphur	5.042	0	93.0

sulphate in the flasks containing living cells. In a second experiment, dense or dilute bacterial suspensions oxidized $\text{Na}_2(^{35}\text{S} \cdot \text{SO}_3)$ with a sulphur pool (Fig. 4) and total analyses of ^{35}S distribution in the reaction mixtures were made both during

Fig. 4. Simultaneous oxidation of $\text{Na}_2(^{35}\text{S} \cdot \text{SO}_3)$ and elementary sulphur by dense and dilute suspensions of *Thiobacillus* strain C. Experimental details were as given in Table 5. Thiosulphate was added at 30 min. Curve 1, dense suspensions; curve 2, dilute suspensions; curve 3, boiled bacteria, both densities.

rapid oxidation and immediately after its cessation (Table 5). No $[^{35}\text{S}]$ sulphur was formed, but with the dilute suspensions labelled trithionate accumulated as usual and ^{35}S was present in the oxidized atoms of trithionate and thiosulphate, just as it was in the absence of elementary sulphur.

DISCUSSION

Since no elementary $[^{35}\text{S}]$ sulphur was precipitated during $[^{35}\text{S}]$ thiosulphate oxidation nor was any trapped in a pool of added sulphur, either sulphur is not an intermediate in the oxidation of thiosulphate by *Thiobacillus* strain C or, if it is, it failed to exchange at all with exogenous sulphur even though this was being oxidized simultaneously.

A preferential formation of sulphate from the oxidized sulphur atom of thiosulphate (Table 2 and Fig. 2) has also been observed by Trudinger (1964c) with *Thiobacillus* X and by Smith (1965) with *Chromatium*. This finding is consistent with

Table 5. Oxidation of (³⁵S·SO₃)²⁻ and the formation of [³⁵S]trithionate in the presence of a pool of elementary sulphur

Warburg flasks contained (in a final volume of 2.9 ml.) 0.4 mg. dry wt. (dilute) or 4.0 mg. dry wt. (dense) of *Thiobacillus* strain C, 200 μmoles of sodium phosphate buffer, pH 7.0, and 40 mg. of elementary sulphur; after preincubation at 30°, 10.9 μmoles (4 μC) of Na₂(³⁵S·SO₃) were added and oxidation was followed manometrically (Fig. 4). Duplicate flasks were removed during (dilute) and at the end of (dilute and dense) rapid oxidation and the sulphur was separated by centrifugation, washed, dried and dissolved in CS₂. The supernatants were mixed with ethanol for chromatography, and sampled for ³⁵S content. Thiosulphate and trithionate were purified from mixtures for degradation. Average values for duplicates are given.

(a) Distribution of ³⁵S in total samples

Treatment	Sample time (see Fig. 4) (min.)	10 ⁻³ × ³⁵ S (counts/100 sec./flask)		Recovery of added ³⁵ S (%)	Distribution of ³⁵ S in supernatants (%)			
		Supernatant	S ⁰ pool		S ₂ O ₃ ²⁻	SO ₄ ²⁻	S ₃ O ₆ ²⁻	S ₄ O ₆ ²⁻
Boiled (dense)	80	282	0	99.0	84.7	3.1	5.5	6.9
Living (dense)	80	299	0	105.0	0	99.4	0.6	0
Boiled (dilute)	105	260	0	91.0	86.1	1.5	5.5	6.9
Living (dilute)	54	277	0	98.0	46.9	27.0	20.4	5.8
Living (dilute)	105	286	0	99.0	0	81.5	18.5	0

(b) Distribution of ³⁵S in the isolated thiosulphate and trithionate

Treatment	Compound degraded	Distribution (% of recovered ³⁵ S)		³⁵ S recovery in assay (%)
		Reduced atom	Oxidized atom	
Boiled (dilute) 105 min.	Thiosulphate	92.2	7.8	89.6
	Trithionate	81.0	19.0	83.3
Living (dilute) 54 min.	Thiosulphate	63.9	36.1	100.3
	Trithionate	51.3	48.7	89.3

a number of different possible mechanisms of thiosulphate oxidation. In the mechanism proposed by Peck (1960, 1962), thiosulphate undergoes an initial reductive cleavage by which the oxidized sulphur atom appears as sulphite and is oxidized to sulphate by two further reactions, whereas the reduced atom appears as sulphide whose oxidation pathway is unknown. Similarly, by any mechanism of thiosulphate metabolism in which the first step is its oxidation to tetrathionate or a related S₄ compound, it seems clear that more reactions will be required to oxidize the reduced sulphur atom to sulphate than to complete the oxidation of the oxidized sulphur atom, which is already combined with three oxygen atoms. Thus, on either hypothesis, preferential formation of sulphate from the inner, oxidized sulphur atom of thiosulphate might be expected.

When thiosulphate was supplied as (S·³⁵SO₃)²⁻ rather than as (³⁵S·SO₃)²⁻, not only was [³⁵S]sulphate formed more rapidly but [³⁵S]thiosulphate disappeared more quickly. It is not at once obvious why this should be so, since any reaction of thiosulphate must result in the disappearance of the whole molecule. A possible explanation comes from the observation that thiosulphate labelled in both sulphur atoms appeared within 20 sec. when

(³⁵S·SO₃)²⁻ was oxidized. This suggests that, after an initial reaction, the reduced sulphur atom of (³⁵S·SO₃)²⁻ is oxidized, forming uniformly labelled thiosulphate. Thus the lag in the disappearance of [³⁵S]thiosulphate when (³⁵S·SO₃)²⁻ is the substrate would be due to the resynthesis of labelled thiosulphate. Indeed, from Fig. 2, it can be deduced that between the fourth and fifth minutes of this experiment the thiosulphate remaining in solution must have been derived almost entirely from the reduced sulphur atom of the initial thiosulphate, since during the first 4 min. all the radioactivity supplied as (S·³⁵SO₃)²⁻ had been converted into sulphate or trithionate. If the initial reaction of thiosulphate is its reductive cleavage to sulphide and sulphite, uniformly labelled thiosulphate could arise by the oxidation of the sulphide moiety to thiosulphate, as Peck & Fisher (1962) have suggested. On the other hand, it could arise from the two central atoms of a tetrathionate molecule formed by the initial oxidation of thiosulphate.

As Trudinger (1964c) has pointed out, polythionate formation during thiosulphate oxidation by thiobacilli is variable and dependent on a number of factors including the way in which the organisms were grown. Thus not too much significance should be attached to the accumulation of trithionate,

rather than other polythionates, in our experiments. Nevertheless an analysis of the course of [^{35}S]trithionate production from [^{35}S]thiosulphate throws some light on the mechanism of thiosulphate oxidation.

During the first 30 sec. of thiosulphate oxidation, [^{35}S]trithionate was formed more quickly from $(\text{S} \cdot ^{35}\text{SO}_3)^{2-}$ than from $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ (Table 2). Thereafter the rate of [^{35}S]trithionate formation was the same from either substrate. These facts rule out the possibility that trithionate was formed by the simple oxidation of tetrathionate:

because, by this mechanism, the rate of ^{35}S appearance in trithionate would be twice as rapid when ^{35}S was supplied in the reduced sulphur atom of thiosulphate rather than in the oxidized one.

Moreover, such a mechanism fails to explain the distribution of ^{35}S within the trithionate molecules. The results show that, when $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ is supplied, the first-formed trithionate is labelled predominantly in the central, reduced sulphur atom, but as oxidation proceeds the proportion of ^{35}S in the outer, oxidized atoms of trithionate increases and, when thiosulphate oxidation ceases, the two oxidized atoms together contain about half of the total ^{35}S in the trithionate (Table 3).

These results can be accounted for in at least two different ways. First, they are consistent with the hypothesis of Peck (1960) provided that the reduced sulphur atom of thiosulphate, which, by his mechanism, appears first as sulphide, is oxidized to sulphate with sulphite as an intermediate. Evidence that suggests that sulphite is formed from both atoms of thiosulphate has, in fact, been presented by Santer (1959) and by Peck & Stulberg (1962) from ^{18}O -labelling experiments. Trithionate might then arise by an enzyme-catalysed oxidative reaction between thiosulphate and sulphite (Scheme 1). By this mechanism one would expect that, at first, trithionate would be formed by reaction (a), since sulphite would be produced more quickly initially from the oxidized sulphur atom of thiosulphate; this initial trithionate would be labelled only in the central sulphur atom when $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ was the substrate. As the reduced sulphur atom of thiosulphate also appeared as sulphite, reaction (b) would take place and, when both reactions (a) and (b) were equally rapid, the rate of incorporation of ^{35}S into trithionate would be the same whether the substrate was $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ or $(\text{S} \cdot ^{35}\text{SO}_3)^{2-}$. Moreover, ^{35}S from $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ would appear in increas-

Scheme 1. Hypothetical reactions explaining trithionate formation.

ing amounts in the oxidized sulphur atoms of trithionate and one would expect, finally, two-thirds of the ^{35}S in trithionate to be in the reduced central atom and one-third in the oxidized atoms. However, when $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ was supplied, thiosulphate labelled in both sulphur atoms appeared (Table 5 and Trudinger, 1964c). If this doubly labelled thiosulphate were to participate in the reactions postulated in Scheme 1, a greater proportion of ^{35}S would be introduced into the oxidized sulphur atoms of trithionate when $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$ was the original substrate. A more equal distribution of ^{35}S between the reduced and oxidized sulphur atoms of trithionate would then be expected, in agreement with the observed result (Table 3). One would expect, too, that [^{35}S]trithionate formation from $(\text{S} \cdot ^{35}\text{SO}_3)^{2-}$ would terminate before that from $(^{35}\text{S} \cdot \text{SO}_3)^{2-}$, as Fig. 2 shows. Thus the mechanism of Scheme 1 is consistent with our results. On this view trithionate arises, by oxidation, as a by-product of the main sequence of thiosulphate oxidation. On the Peck mechanism, the initial attack of thiosulphate is reductive. Both tetrathionate formation and trithionate formation from thiosulphate are oxidative processes; their function may be to provide electrons for the reductive cleavage of thiosulphate. The subsequent oxidation of the polythionates might involve their prior reduction to thiosulphate and sulphite (Imai, Okuzumi & Katagiri, 1962; Trudinger, 1964a).

Other mechanisms of trithionate formation are, however, possible and a second way in which our

results could be accounted for is by a reaction between thiosulphate and tetrathionate, giving trithionate as a product. Pankhurst (1964) suggests that trithionate is formed like this by a purely chemical, i.e. non-enzyme-catalysed, reaction. However, experiments have shown that the rate of formation of labelled trithionate when [³⁵S]thiosulphate and tetrathionate are shaken together is far too low to account for [³⁵S]trithionate formation in our cultures (D. P. Kelly, unpublished work). Moreover, the amount of tetrathionate present at any time is very small. Nevertheless, trithionate might be formed by an enzyme-catalysed reaction between thiosulphate and tetrathionate and, if a series of reactions like those of Scheme 2 took place, both the rate of formation of [³⁵S]trithionate from (³⁵S·SO₃)²⁻ or (S·³⁵SO₃)²⁻ and the distribution of ³⁵S within the trithionate molecules would be the same as those predicted by the mechanism of Scheme 1. Thus our results, though eliminating

some mechanisms of trithionate formation, do not distinguish unequivocally between mechanisms of thiosulphate oxidation based on an initial reductive attack, as in Scheme 1, and mechanisms based on an initial oxidation, as in Scheme 2.

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Scheme 2. Alternative hypothetical reactions explaining trithionate formation.