The Oxidation of Inorganic Compounds of Sulphur by various Sulphur Bacteria

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SUMMARY: Cultures of *Thiobacillus thiooxidans*, *Th. thioparus*, *Th. novellus*, *Thiobacillus B* (Waksman, 1922*a*), strains 'T' and 'K' (Trautwein, 1921) and *Th. concretivorus*, *Thiobacillus X* and the 'M' strains, organisms isolated from concrete (Parker, 1945, 1947), were examined to elucidate the mode of oxidation and to establish the identity of the organisms recently isolated from corroded concrete.

Thiosulphate was oxidized by all these bacteria. *Th. thiooxidans*, *Th. concretivorus* and *Thiobacillus X* first converted it to tetrathionate and sulphate and then oxidized the tetrathionate to sulphate and free sulphuric acid. *Thiobacillus X* differed from the other two in that, owing to a lesser acid tolerance, some tetrathionate was found in the final products of oxidation. *Th. thioparus* converted thiosulphate to sulphate and sulphur, followed by partial oxidation of the sulphur to sulphuric acid. *Th. novellus* produced sulphate and sulphuric acid. *Thiobacillus B*, the 'T' and 'K' strains and the 'M' strains formed sulphate and tetrathionate with temporary increase in pH value; only *Thiobacillus X* oxidized tetrathionate, yielding sulphate and sulphuric acid.

Elementary sulphur was oxidized by *Th. thiooxidans*, *Th. concretivorus*, *Thiobacillus X* and *Th. thioparus*; the rates of oxidation decreased in that order, and the only product was sulphuric acid.

Hydrogen sulphide was oxidized only at low concentrations and only by *Th. concretivorus* and *Thiobacillus X*; sulphuric acid was the end-product, and elementary sulphur may have been an intermediate.

Thiobacillus X differed from *Th. thiooxidans* in pH range for growth and from *Th. thioparus* in its method of oxidation of thiosulphate, tetrathionate and H₂S. It appeared to fit earlier descriptions of *Th. thioparus* by Nathansohn (1902) and Beijerinck (1904) more closely than the strain described as *Th. thioparus* by Starkey (1934*a*). The 'M' strains were similar to *Thiobacillus B* and the 'T' and 'K' strains of Trautwein.

Species of micro-organisms capable of oxidizing inorganic sulphur compounds fall into three main groups: those which oxidize hydrogen sulphide autotrophically and deposit sulphur intra- or extracellularly, including members of the Beggiatoaceae, Thiorhodaceae and Chlorobacteriaceae families of the classification of *Bergey's Manual* (1948); members of the genus *Thiobacillus* which oxidize a wide variety of sulphur compounds; various heterotrophic organisms such as *Pseudomonas* spp. which appear to have a much more limited ability for oxidation.

Difficulty in isolating pure cultures of members of the first group has retarded the elucidation of their metabolic activities. However, pure culture studies of the Thiorhodaciae and Chlorobacteriaceae by van Niel (1931) have thrown considerable light on the mechanism of photosynthesis by these organisms. Detailed studies of the oxidative ability of organisms of the second and third

groups were made by Starkey (1925, 1934*b*, 1935*a*). More recently, the work of Umbreit and colleagues (Vogler, 1942; Vogler & Umbreit, 1942; LePage & Umbreit, 1943) with *Thiobacillus thiooxidans*, has revealed something of the nature of the chemosynthetic process.

However, the ability of several species of the second and third groups to oxidize some forms of sulphur remains uncertain, and the exact course of the oxidation is still open to doubt. To help resolve these points and to establish the identity of species of sulphur bacteria recently isolated from corroded concrete (Parker, 1945, 1947) an examination of several species of bacteria able to oxidize inorganic sulphur compounds has been made and some of Starkey's work has been repeated in greater detail. Three types of organism isolated from concrete were examined: the autotrophic *Th. concretivorius* (Parker, 1945); the *Th. thioparus*-like autotrophic type (Parker, 1947) here referred to as *Thiobacillus X*; a miscellaneous collection of heterotrophic types (Parker, 1947) here referred to as the 'M' strains; strains of *Th. thiooxidans*, *Th. thioparus*, *Th. novellus*, *Thiobacillus B* (Waksman); strains 'T' and 'K' of Trautwein. These latter were kindly supplied by Dr R. L. Starkey and, except for *Th. thioparus*, were subcultures of those originally isolated. The strain of *Th. thioparus* was a subculture of an organism isolated by Starkey (1934*a*) and considered by that author to be identical with the original strain isolated by Nathansohn (1902) and named by Beijerinck (1904).

METHODS

Media. The constituents of the basal media used are shown in Table 1. To study the oxidation of the various inorganic sulphur compounds, appropriate alterations were made in the sulphur compound added.

Table 1. *Composition of media used in study of oxidation by various sulphur bacteria*

	Medium					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
	(g./l.)					
(NH ₄) ₂ SO ₄	0.1	0.1	0.1	—	—	—
NH ₄ Cl	—	—	—	0.1	0.1	0.1
K ₂ HPO ₄	2.0	4.0	2.0	0.2	—	—
KH ₂ PO ₄	—	4.0	—	—	3.0	3.0
MgSO ₄ ·7H ₂ O	0.1	0.1	0.1	—	—	—
MgCl ₂ ·6H ₂ O	—	—	—	2.5	0.1	0.1
CaCl ₂	0.1	0.1	0.1	—	0.1	0.1
FeCl ₃ ·6H ₂ O	0.02	0.02	0.02	—	—	—
MnSO ₄ ·4H ₂ O	0.02	0.02	0.02	—	—	—
NaHCO ₃	—	—	—	1.0	—	—
CaCO ₃	—	—	Excess	—	—	—
Sulphur	—	—	—	—	—	10.0
Na ₂ S ₂ O ₃ ·5H ₂ O	10.0	10.0	10.0	5.0	5.0	—
pH value	8.5	6.6	9.2	9.1	4.2	4.2

Media 1 and 2 are those used by Starkey (1934*a*); medium 3 is similar to his no. 3. Media 4, 5 and 6 are similar to those used by Beijerinck (1904).

The rate of oxidation could be varied by altering the ratio of surface area exposed to air to volume of culture medium, so appropriate volumes of medium were placed in Erlenmeyer flasks depending on the speed of reaction desired. In general, 100 ml. medium was used in a 300 ml. Erlenmeyer flask. Separate flasks were taken for analysis at intervals. In some experiments, however, 1.5 l. medium was used in a 2 l. flask and samples were taken at intervals from it.

Media containing thiosulphate were steamed in bulk on three successive days and then distributed aseptically in 100 ml. lots into sterile 300 ml. Erlenmeyer flasks. Medium 6 was autoclaved in separate flasks and the sulphur was steamed in test tubes and then added to the medium.

Media containing tetrathionate were incubated in 50 ml. lots in 100 ml. Erlenmeyer flasks. Potassium tetrathionate was prepared in this laboratory as follows: Wackenroder's solution was prepared by passing H_2S through a saturated solution of sulphurous acid held just above 0° (Debus, 1888). When the reaction was completed sulphur was filtered off and the solution concentrated to sp.gr. 1.82 by evaporation on a water-bath. Potassium salts of the mixed thionic acids were then prepared by the addition of a saturated solution of potassium acetate to the concentrated Wackenroder's solution. The potassium salts were allowed to crystallize for 24 hr. and crystalline pentathionate and tetrathionate filtered off. The mixed crystalline mass was recrystallized from water slightly acidified with sulphuric acid and the potassium tetrathionate separated by hand picking typical crystals. It was possible in this way to separate material which appeared to be pure potassium tetrathionate; this was again recrystallized from 90% (v/w) ethanol. The final product was shown to be free from thionates other than tetrathionate by the analytical methods described below.

Inoculum. Suspensions of the various strains were obtained by washing the growth from an agar slope with 1–2 ml. sterile distilled water containing thiosulphate; 0.25 ml. of this suspension was used to inoculate each flask. With cultures which grew well on agar medium the number of organisms in the culture medium immediately after inoculation was about 10^6 /ml., while cultures which grew poorly on agar medium, such as *Th. novellus*, gave an initial viable count of about 10^3 /ml.

Incubation. Cultures were incubated at 28° , except for those grown in an atmosphere of hydrogen sulphide. Loss of water during prolonged incubation was minimized by using small incubators each containing a shallow vessel of water. When hydrogen sulphide oxidation was examined, the inoculated flasks were incubated at room temperature (about 18°) in a glass box, through which was passed air containing approximately 200 p.p.m. H_2S .

Analyses. Usually a whole flask was taken for analysis; when significant evaporation had occurred, the contents were first transferred to a standard flask and diluted with sterile distilled water to the original volume. The bacterial count was determined by serial tenfold dilution of 1 ml. medium in sterile distilled water and inoculation of the dilutions into appropriate culture medium. This procedure was only accurate to a power of 10, but was sufficiently

accurate to indicate the major changes in population during incubation. The pH values were determined electrometrically.

Cultures were filtered through asbestos in Gooch crucibles into dry flasks, to remove elementary sulphur. The sulphur adhering to the incubated flask was later removed and washed into the same crucible. Samples from the filtered culture were quickly measured and the analyses begun at once, as in some cases decomposition of the solution, shown by clouding, took place very soon after filtration.

Thiosulphate, polythionate, tri-, tetra- and pentathionate, and elementary sulphur were determined by the methods of Starkey (1935*a*). The term 'elementary sulphur' is used to describe any form of insoluble sulphur precipitated during growth. Sulphates were determined gravimetrically as BaSO₄. *Sulphide* was determined by the methylene-blue method of Pomeroy (1936), except in media where it was inapplicable. In these cases the antimony sulphide method (*Standard Methods of Water Analysis*, American Public Health Association, 8th ed.) was used. *Sulphide* was determined as 'sulphide after acidification' without reference as to whether the sulphide was present as dissolved hydrogen sulphide, hydrosulphide, or polysulphide. The analytical results for the various forms of sulphur are recorded in the Tables as mg. S/l. culture medium.

RESULTS

Thiobacillus concretivorus

Thiosulphate oxidation. The method of oxidation of thiosulphate by this strain has been described (Parker, 1945). In the present investigation a more detailed examination of the course of reaction was made. Flasks of medium 5 were inoculated with a young culture and analysed at frequent intervals. Two different strains were examined, but, as they behaved similarly, the results with one strain only are shown (Table 2). The chemical changes fell into two

Table 2. *Oxidation of thiosulphate by Thiobacillus concretivorus*

Cultures in medium 5 were analysed at intervals after inoculation

Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ml.)
		S ₂ O ₃ ^{''}	S ₃ O ₄ ^{''}	SO ₄ ^{''}	S (element)	
		Concentration (mg.S/l.)				
0	4.4	1030	0	40	nt	10 ⁶
42	4.3	1020	0	30	nt	10 ⁶
114	4.4	800	200	110	nt	10 ⁷
138	4.1	275	560	240	20	10 ⁸
162	2.3	0	10	1000	60	10 ⁷

nt = not tested.

stages: (1) during oxidation of the thiosulphate and before its complete disappearance, substantial quantities of polythionate were detected; (2) quantitative examination indicated that the polythionate was almost entirely tetrathionate. Tetrathionate persisted until all the thiosulphate had been

oxidized. Owing to the rapidity of oxidation, polythionates could only be detected over a period of 2 or 3 days. This transitory occurrence of polythionate was no doubt responsible for the failure to detect it in the previous work. During the conversion of thiosulphate to tetrathionate, there was very little change in pH value of the medium. There was a definite increase in sulphate and the bacterial count also increased. Very small amounts of elementary sulphur were formed.

After the complete disappearance of thiosulphate, the tetrathionate disappeared, this change being completed within 24 hr. It was accompanied by a further increase in sulphate concentration, a very marked decrease in pH value and the formation of a further small amount of elementary sulphur. The bacterial count remained high during this stage.

No further chemical change of any magnitude occurred after the complete oxidation of tetrathionate to sulphate, but the culture remained viable for at least a further 6 days. This was probably due to the presence of the small amounts of elementary sulphur which served as a further source of energy after thiosulphate and tetrathionate had disappeared.

Starkey (1925) found that the course of oxidation of thiosulphate by *Th. thiooxidans* was influenced by the thiosulphate concentration. The influence of concentration of thiosulphate on the course of oxidation was therefore examined with *Th. concretivorus*. Batches of medium 5, with thiosulphate

Table 3. *Effect of initial thiosulphate concentration on oxidation of thiosulphate by Thiobacillus concretivorus*

Cultures in medium 5 with various initial concentrations of $\text{Na}_2\text{S}_2\text{O}_3$ were analysed at intervals after inoculation.

Initial thiosulphate concentration (% Na ₂ S ₂ O ₃)	Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ ml.)
			S ₂ O ₃ ^{''}	S ₄ O ₆ ^{''}	SO ₄ ^{''}	S element	
			Concentration (mg. S/litre)				
1	0	4.6	2,580	0	30	0	10 ⁶
	42	4.2	2,510	40	nd	0	10 ⁶
	114	4.7	1,545	370	nd	10	10 ⁷
	162	2.6	0	870	nd	100	10 ⁷
	210	nd	0	20	nd	240	10 ⁷
	306	nd	0	0	2,590	10	10 ³
2	0	4.6	5,250	0	60	0	10 ⁶
	144	4.6	4,370	400	nd	60	10 ⁶
	240	4.6	3,250	940	nd	nd	nd
	264	3.3	180	680	nd	120	10 ⁷
	336	1.3	0	10	nd	380	10
	600	1.6	0	0	5,200	130	10
4	0	4.7	10,360	0	60	0	10 ⁶
	120	4.9	9,580	0	nd	60	10 ⁵
	288	4.9	8,490	0	nd	550	10 ⁷
	480	4.6	3,320	2,770	nd	nd	10 ⁶
	624	3.7	2,660	1,190	nd	1,010	10 ⁴
	840	1.8	0	0	8,770	1,290	0

nd=not determined.

concentrations of 1.0 % (w/v), 2.0 % (w/v) and 4.0 % (w/v) were inoculated and the reaction followed. Thiosulphate oxidation was not inhibited by 4 % (w/v) sodium thiosulphate, although the time required for complete oxidation increased with concentration of thiosulphate. The decrease in pH value was delayed with the higher concentrations of thiosulphate, though ultimately it fell to less than pH 2.0 with all concentrations. (Starkey noted a similar phenomenon with *Th. thiooxidans*.) Tetrathionate was formed during thiosulphate oxidation at each concentration but disappeared before the cultures became sterile. Elementary sulphur formation was observed at each concentration, and was greater the higher the concentration. At the end of the incubation, the sulphur was present predominantly as sulphate at each concentration.

Tetrathionate oxidation. The finding that tetrathionate was an intermediate product of thiosulphate oxidation by *Th. concretivorus* made it desirable to examine its ability to oxidize tetrathionate. Quite high concentrations of tetrathionate were produced as an intermediate from thiosulphate, but the tetrathionate so formed was quickly oxidized further to sulphate and free sulphuric acid.

Medium 5, in which 0.5 % (w/v) potassium tetrathionate replaced thiosulphate, was inoculated with this strain, but, despite repeated attempts, neither growth nor oxidation of the tetrathionate occurred. As growth, tetrathionate formation and oxidation occurred in medium 5 when potassium thiosulphate replaced sodium thiosulphate, the absence of growth with potassium tetrathionate cannot be attributed to an absence of sodium ions. The inability of this organism to oxidize tetrathionate, despite its destruction by cultures in thiosulphate medium, is hard to understand, and no satisfactory reconciliation of the two observations can be made at present.

Oxidation of elementary sulphur. The method of oxidation of elementary sulphur by this organism was studied by Parker (1945). The sole product of oxidation in the presence of excess sulphur was sulphuric acid which reached concentrations of over 5 % (w/v).

H₂S oxidation. Parker (1945) reported that this organism did not utilize H₂S when cultures were exposed to air containing 1 % (v/v) H₂S. In the present work the organism was inoculated into liquid mineral salt medium containing no oxidizable form of sulphur and incubated in air containing 200 p.p.m. H₂S; initial pH value of medium 4.5. The results of analyses during incubation are shown in Table 4.

Metabolism was indicated by the continuous viable count throughout the period of incubation and was accompanied by a rapid drop in pH value from 4.5 to 1.8 in the inoculated flasks. The increase in the total sulphur content of the inoculated compared with the sterile flasks shows that H₂S was utilized. The end-product of oxidation was mainly sulphuric acid, but the presence of a small amount of elementary sulphur towards the end of the incubation period in the inoculated flasks, and its absence from the controls, suggested that sulphur was first formed and then oxidized. Thiosulphates and polythionates were not detected at any stage of the incubation. The growth of the

organism in air containing 200 p.p.m. H_2S compared with its lack of growth in 1 % (v/v) H_2S suggests that the higher concentration was toxic.

Table 4. *Oxidation of H_2S by Thiobacillus concretivorus*

Cultures in medium 5 without thiosulphate, but with an atmosphere of air containing 200 p.p.m. H_2S were analysed at intervals after inoculation.

Period of incubation (days)		pH value	Form of S			Viable count (organisms/ml.)
			SO ₄ ²⁻	S (element)	Sulphide	
			Concentration (mg. S/l.)			
Inoculated culture	6	3.1	45	10	0	10 ⁷
	11	2.3	158	73	0	10 ⁷
	18	2.2	301	135	0	10 ⁴
	21	2.0	389	nd	0	10 ⁴
	29	1.8	481	405	0	10 ⁵
Sterile control	6	4.5	0	0	0.9	0
	11	4.4	0	0	0.6	0
	18	4.4	0	0	0.6	0
	21	4.4	0	0	0.6	0
	29	4.4	0	0	0	0

nd = not determined.

Thiobacillus thiooxidans

Thiosulphate oxidation. The route of oxidation of thiosulphate by this organism was described by Waksman & Starkey (1923). Since tetrathionate was detected during the oxidation of thiosulphate by *Th. concretivorus*, a more detailed examination of its oxidation by *Th. thiooxidans* was made.

Table 5. *Oxidation of thiosulphate by Thiobacillus thiooxidans*

Cultures in medium 5 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ml.)
		$\text{S}_2\text{O}_3''$	$\text{S}_4\text{O}_6''$	SO_4''	S (element)	
		Concentration (mg. S/l.)				
0	4.4	1030	0	40	0	10^8
42	4.3	1020	10	30	nt	10^8
114	4.3	945	70	80	nt	10^7
138	4.0	120	680	310	tr.	10^7
162	2.2	0	0	1070	tr.	10^8

nt = not tested.

The results given in Table 5 show that during the destruction of thiosulphate, tetrathionate and sulphate were formed, but that there was little change in pH value until thiosulphate had completely disappeared. Tetrathionate was only found during a period of 24 hr. at the fifth to sixth day of incubation. After the complete disappearance of thiosulphate, tetrathionate was converted to sulphate with a sharp drop in pH value.

The effect of concentration of thiosulphate on the course of reaction was studied in the manner described for *Th. concretivorus*. Table 6 records that, as noted by Starkey, the time taken for the pH value to decrease increases with concentration of thiosulphate. Thiosulphate was completely oxidized in media containing concentrations of 0.5 % (w/v) and 1 % (w/v), but with concentra-

Table 6. *Effect of thiosulphate concentration on the oxidation of thiosulphate by Thiobacillus thiooxidans*

Cultures in medium 5, containing various concentrations of sodium thiosulphate were analysed at intervals after inoculation.

Initial thiosulphate concentration (% Na ₂ S ₂ O ₃)	Period of incubation (hr.)	pH value	Form of S				Viable count (organisms/ ml.)
			S ₂ O ₃ ^{''}	S ₄ O ₆ ^{''}	SO ₄ ^{''}	S (element)	
			Concentration mg. S/l.				
1	0	4.6	2,580	0	30	0	10 ⁶
	42	4.2	2,455	70	110	nd	10 ⁶
	114	4.2	2,080	220	250	10	10 ⁵
	162	4.3	1,785	280	350	90	10 ⁷
	210	4.3	885	780	710	90	10 ⁸
	306	1.8	0	0	2,430	190	10 ⁷
	618	nd	nd	nd	nd	10	10 ⁸
2	0	4.6	5,250	0	0	0	nd
	144	4.2	4,990	130	90	40	10 ³
	240	4.3	4,965	90	155	nd	nd
	264	4.1	4,870	80	250	50	10 ⁵
	600	4.0	4,790	130	250	80	10 ⁵
4	0	4.9	10,510	0	0	0	nd
	144	4.5	10,485	0	25	10	10 ⁴
	264	4.5	10,075	0	405	nd	10 ⁵
	408	4.5	9,830	0	640	40	10 ⁴
	600	3.9	9,175	70	1,025	310	0

nd = not determined.

tions of 2 % (w/v) and 4 % (w/v), only about one-tenth was oxidized, even after 25 days' incubation. The low viable counts indicate that the higher concentrations of thiosulphate exerted an inhibitory effect. Tetrathionate was found, but the amount produced was less with the higher concentrations of thiosulphate. With a 1.0 % (w/v) thiosulphate the course of oxidation was similar to that described for 0.5 % (w/v); thiosulphate was first oxidized to tetrathionate and sulphate with little change in pH value. In this case, however, tetrathionate was detectable during a much longer period (168 hr.). It reached a maximum concentration when all thiosulphate had been oxidized, and was then converted to sulphate with rapid decrease in pH value. Proportionately much more elementary sulphur was formed from 1 % than from 0.5 % thiosulphate, suggesting that tetrathionate was partly oxidized to acid before the complete disappearance of thiosulphate. The low and diminishing tetrathionate/sulphate ratio during the period of thiosulphate destruction supports this view.

Tetrathionate oxidation. Medium 5, in which 0.5% (w/v) potassium tetrathionate replaced thiosulphate, was repeatedly inoculated, but neither growth nor tetrathionate oxidation could be established. With this organism, like *Th. concretivorus*, it is difficult to understand its inability to metabolize tetrathionate as a primary source of sulphur, while the tetrathionate formed during oxidation of thiosulphate media is rapidly destroyed.

Oxidation of elementary sulphur by Th. thiooxidans was studied by Lipman, Waksman & Joffe (1921), Waksman (1922*a, b*), Waksman & Starkey (1923), Starkey (1925). Our re-examination of this oxidation process in medium 6 confirmed the findings of the above authors that sulphur is converted directly to free sulphuric acid without the formation of any intermediate products.

H₂S oxidation. *Th. thiooxidans* was inoculated into medium 5 without thiosulphate, and incubated in air containing H₂S. A viable count was obtained even after 48 days incubation, by which time the pH value had decreased to 2.2. H₂S was oxidized as shown by the increase in total dissolved sulphur from 0 to 472 mg./l.; the main product was sulphuric acid. As with *Th. concretivorus*, a little elementary sulphur accumulated towards the end of the incubation period; this suggests that the oxidation goes through elementary sulphur. The amount of sulphur formed by *Th. thiooxidans* was much less than that formed by *Th. concretivorus*. The ability of *Th. thiooxidans* to oxidize H₂S has been the subject of contradictory statements in the literature, but the present experiments definitely establish its ability to utilize H₂S under the conditions described. Its mode of oxidizing sulphide appears to be identical with that of *Th. concretivorus*.

Thiobacillus X

Thiosulphate oxidation. Oxidation of thiosulphate by two strains was studied. As they oxidized thiosulphate in essentially the same way, results for one strain only (X44) are given in Fig. 1. The final products of oxidation were tetrathionate, sulphate, free sulphuric acid, and very small amounts of free sulphur and pentathionate; the pH value fell from 6.6 to 3.3. The oxidation occurred in three stages. During the first 45 hr. there was a slow increase in sulphate and fall in pH value, but no polythionates could be detected, nor was any free sulphur present. About 10% of the thiosulphate was oxidized during this period and the bacterial count increased from 10⁶ to 10⁸ organisms/ml. In the next 24 hr. the remaining 90% of the thiosulphate was oxidized, the bacterial count increased to 9/ml. and there was little fall in pH value. There was accelerated production of sulphate and rapid production of polythionate (almost entirely tetrathionate). The third stage immediately followed the disappearance of thiosulphate. The pH value decreased from 6.6 to *c.* 3, tetrathionate concentration decreased and the sulphate concentration increased (although more slowly than in the previous period). A thin pellicle, presumably of sulphur, became visible. This third stage occupied 27 hr., during which the bacterial count fell from 10⁹ to 10⁷ organisms/ml. Following the sharp decrease in pH value there was a further slow decrease to an ultimate value of pH 3.0, accompanied by a slow decrease in polythionate and an increase in sulphate

concentration. The bacterial count decreased and the culture became sterile after 120 hr., although a considerable amount of polythionate remained. The inability of this organism to carry tetrathionate oxidation to completion suggests that it is intolerant of pH values less than 3.0. This point was examined by adding a suspension of organisms to sterile saline buffered to different pH values; it was found that on subculture into medium 2, organisms exposed to

Fig. 1. Oxidation of thiosulphate by *Thiobacillus X* (strain X44). Cultures in medium 2 containing 1 % (w/v) $\text{Na}_2\text{S}_2\text{O}_3$ were analysed at intervals after inoculation.

pH values less than 3.0 for 30 min. failed to grow. Further support for this view was obtained by making the usual analyses in a thiosulphate culture in which the pH value, by continuous readjustment, was prevented from becoming less than 4.7 during the whole period of incubation. Under these conditions tetrathionate was completely converted to sulphate, and the rate of increase of sulphate concentration following thiosulphate disappearance was much greater than in the unadjusted culture. Whereas the unadjusted culture was sterile within 5 days of completion of thiosulphate oxidation, a count of 10^7 viable organisms/ml. was found 12.5 days after thiosulphate oxidation had ceased in the adjusted culture.

Tetrathionate oxidation. Tetrathionate was formed as an intermediate in thiosulphate oxidation, and was subsequently converted partially to sulphate and free acid. The strain used was inoculated into medium 2, containing 0.5 % (w/v) tetrathionate, instead of thiosulphate, and analysed at intervals as before (Table 7). The strain grew well and oxidized tetrathionate rapidly, the final products being sulphate and some pentathionate; some tetrathionate

remained unoxidized. The oxidation was accompanied by a decrease in pH value from 6.7 to 3.3. Within 30 hr. after inoculation, rapid oxidation of tetrathionate began, and its destruction was associated with rapid increase in sulphate concentration and a sharp decrease in pH value from 6.6 to 3.5. A little thiosulphate appeared between 20 and 45 hr. but later disappeared.

Table 7. *Oxidation of tetrathionate by Thiobacillus X (strain X 44)*

Cultures in medium 2 with potassium tetrathionate instead of thiosulphate were analysed at intervals after inoculation.

		Form of S					
Period of incubation (hr.)	pH value					S	Viable count (organisms/ml.)
		S ₂ O ₃ ²⁻	S ₄ O ₆ ²⁻	S ₅ O ₆ ²⁻	SO ₄ ²⁻	(element)	
		Concentration mg. S/l.					
20	6.6	5	2100	0	80	0	10 ⁶
45	6.2	55	1810	0	380	tr.	10 ⁸
68	3.9	0	1510	0	590	10	10 ⁸
74	3.8	0	nd	nd	nd	nd	10 ⁷
93	3.6	0	1540	0	600	tr.	10 ⁷
141	3.6	0	1560	0	660	tr.	10 ⁶
237	3.5	0	1050	570	640	tr.	10 ³
573	3.3	45	1150	170	850	20	Sterile
Control							
45	6.7	5	2080	0	140	0	Sterile
573	6.5	205	1290	540	200	0	Sterile

tr. = trace; nd = not determined.

When the pH value had decreased to 3.5, oxidation of tetrathionate, sulphate formation, and further fall in pH value all practically ceased. The bacterial count decreased shortly after and eventually the culture became sterile. These results are essentially the same as those described for the third stage of thio-sulphate oxidation by this organism. The appearance of pentathionate after *c.* 10 days incubation may have been due to a chemical conversion of tetrathionate after bacterial activity had ceased, as the viable count had then fallen to 10^3 organisms/ml. The presence of pentathionate in the sterile control after 24 days incubation indicated that chemical conversion of tetrathionate to pentathionate could occur. These results confirm the ability of this organism to oxidize tetrathionate with the formation of sulphate and acid.

Oxidation of elementary sulphur. The strain was inoculated into medium 2 containing 10 g. sulphur/l. in place of thiosulphate and analysed at intervals. The organism grew with elementary sulphur which was oxidized directly to sulphuric acid as indicated by an increase in sulphate and a decrease in pH value. No immediate products were detected at any stage of the incubation.

The rate of oxidation, indicated by the rate of acid formation, during the period of maximum activity of this organism was only about 10 % of that exhibited by *Th. concretivorus* and *Th. thiooxidans*. The oxidation of elementary sulphur by this organism also differed from those organisms, in that the amount of acid formed before the culture became sterile and oxidation ceased was less. Only 0.061 % (w/v) SO_4^{2-} was formed by *Thiobacillus X*, compared with values

between 1 and 10 % with the two high acid-formers; the pH value in this case fell only to 3.1, whereas with the other two organisms it became less than 1.0.

H₂S oxidation. Medium 2 lacking any form of oxidizable sulphur was inoculated and incubated in air containing 200 p.p.m. of H₂S. A high viable count was maintained for 24 days; thereafter the count decreased and the culture was sterile after 47 days. Sterility was again associated with decrease in pH value to 3.0, as happened with elementary sulphur and thiosulphate. During the early stage of oxidation only sulphate sulphur increased (owing to the formation of sulphuric acid), but towards the end of the incubation period, when the viable count had decreased, there was also an increase in elementary sulphur. This suggests that the conversion of H₂S to sulphate was through the intermediate formation of elementary sulphur. There was no evidence that thiosulphate or polythionates were formed at any stage.

Thiobacillus thioparus

Thiosulphate oxidation. The strain *Thiobacillus X* was described (Parker, 1947) as *Th. thioparus*-like principally because of (a) its ability to form a pellicle when grown with thiosulphate in liquid culture in a test tube, (b) its very slow oxidation of elementary sulphur and (c) the fact that growth ceased when the pH value fell to 3–4. A detailed examination of an authentic strain of *Th. thioparus* was therefore made for comparison with *Thiobacillus X*. Medium 2, containing 1 % (w/v) sodium thiosulphate, was inoculated from a fresh slope culture, and observations were made as before (Table 8). The

Table 8. *Oxidation of thiosulphate by Thiobacillus thioparus*

Cultures in medium 2 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S			Viable count (organisms/ml.)
		S ₂ O ₃ ^{''}	SO ₄ ^{''}	S (element)	
		Concentration mg. S/l.			
0	6.6	2670	40	0	10 ⁶
87	6.5	2605	110	0	10 ⁷
137	6.4	2355	290	80	10 ⁷
186	6.3	1005	1008	680	10 ⁷
208	6.3	570	1260	870	10 ⁷
257	6.0	0	1630	1090	10 ⁷
329	5.5	0	1660	1040	10 ⁷
521	4.9	0	1720	980	10 ⁶
689	4.7	0	1720	970	10 ⁶

ultimate products of oxidation of thiosulphate were sulphate and elementary sulphur; polythionates were not detected at any stage of incubation. A decrease in pH value from 6.6 to 4.7 indicated that some of the sulphate existed as H₂SO₄. During the oxidation of thiosulphate, sulphate and sulphur were formed in the ratio of 6 to 4, and the pH value decreased from 6.5 to 6.0. This supports the observations of Starkey (1935*a*). After the complete disappearance of thiosulphate, there was a further decrease in pH value to 4.7, accompanied

by a decrease in the amount of elementary sulphur and a corresponding rise in sulphate concentration. This confirmed the suggestion of Starkey (1935*a*) that the decrease in pH value which follows the disappearance of thiosulphate is due to oxidation of sulphur. The continued viability of the culture for at least 18 days after the disappearance of the thiosulphate, when elementary sulphur was the only available sulphur source, suggests that this conversion of elementary sulphur to sulphuric acid is biological. The slow oxidation of elementary sulphur eventually ceased when the pH value dropped to 4.5. This occurs when only a small proportion of the sulphur first formed had been converted into sulphuric acid. This organism did not produce tetrathionate from thiosulphate.

Tetrathionate oxidation. Medium 2 without thiosulphate, but containing 0.5 % (w/v) tetrathionate, was inoculated with *Th. thioparus*. After 24 days of incubation less tetrathionate had disappeared from the incubated flasks than from the sterile controls, although a high viable count in the inoculated flask was always found. A decrease in pH value from 6.6 to 5.9 in the inoculated flasks, compared with a stable pH 6.4 in the sterile control, suggested that oxidation of some sulphur compound had occurred. The presence of 205 mg. thiosulphate sulphur/l. in the sterile controls suggests that thiosulphate was formed by chemical decomposition of tetrathionate and then oxidized by the organisms. It seems probable that this organism does not oxidize tetrathionate.

Oxidation of elementary sulphur. Starkey (1934*a*) stated that *Th. thioparus* oxidizes elementary sulphur. Re-examination of the strain used during the present work confirmed that finding. Sulphur was oxidized to sulphuric acid without the formation of detectable intermediate products. The rate of oxidation was similar to that of *Thiobacillus X* and was much less than for *Th. thiooxidans* and *Th. concretivorus*; the amount of sulphur oxidized was also much less (650 mg./l.). With this organism oxidation ceased when the pH value dropped to 4.4, as happened with thiosulphate.

H₂S oxidation. *Th. thioparus* was inoculated into media 1, 2, 3 and 4, containing no oxidizable form of sulphur, and incubated in air containing 200 p.p.m. H₂S. No growth occurred, so this organism cannot oxidize hydrogen sulphide. Starkey did not specifically mention gaseous hydrogen sulphide, but stated that when examined by him the organism did not utilize sulphides. Beijerinck (1904) recorded that both calcium and hydrogen sulphides were utilized by the original culture of *Th. thioparus* isolated by Nathansohn.

Thiobacillus novellus

Thiosulphate oxidation. The organism was examined in the usual way, using medium 2 with 1 % (w/v) thiosulphate. The rate of oxidation by this strain used was very much slower than with any of the previous four organisms; less than 20 % of the thiosulphate was oxidized in 40 days. The only product of oxidation was sulphate, and its formation was accompanied by a slow decrease in pH value; no tetrathionate was formed. The bacterial count indicated a low degree of bacterial activity. These results confirm Starkey's observations (1935*a*) on this organism.

Tetrathionate oxidation. When medium 2, without thiosulphate, but containing 0.5 % (w/v) potassium tetrathionate, was inoculated with this organism it became sterile within 15 days, indicating inability to survive or grow with tetrathionate.

Oxidation of elementary sulphur. Inoculation into medium 2 with elementary sulphur in place of thiosulphate indicated that, while viable organisms could be recovered 32 days later, no detectable oxidation of sulphur to sulphate took place. This agrees with Starkey's finding (1935*b*) that the organism does not oxidize free sulphur.

H₂S oxidation. As Starkey found, the organism did not oxidize hydrogen sulphide when inoculated into medium 2 or 4 with hydrogen sulphide as the sole source of sulphur.

'M' cultures

Thiosulphate oxidation. Three strains of this group, M.20, M.77 and M.79, were selected for detailed study because they were the best oxidizers of thiosulphate. As the course of the reaction with the three strains was similar the results from one strain only (M.79) are shown in Table 9.

Table 9. *Oxidation of thiosulphate by 'M' organism, strain M.79*

Cultures in medium 2 were analysed at intervals after inoculation.

Period of incubation (hr.)	pH value	Form of S						Total S (by addition)	Viable count (organisms/ml.)
		S ₂ O ₃ ''	S ₄ O ₆ ''	S ₅ O ₆ ''	S ₈ O ₆ ''	SO ₄ ''	S (element)		
		Concentration mg. S/l.							
0	6.6	2640	0	0	0	80	0	2720	nd
22	6.6	2630	0	nd	nd	100	0	nd	10 ⁶
46	6.5	nd	nd	nd	nd	nd	nd	nd	nd
71	6.6	2440	nd	nd	nd	150	nd	nd	10 ⁶
166	7.3	1255	880	470	0	410	10	3025	10 ⁷
238	7.5	1020	1000	330	0	360	20	2730	10 ⁷
384	7.5	815	1680	30	180	nd	20	nd	10 ⁶
502	7.8	535	1230	330	240	420	30	2785	10 ⁶
695	7.6	395	nd	nd	nd	nd	nd	nd	10 ⁶
1367	7.0	365	490	590	460	7.80	75	2755	10 ⁷
1775	6.8	nd	nd	nd	nd	nd	nd	nd	nd

nd = not determined.

Strain M.79 destroyed 87 % of the thiosulphate during 57 days. The products of oxidation after 56 days of incubation were a mixture of tri-, tetra- and pentathionate, some sulphate and a little elementary sulphur. The pH value at first increased from 6.6 to 7.8 and then fell slowly to 7.0. The bacterial count remained high throughout the period of incubation. There was a rapid disappearance of thiosulphate early in the incubation, becoming slower after *c.* 8 days and stopping before all the thiosulphate had disappeared. The period of rapid thiosulphate oxidation was associated with the appearance of a considerable amount of tetrathionate and a sharp increase in pH value; this increase distinguishes these strains from those organisms already described. There was a slight increase in sulphate concentration and some pentathionate formation, but no elementary sulphur was formed in this stage. The subsequent stage of slower thiosulphate oxidation was associated with a slow decrease in

tetrathionate, the formation of tri- and pentathionate, and a slow increase in sulphate. Only minute amounts of elementary sulphur were formed, even after prolonged incubation.

Tetrathionate oxidation. The 'M' strains produced tetrathionate from thio-sulphate, but showed little ability to destroy it. They were inoculated into medium 2 in which 0.5 % (w/v) tetrathionate replaced thiosulphate, but could only be recovered in small numbers after 14 days.

Oxidation of elementary sulphur. Strain M.79 was incubated in medium 2 with elementary sulphur in place of thiosulphate. It persisted in the medium for at least 32 days. Although no change occurred in the pH value of the medium, there was a very slight increase (from 49 to 106 mg./l.) in the sulphate content, which suggests that this strain could oxidize sulphur very slowly. The pH value did not decrease, probably because the amount of acid formed was too small to affect the phosphate-buffered medium. The rate of sulphate formation was lower than with *Thiobacillus X* and *Th. thioparus*.

H₂S oxidation. Although these 'M' strains remained viable for periods up to 44 days when inoculated into medium 4 with hydrogen sulphide as source of sulphur, there was no evidence of hydrogen sulphide oxidation.

Thiobacillus B (Waksman) and strains 'T' and 'K' (Trautwein)

Thiosulphate oxidation. *Thiobacillus B* (Waksman) and strains 'T' and 'K' (Trautwein) were examined in medium 2. The results confirm Starkey's observations (1935*a*) that these cultures oxidize thiosulphate primarily with the formation of alkali and tetrathionate, accompanied by an increase in pH value. This was followed later by the appearance of trithionate, pentathionate and some sulphate, with a slow decrease in pH value.

Tetrathionate oxidation. These strains were similarly examined to determine their ability to survive in medium 2 in which 0.5 % (w/v) tetrathionate replaced thiosulphate. All three strains were recovered after 15 days incubation, but since the pH value of the cultures did not differ from that of the control, it seems unlikely that they were capable of oxidation of tetrathionate.

Oxidation of elementary sulphur. These strains were inoculated into medium 2 with elementary sulphur in place of the thiosulphate. Strains 'B' and 'T' survived for 32 days, but strain 'K' was not viable after 7 days. There was no analytical evidence to suggest that any of the strains oxidized sulphur to sulphate, which confirms Starkey's findings (1934*a*). Their behaviour is thus similar to that of *Th. novellus* in this respect.

H₂S oxidation. *Thiobacillus B* and strains 'T' and 'K' were inoculated into medium 4 with hydrogen sulphide as source of sulphur. Cultures of *Thiobacillus B* and strain 'K' became sterile within 24 hr. It was earlier suggested that H₂S in high concentration is toxic to *Th. concretivorus* and the rapid death of the cultures of *Thiobacillus B* and of strain 'K' suggests that H₂S may be toxic to these organisms also. Strain 'T', however, grew. Parker (1947) showed that H₂S in air, passed over an alkaline solution, forms small amounts of thiosulphate and polythionate. It seems probable that a small amount of thiosulphate was formed chemically, in the 'T' culture medium, and that this

enabled strain 'T' to grow. With *Thiobacillus B* and strain 'K', however, the toxic effect of H₂S would appear to have prevented growth, despite the presence of available thiosulphate.

DISCUSSION

Oxidation of thiosulphate

Thiosulphate is the form of oxidizable sulphur most widely utilized by *Thiobacillus* spp.; the nine strains studied all oxidized thiosulphate readily. The course of oxidation, however, varies with the strain, four different courses being observed.

(1) Thiosulphate is oxidized by *Th. concretivorus* and *Th. thiooxidans* first to tetrathionate and sulphate. In a further stage the tetrathionate is oxidized to sulphate and free sulphuric acid according to equations such as

The production of free acid in the second stage explains the rapid decrease in pH value after the disappearance of thiosulphate.

Waksman & Starkey (1923) stated that *Th. thiooxidans* oxidized thiosulphate directly to sulphate and sulphuric acid; the present examination revealed that there is an intermediate production of tetrathionate. Waksman & Starkey (1923) only made observations at infrequent intervals, and Starkey (1934*b*), when examining this organism for polythionate formation, also only made observations after 7 days incubation, when 7 % of the thiosulphate had been oxidized, and after 28 days, when the whole of it had disappeared. In view of the transient occurrence of the tetrathionate during incubation it is not surprising that its formation was overlooked.

Th. concretivorus differs from *Th. thiooxidans* in that the former oxidizes thiosulphate completely from initial concentrations up to 4 %, whereas *Th. thiooxidans* is markedly inhibited by 2 % and almost completely inhibited by 4 %.

With *Th. concretivorus* in concentrations of 2 and 4 % thiosulphate the decrease in pH value and the occurrence of the maximum tetrathionate concentration before all the thiosulphate has disappeared suggests that towards the end of the thiosulphate oxidation, thiosulphate and tetrathionate oxidation are taking place simultaneously. With the 2 and 4 % concentrations, the amount of elementary sulphur formed is proportionately much greater than with the lower concentrations. The formation of 100 mg. S/l. after 26 days from the 4 % concentration before complete disappearance of thiosulphate is also consistent with the simultaneous oxidation of thiosulphate and tetrathionate; the acid formed from tetrathionate oxidation could react with unchanged thiosulphate to precipitate sulphur. As a final stage the sulphur so formed may be oxidized to sulphuric acid as, with the 1.0 and 2 % initial concentrations of thiosulphate, the concentration of sulphur at the end of the incubation period further decreased.

Oxidation by *Th. thiooxidans* in 2 % thiosulphate, although less in extent,

is similar in nature to that by *Th. concretivorus* at concentrations of 2 and 4 %. Over the whole period of observation tetrathionate and elementary sulphur could be detected; the sulphate showed a slow increase and the pH value a slow decrease. Although only a little thiosulphate was oxidized it would appear that thiosulphate and tetrathionate oxidation occurred simultaneously.

Thiosulphate oxidation by *Thiobacillus X* appears also to occur in two stages according to equations such as (1) and (2) above. With this organism, however, the second stage does not go to completion; the culture becomes sterile at a pH value of 3.0, although some tetrathionate remains unoxidized. The increase in sulphate without any tetrathionate accumulation at the beginning of the incubation, and the fact that the pH value does not increase during the rapid oxidation of thiosulphate in the second stage, may be because the two reactions occur simultaneously from the beginning of the incubation, although the formation of the tetrathionate oxidase would appear to be slower than that of the thiosulphate oxidase.

(2) Thiosulphate is oxidized by *Th. thioparus* to sulphate and elementary sulphur, followed by a slight and slow oxidation of the sulphur to sulphuric acid, with a decrease in pH value to 4.3, according to equations such as (3) and (4):

As with *Thiobacillus X* the extent of acid formation appears to be limited by acid tolerance of the organisms; this was confirmed by examination of the pH tolerance of the organism in buffered saline. The first stage of this oxidation by *Th. thioparus* was described by Starkey (1935*a*); it was confirmed in the present work. Starkey also suggested that the decrease in pH value was probably due to oxidation of the sulphur first formed; this suggestion has been confirmed by the present work.

(3) Thiosulphate may be converted by *Th. novellus* directly to sulphate and sulphuric acid, with decrease in pH value according to an equation such as (5):

As with *Thiobacillus X* and *Th. thioparus*, the amount of acid formed appears to be limited by the acid tolerance of the organism; this was confirmed by separate examination in buffered saline. The present observations confirm those of Starkey (1935*a*) with regard to the route of thiosulphate oxidation by *Th. novellus*. It had been considered that the thiosulphate oxidation by *Th. novellus* was identical with that produced by *Th. thiooxidans*, but the present observations concerning the intermediate production of tetrathionate by *Th. thiooxidans* indicates a marked difference between the two organisms.

(4) Thiosulphate can also be converted to sulphate and tetrathionate, with an increase in pH value, according to an equation such as (6):

followed by a further small conversion of tetrathionate to tri- and penta-thionate, and a decrease in pH value, sometimes back to the initial value at

inoculation. Oxidation in this way by the 'T' and 'K' strains of Trautwein and by *Thiobacillus B* was described by Starkey (1935*a*); the present results are in agreement with those observations. Starkey suggested that the conversion of tetra- to tri- and pentathionate was a purely chemical change; the findings of high viable counts throughout the incubation period in the present work suggests that the organisms may be involved. However, the detection of pentathionate in sterile controls of medium 2 containing tetrathionate instead of thiosulphate, indicates that pentathionate may arise from tetrathionate by chemical change. The facultative autotrophic 'M' strains isolated from concrete, attack thiosulphate in the same way as the 'B', 'T' and 'K' strains. The products of oxidation are identical, but the cultures vary in their rate of oxidation.

Oxidation of tetrathionate

Tetrathionate as a sole source of oxidizable sulphur is oxidized only by *Thiobacillus X*; the products of oxidation are sulphate and sulphuric acid according to equation (2). Oxidation of tetrathionate is incomplete because of the acid intolerance of the organism. In view of the intermediate formation of tetrathionate from thiosulphate by *Th. thiooxidans*, *Th. concretivorus*, the 'M', 'B', 'T' and 'K' strains as well as by *Thiobacillus X*, it is of interest that only *Thiobacillus X* can oxidize tetrathionate when it is supplied as the sole sulphur source.

Oxidation of elementary sulphur

Elementary sulphur is oxidized direct to sulphuric acid; this is brought about only by the four strictly autotrophic strains *Th. thiooxidans*, *Th. concretivorus*, *Thiobacillus X* and *Th. thioparus*. The rate of acid formation and ultimate concentration of acid formed differs among the strains, and the latter is dependent on the acid tolerance of the strain. *Th. thiooxidans* and *Th. concretivorus* give the highest rates and the highest final concentration of acid, *Thiobacillus X* shows a slower rate and lower final concentration, while the rate of sulphur oxidation by *Th. thioparus* and the final acid concentration is even less.

Oxidation of H₂S

Oxidation of gaseous H₂S in air passed over appropriate liquid cultures is only brought about by *Th. thiooxidans*, *Th. concretivorus* and *Thiobacillus X*. The only product of oxidation of H₂S is sulphuric acid, although the accumulation of small amounts of elementary sulphur towards the end of incubation in acid media, and of thiosulphate in alkaline media, suggests that the formation of sulphur and thiosulphate by purely chemical means occurs first, this sulphur or thiosulphate then being oxidized to sulphuric acid by the organism. Oxidation of H₂S by *Th. thiooxidans* and *Th. concretivorus* produces only enough acid to give a pH value of 2.0 under the given conditions, while oxidation of elementary sulphur and thiosulphate by the same organisms produces an acidity equivalent to a pH value of less than 1.0. As the same organisms are involved, difference in acid tolerance cannot be responsible. If elementary sulphur formed chemically from H₂S be an essential preliminary product in

acid media, it is possible that this reaction does not proceed below pH 2.0, and the activity of the organisms ceases at this pH because no more sulphur becomes available. Some support for this hypothesis is derived from other investigations in these laboratories which indicate that H_2S dissolved in water is chemically oxidized by dissolved oxygen at very much slower rates under acid conditions than under alkaline conditions. It is, of course, also possible that the pH range of the enzyme systems involved in H_2S oxidation is different from those involved in the oxidation of elementary sulphur and thiosulphate. In contrast to the behaviour of *Th. concretivorus* and *Th. thiooxidans* in H_2S the pH value of the medium decreases to 3.0 with *Thiobacillus X*; this value is the limit of its acid tolerance with elementary sulphur or thiosulphate as sulphur source.

Identity of organisms isolated from concrete

Th. concretivorus. This organism oxidizes thiosulphate, elementary sulphur and hydrogen sulphide according to the same reactions as *Th. thiooxidans*, and like *Th. thiooxidans* fails to utilize tetrathionate. It differs from *Th. thiooxidans* in being inhibited by higher concentrations of thiosulphate. The two organisms are closely related with regard to oxidation of sulphur compounds, but, as described earlier (Parker, 1945), they differ in their nitrogen requirements. *Th. thiooxidans* utilizes ammonia but is inhibited by nitrate, whereas *Th. concretivorus* utilizes ammonia or nitrate.

Thiobacillus X. This organism was previously considered to be closely related to *Th. thioparus*, but the present study reveals that the two organisms exhibit marked differences with regard to their oxidation of sulphur compounds. *Th. thioparus* oxidizes thiosulphate with formation of elementary sulphur and sulphate, whereas *Thiobacillus X* converts thiosulphate first to sulphate and tetrathionate followed by a partial destruction of tetrathionate, forming sulphate and free sulphuric acid. *Th. thioparus* does not oxidize tetrathionate or H_2S , whereas *Thiobacillus X* readily oxidizes both compounds. The only point of similarity is their common power to oxidize elementary sulphur slowly to sulphuric acid. *Thiobacillus X* must therefore be considered a different species from *Th. thioparus*, and with regard to oxidation of sulphur compounds is much more closely related to *Th. thiooxidans* and *Th. concretivorus*. All three organisms utilize thiosulphate, elementary sulphur or H_2S , and the route of oxidation of thiosulphate is by the same two reactions in each case. *Thiobacillus X* differs from *Th. thiooxidans* and *Th. concretivorus*, however, in its acid tolerance. Oxidation of thiosulphate ceases with *Thiobacillus X* at pH 3.0, leaving sulphate, free sulphuric acid and some unchanged tetrathionate as the products of oxidation, while oxidation by *Th. thiooxidans* and *Th. concretivorus* only ceases at a pH value less than 1.0, the only products of oxidation being sulphate and free sulphuric acid. *Thiobacillus X* must therefore be considered as distinct from *Th. thioparus* on the one hand and *Th. thiooxidans* and *Th. concretivorus* on the other. Before classing *Thiobacillus X* as a new species it is desirable to review the published literature on *Th. thioparus*. The strain of *Th. thioparus* examined in these studies was one isolated by Starkey in 1935, and Starkey (1935*a*) considered it identical with the organism

isolated by Nathansohn (1902) and which was re-examined and named by Beijerinck (1904).

The original organism of Nathansohn was said to produce tetrathionate by oxidation of thiosulphate but Starkey was unable to detect tetrathionate as a product of thiosulphate oxidation by his strain, and suggested that the original Nathansohn strain was contaminated with an organism similar to the 'B', 'T' and 'K' strains. Production of acid was not specifically mentioned by Nathansohn or Beijerinck, but slight acid formation by the original culture cannot be excluded.

Nathansohn's original strain was said to produce sulphur from thiosulphate, and Starkey's strain produces sulphur and sulphate in the ratio of 4:6. *Thiobacillus X* produces a thin pellicle of sulphur, but very much less than that produced by Starkey's organism. It is possible that the sulphur formed by Nathansohn's original organism and by *Thiobacillus X* is due to chemical reaction between thiosulphate and tetrathionate, whereas the sulphur formed by Starkey's strain is directly produced from thiosulphate.

According to Beijerinck (1904), *Th. thioparus* can oxidize thiosulphate, tetrathionate, sulphur, sulphides and H_2S . In view of the production of tetrathionate from thiosulphate by *Thiobacillus X* and its ability to oxidize thiosulphate, tetrathionate, sulphur and H_2S , it seems more likely that the original strain of Nathansohn was not impure; that its oxidizing powers as described by that author were correct; and that the strain *Thiobacillus X* isolated from concrete is identical with or very similar to the original *Th. thioparus*. Starkey's strain, which he considered identical with the original *Th. thioparus*, would appear to differ from both *Th. thioparus* as isolated by Nathansohn and *Thiobacillus X*.

Since the completion of the present work, Vishniac (1952) published a manometric study of the oxidation of thiosulphate by a strain considered identical with Starkey's strain of *Th. thioparus*. It was found that this organism gave qualitative tests for tetrathionate, and that its oxygen uptakes were consistent with tetrathionate formation. Vishniac considered that oxidation of thiosulphate by the strain used proceeded through tetrathionate, trithionate and dithionate to sulphate. It appears likely that Vishniac's strain was very similar to *Thiobacillus X* and to *Th. thioparus* as originally described by Nathansohn; although Vishniac stated that a culture of Starkey's strain of *Th. thioparus* appeared identical with his own, if examined closely it seems probable that they would differ in the same way as *Thiobacillus X* and *Th. thioparus* differed in the present work.

'M' strains. Those cultured from concrete appear to be closely related to the 'T' and 'K' strains of Trautwein and *Thiobacillus B* with regard to oxidation of sulphur compounds. All strains oxidize thiosulphate with the formation of tetrathionate, and with an increase in pH value they are similar in their action on the other forms of sulphur examined.

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REFERENCES

- BEIJERINCK, M. W. (1904). Über die Bakterien welche sich im Dunklen mit Kohlensäure als Kohlenstoffquelle ernähren können. *Zbl. Bakt.* (2 Abt.), **11**, 593.
- Bergey's Manual of Determinative Bacteriology* (1948). 6th ed. Edited by BREED, R. S., MURRAY, E. G. D. & HITCHENS, A. P. Baltimore. The Williams & Wilkins Co.
- DEBUS, H. (1888). Chemical investigation of Wackenroder's solution, and explanation of the formation of its constituents. *J. chem. Soc.* **53**, 278.
- JACOBSEN, H. C. (1914). Die Oxydation von Schwefelwasserstoff durch Bakterien. *Folia microbiol., Delft*, **3**, 155.
- LE PAGE, G. A. & UMBREIT, W. W. (1943). Phosphorylated carbohydrate esters in autotrophic bacteria. *J. biol. Chem.* **147**, 263.
- LIPMAN, J. G., WAKSMAN, S. A. & JOFFE, J. S. (1921). The oxidation of sulphur by soil micro-organisms. *Soil Sci.* **12**, 475.
- NATHANSOHN, A. (1902). Über eine neue Gruppe von Schwefelbakterien und ihren Stoffwechsel. *Mitt. zool. Sta. Neapel*, **15**, 665.
- NIEL, C. B. VAN (1931). On the morphology and physiology of the purple and green sulphur bacteria. *Arch. Mikrobiol.* **3**, 1.
- PARKER, C. D. (1945). The corrosion of concrete. I. The isolation of a species of bacterium associated with the corrosion of concrete exposed to atmospheres containing hydrogen sulphide. *Aust. J. exp. Biol. med. Sci.* **23**, 81.
- PARKER, C. D. (1947). Species of sulphur bacteria associated with the corrosion of concrete. *Nature, Lond.* **159**, 439.
- POMEROY, R. (1936). The determination of sulphides in sewage. *Sewage Wks. J.* **8**, 572.
- STARKEY, R. L. (1925). Concerning the physiology of *Thiobacillus thiooxidans* an autotrophic bacterium oxidizing sulphur under acid conditions. *J. Bact.* **10**, 135.
- STARKEY, R. L. (1934a). Cultivation of organisms concerned in the oxidation of thiosulphate. *J. Bact.* **28**, 365.
- STARKEY, R. L. (1934b). The production of polythionates from thiosulphate by microorganisms. *J. Bact.* **28**, 387.
- STARKEY, R. L. (1935a). Products of the oxidation of thiosulphate by bacteria in mineral media. *J. gen. Physiol.* **18**, 325.
- STARKEY, R. L. (1935b). Isolation of some bacteria which oxidize thiosulphate. *Soil Sci.* **39**, 197.
- TRAUTWEIN, K. (1921). Beitrag zur Physiologie und Morphologie der Thionsäurebakterien (Omelianski). *Zbl. Bakt.* (2 Abt.), **53**, 513.
- VISHNIAC, W. (1952). The metabolism of *Thiobacillus thioparus*. I. The oxidation of thiosulphate. *J. Bact.* **64**, 363.
- VOGLER, K. H. (1942). Studies on the metabolism of the autotrophic bacteria. II. The nature of the chemosynthetic mechanism. *J. gen. Physiol.* **26**, 103.
- VOGLER, K. G. & UMBREIT, W. W. (1942). Studies on the metabolism of the autotrophic bacteria. III. The nature of the energy storage material active in the chemosynthetic process. *J. gen. Physiol.* **26**, 157.
- WAKSMAN, S. A. (1922a). Micro-organisms concerned in the oxidation of sulphur in the soil. *J. Bact.* **7**, 231.
- WAKSMAN, S. A. (1922b). Micro-organisms concerned in the oxidation of sulphur in the soil. III. Media used for the isolation of sulphur bacteria from the soil. *Soil Sci.* **13**, 329.
- WAKSMAN, S. A. & STARKEY, R. L. (1923). On the growth and respiration of sulphur oxidizing bacteria. *J. gen. Physiol.* **5**, 285.

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