FORMATION OF SULFIDE BY SOME SULFUR BACTERIA[1]

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Previous studies with the strictly autotrophic sulfur bacterium, *Thiobacillus thiooxidans*, growing on elemental sulfur have shown that the sulfur is rapidly oxidized to sulfate without the accumulation of intermediate products (Waksman and Starkey, 1923). However, there is no evidence that the transformation does not take place in several steps (Buchanan and Fulmer, 1930). The possibility has been suggested that the sulfur undergoes some change before entering the bacterial cells, being either reduced to sulfide or oxidized to one of the thionic acids.

The strictly autotrophic sulfur bacterium, *Thiobacillus thioparus*, transforms thiosulfate to sulfate and a material generally considered to be elemental sulfur (Starkey, 1935b). Recently von Deines (1933b) claimed that the sulfur-like substance secreted by the larger sulfur bacteria is not elemental sulfur, but a highly sulfured polysulfide. This substance has long been considered to be the same as that formed by *T. thioparus*. In previous studies with this organism and other bacteria which oxidize thiosulfate, no evidence of sulfide formation was detected (Starkey, 1935b).

In this report consideration is given to the possibility of some transformation of elemental sulfur preceding its passage into the cells of sulfur bacteria and to the possibility that the precipitate formed by *T. thioparus* is a sulfide (Starkey, 1936).

CHARACTERIZATION OF THE SULFUR MATERIAL

In 1870, Cramer first reported on the chemical properties of the globules occurring in cells of some of the large, filamentous,

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sulfur bacteria. They were insoluble in HCl but soluble in absolute alcohol and in carbon bisulfide; he concluded that the material was sulfur. F. Cohn (1875) observed further that when the cells were heated dry on a glass slide, the globules coalesced to form larger yellow drops giving off sulfurous vapors. Étard and Olivier (1882) found that the globules were soluble in ether and in chloroform. Zopf (1884) characterized the sulfur occurring in filaments of *Beggiatoa* as highly refractive, glistening, round granules, soluble in absolute alcohol, carbon bisulfide, warm potash and warm sodium sulfite, and in nitric acid and potassium chlorate at ordinary temperatures.

Winogradsky (1887) referred to the cells of *Beggiatoa* as follows: "They oxidize H_2S and store up sulfur in the form of small globules which consist of soft amorphous sulfur that never is transformed into the crystalline condition in the living cells" (p. 589).[2] He concluded that these oily-appearing globules are pure sulfur; the globules changed to crystalline sulfur after death of the organism. By heating the cells in water at 70°C., the numerous small globules contained in each cell coalesced to form a single large droplet; in the living cells, plasma membranes keep the globules apart. This was considered evidence that the material was not solid sulfur since the latter melts only above 100°C.

Gasperini (1898) noted that the globules disappeared when treated with acetic acid, and concluded therefore that the material was not pure sulfur. Corsini (1905) discovered that the acetic acid caused the soft oily globules to change to crystals of rhombic sulfur. He confirmed most of the reactions of the sulfur with the previously mentioned reagents and noted further that it dissolved in benzene and in xylol, that it was not affected by strong acids (HCl, H_2SO_4, and HNO_3) in the cold, but was oxidized by hot nitric acid.

When the cells are treated with aceto-carmine, the sulfur crystallizes outside of the cells (Ellis, 1932) the same as during treatment with acetic acid or other materials which cause death of the organism (Winogradsky, 1887). The same author found that watery picric acid also causes the sulfur to crystallize. When

[2] Translated.

treated with a solution of silver nitrate, the sulfur droplets change to Ag_2S (Monti, 1935).

The globules produced by *T. thioparus* from thiosulfate have the same appearance as those produced by the larger sulfur bacteria (Starkey, 1935a). Nathansohn (1902) described the material as droplets of oily amorphous sulfur occurring outside of the small bacterial cells. The globules dissolved in chloroform and in carbon bisulfide and gave off sulfur dioxide when heated. Beijerinck (1904a and b) dissolved the globules in benzene. They do not appear to be affected by strong acids in the cold, but are decomposed by strong alkalis and warm nitric acid. There is no evidence that the globules produced by the larger colorless sulfur bacteria, by the purple bacteria, or by *T. thioparus* differ chemically.

The reactions of the globules with mineral acids, alkalis, acetic acid, and the various solvents were confirmed by von Deines (1933b). His claim that the material is a polysulfide depends principally upon the following observations: by microchemical tests the material was found to be 99 per cent sulfur; by repeated washing it became emulsified, a property which he believed was characteristic of highly sulfured polysulfide and not elemental sulfur; when the sulfur was washed with dilute hydrochloric acid and then held under reduced pressure, it puffed up with bubbles of hydrogen sulfide gas.

EXPERIMENTAL

Medium I was used for cultivation of *T. thioparus* and medium II for *T. thiooxidans*.

	Medium I grams	*Medium II* grams
Distilled water	1000.0	1000.0
Sulfur		10.0
$Na_2S_2O_3 \cdot 5H_2O$	10.0	
$(NH_4)_2SO_4$	0.1	0.3
K_2HPO_4	2.0	
KH_2PO_4		3.0
$CaCl_2 \cdot 2H_2O$	0.1	0.25
$MgSO_4 \cdot 7H_2O$	0.1	0.5
$FeCl_3 \cdot 6H_2O$	0.02	0.02
$MnSO_4 \cdot 2H_2O$	0.02	
pH	7.8	4.8

For medium I the thiosulfate and ammonium sulfate were sterilized separately; all of the constituents were sterilized under pressure. The flasks of medium II were sterilized in flowing steam for thirty minutes on each of three successive days. Thiosulfate was determined by titration of aliquot portions (from 5 to 25 cc.) with 0.01 normal iodine solution.

Ash content of the precipitate

The following experiment was performed to determine whether or not the material precipitated by *T. thioparus* contains any substance, such as sulfide, which will combine with appreciable amounts of metallic cations.

TABLE 1

Residues from sulfur material precipitated from thiosulfate by T. thioparus

	SOLUTION MADE ALKALINE	SOLUTION MADE ACID
pH before inoculation	7.8	7.8
pH after growth	5.2	5.2
	mgm.	*mgm.*
Initial thiosulfate-sulfur in 900 cc	2,286.3	2,286.3
Thiosulfate-sulfur in 900 cc. after growth	4.8	9.0
Weight of material extracted by CS_2	770.1	772.1
Weight of residue after ignition	0.1	−0.8

The bacterium was grown in medium I in 300-cc. amounts in 1-liter Erlenmeyer flasks. After 28 days, the thiosulfate was practically all decomposed and the reaction had changed from the initial pH 7.8 to 5.2 as indicated in table 1. The solutions in three flasks were mixed together, adjusted to pH 9.0 with NaOH, and kept for one hour before filtration. The precipitate was then collected on a filter paper and washed with 500 cc. of distilled water. The solutions of three other flasks were mixed together and then adjusted to pH 3.0 with acetic acid. After standing for one hour, the solution was filtered and the precipitate washed with 500 cc. of distilled water.

It was assumed that the acid treatment would remove bases

which were associated with any sulfide contained in the precipitate. Upon subsequent ignition there should be no ash. Any sulfide which was present in the solutions which were made alkaline should form compounds which would leave a residue on ignition.

The papers and contents were air-dried and the major portion of the precipitate was transferred to flasks and treated with 100-cc. portions of CS_2 for 24 hours. The solution was filtered, the filtrate evaporated in small crucibles, and the residue dried at 65C°. The material had the appearance of crystals of pure rhombic sulfur. After ignition there was no residue in the case of the material originating either from the acid or alkaline media as shown by table 1. The slight positive or negative differences are within the error of the determination using crucibles weighing about 15 grams each. If any sulfide was contained in the sulfur material, it was not detected by this method. If there had been any appreciable amount of sulfide, it should have been detected in the relatively large amount of material used. Sulfides of the alkalis are not appreciably soluble in CS_2, but the fact remains that, although the sulfur was readily soluble in CS_2, no material leaving an ash residue was dissolved. Since in this experiment it is only the globules which are being characterized, any sulfide which might have been removed during the washing need not be considered.

Influence of the precipitate on the iodine titration of the medium

Medium I, in 100-cc. portions in 250-cc. Erlenmeyer flasks, was inoculated with *T. thioparus*. After 23 days, 20-cc. portions were titrated with standard iodine solution. Similar portions were titrated after filtration to remove the precipitate. As shown in table 2, there was no material reacting with iodine after the thiosulfate had been decomposed; the amount of iodine required to give a blue color to the starch indicator was only one drop for 20 cc. of culture solution.

Twenty cubic centimeters of the uninoculated medium contained 45.6 mgm. of thiosulfate-sulfur. If, after decomposition by the bacterium, two-fifths of this was precipitated sulfur as

determined in previous studies (Starkey, 1935b), there would be 18.2 mgm. of the precipitate in the aliquot used for titration. If it is assumed that only one per cent of this was in the form of sulfide as H_2S, the amount of H_2S would be 0.182 mgm. Since

$$H_2S + I_2 = 2\,HI + S$$

the amount of iodine required for its oxidation would be 1.36 mgm. One cubic centimeter of the iodine solution which was used contained 1.25 mgm. of iodine, consequently 1.09 cc. of the iodine solution would have been required to effect the oxidation. An amount of sulfide reacting with such a quantity of iodine would be detected easily by the method used. It thus appears

TABLE 2

Titration of solutions before and after removal of the precipitate

	CONTROL MEDIUM	INOCULATED MEDIUM
pH	7.8	4.4
	cc.	cc.
Titration of 20 cc. with iodine solution before filtration*	72.20 72.15	0.05 0.05
Titration of 20 cc. with iodine solution after filtration*	72.25 72.25	0.05 0.05

* Iodine solution was 0.00986 normal.

clear that, at least in old cultures, the material contains no appreciable amount of sulfide.

To further test for reducing properties of the precipitate, solutions supporting growth of *T. thioparus* were used at periods preceding complete disappearance of thiosulfate. Medium I was used in 125-cc. amounts in 1-liter Erlenmeyer flasks. Periodically, untreated and filtered portions of one control and one inoculated solution were titrated with standard iodine solution. Since oxidation in the culture continues during the period of analysis, 1 cc. of toluol was added to each flask of medium before starting the analysis. Toluol does not affect the titration. In making the analyses, the 20- or 25-cc. aliquots received 5 cc. of 10-percent acetic acid and an excess of 0.01-normal iodine solution, the

excess being titrated with standard thiosulfate solution. In all cases precipitate appeared in the inoculated solutions, the amount increasing with the oxidation of the thiosulfate. Any sulfide contained in the precipitate should react with the iodine to show differences in the titrations of the solutions before and after filtration. The data in table 3 give no indication that the reaction with iodine was at all affected by the precipitate. Any sulfide formed during growth of the organism was therefore present in very small amounts and must be of little importance in characterizing the precipitate.

TABLE 3

*Amounts of iodine solution used to oxidize equal amounts of unfiltered and filtered culture solutions**

TREATMENT	INCUBATION	UNINOCULATED	INOCULATED
	days	*cc.*	*cc.*
Unfiltered†	5	80.30, 80.35	67.90, 67.80
Filtered†	5	80.20, 80.20	67.85
Unfiltered‡	12	102.00, 102.25	4.15, 4.15
Filtered‡	12	102.00, 102.20	4.15, 4.15
Unfiltered‡	21	103.30, 103.30	5.55, 5.50
Filtered‡	21	103.60, 103.60	5.55, 5.55

* Iodine solution was 0.00986 normal. Duplicate determinations on each solution.

† 20 cc. aliquots.

‡ 25 cc. aliquots.

In further testing for the presence of sulfide, the method of Kurtenacker and Bittner (1924) was used. This involves three procedures: (*a*) One part of solution (in this case 25 cc.) was added to an excess of standard iodine solution, acidulated with acetic acid, and the residual iodine titrated with standard thiosulfate. The iodine reacts with all sulfide, sulfite, and thiosulfate present. (*b*) A second portion (25 cc.) was placed in a 250-cc. glass-stoppered bottle with 15 cc. of 10-per-cent zinc acetate solution and diluted to about 150 cc. Five cubic centimeters of formaldehyde were added to bind any sulfite present, and the solution was acidulated with acetic acid. An excess of stand-

ard iodine solution was added, the solution shaken, and the excess iodine titrated with standard thiosulfate. The iodine reacts with all sulfide and thiosulfate. (*c*) A third portion of the solution was placed in a 100-cc. volumetric flask with 15 cc. of the zinc acetate solution and made up to volume. After thoroughly mixing, the contents were filtered. An aliquot was treated with

TABLE 4

*Amounts of iodine solution required to oxidize unfiltered and filtered solutions in testing for sulfide, sulfite and thiosulfate**

TREATMENT AND SUBSTANCES DETERMINED†	INCUBATION	UNINOCULATED	INOCULATED
	days	*cc.*	*cc.*
Unfiltered for S_2O_3, S, SO_3	6	100.35	78.65
Unfiltered for S_2O_3, S	6	99.90	78.55
Unfiltered for S_2O_3	6	100.30	78.60
Filtered for S_2O_3, S, SO_3	6	100.45	78.65
Filtered for S_2O_3, S	6	99.95	78.50
Filtered for S_2O_3	6	100.40	78.80
Unfiltered for S_2O_3, S, SO_3	13	102.00	3.40
Unfiltered for S_2O_3, S	13	101.50	3.40
Unfiltered for S_2O_3	13	102.00	3.40
Filtered for S_2O_3, S, SO_3	13	102.10	3.40
Filtered for S_2O_3, S	13	101.60	3.35
Filtered for S_2O_3	13	102.00	3.40
Unfiltered for S_2O_3, S, SO_3	19	103.10	0.15
Unfiltered for S_2O_3, S	19	102.65	0.10
Unfiltered for S_2O_3	19	103.05	0.20
Filtered for S_2O_3, S, SO_3	19	103.15	0.10
Filtered for S_2O_3, S	19	102.65	0.10
Filtered for S_2O_3	19	102.95	0.20

* Iodine solution was 0.00986 normal.

† Thiosulfate—S_2O_3; sulfide—S; sulfite—SO_3.

5 cc. of formaldehyde, acidulated with acetic acid, and titrated with standard iodine solution. This determination indicates the amount of thiosulfate. The difference between (*a*) and (*b*) indicates the sulfite content; the difference between (*b*) and (*c*) indicates the sulfide content. In the present case, lack of differences between the three determinations indicates that only thiosulfate was present, and that there was no sulfide or sulfite.

The results in table 4 are reported as amounts of the standard iodine solution used in the titration of equal amounts of culture solutions; corrections have been applied for the amounts of standard thiosulfate used. The data clearly indicate that there are no significant differences in the determinations at any one period, whether the solutions are unfiltered or filtered to remove the precipitate before titration. Neither do the results show any detectable amount of sulfide or sulfite at any of the three periods which represent: (*a*) an early stage of bacterial growth, (*b*) a period when the thiosulfate is nearly all decomposed, and (*c*) a period following complete decomposition of the thiosulfate. If any sulfide was formed in the cultures it failed to accumulate.

Evolution of sulfide by T. thioparus

Although the previous experiments did not indicate that there was any sulfide in solutions supporting growth of *T. thioparus*, it is conceivable that very small amounts of sulfide were produced. Therefore more sensitive tests were sought.

The nitroprusside test was found to give a strong, although quickly fading, violet color in 5 cc. of a solution containing 0.05 mgm. of sulfide-sulfur, or 1 part in 100,000. A light violet test which persisted but a few seconds was detected in 5 cc. of a solution containing slightly more than 1 part in 500,000. This was the limit of effective use of the test. The iodine titration as used would reveal 1 part of sulfide-sulfur in 100,000 with 5-cc. samples, but this was close to the limit of its usefulness.

In making the nitroprusside test, 5 cc. of the solution was made distinctly alkaline with ammonium hydroxide, 0.5 cc. of 5-percent sodium nitroprusside was added, and the color quickly noted. Modifications suggested by Walker (1925) and Giroud and Bulliard (1933) were also tried with similar results.

Lead acetate paper, a very sensitive indicator for sulfide, was used to test for evolution of sulfide from the culture media during growth of the bacterium. A strip of freshly prepared moist paper was held in place by the cotton plug so that the lower end was about 5 mm. from the surface of the solution medium.

In another test, a 10-cc. portion of the culture solution was

boiled for 3 minutes with a lead acetate paper hanging to within 2 cm. of the surface of the gently boiling solution.

Table 5 presents some of the results from cultures of *T. thioparus*. Similar determinations on uninoculated solutions were made regularly. In none of the solutions, either sterile or inoculated, was there any color reaction with nitroprusside. A faint darkening of the lower edge of the lead acetate papers over

TABLE 5

Sulfide production by T. thioparus growing in thiosulfate solutions

INCUBATION	THIOSULFATE DECOMPOSED IN INOCULATED SOLUTIONS	REACTION (pH) OF INOCULATED SOLUTIONS*	NITROPRUSSIDE TEST		BLACKENING OF Pb-ACETATE PAPERS DURING INCUBATION		BLACKENING OF Pb-ACETATE PAPERS BY HEATED SOLUTIONS	
			Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
days	*per cent*							
2	3.5	8.0	–	–	–	–	–	–
3†	6.6	8.0	–	–	–	–	–	+
4	15.6	7.8	–	–	–	±	–	+
5	18.8	7.3	–	–	–	±	–	++
6	23.9	7.3	–	–	–	±	–	++
7	30.8	7.2		–		+		++
9	41.3	6.9	–	–	–	+	–	++
10	66.3	6.8		–		++		++
11	79.7	6.6	–	–	–	++	–	++
12	98.1	6.2		–		++		++
13	100.0	5.0	–	–	–	++	–	+
15	100.0	4.8	–	–	–	++	–	+
16	100.0	4.8		–		++		+
18	100.0	4.8	–	–	–	++	–	–
20	100.0	4.8	–	–	–	++	–	–
23	100.0	4.8	–	–	–	++	–	–

* pH of uninoculated medium was 8.0.

† Definite evidence of precipitation of sulfur in inoculated solutions at this period; the amounts increased with growth at later periods.

the inoculated solutions appeared on the fourth day. The color became darker and gradually spread some distance (4 to 8 cm.) up from the lower end. It was very apparent that sulfide was formed during growth. Sulfide was evolved in small amounts even while the medium was alkaline, and in somewhat greater amounts after the reaction became slightly acid; there was scarcely any change after the reaction had dropped to pH 6.6.

A fresh test paper was substituted for the darkened one in the flask of a culture which had been incubated for 17 days. At this time all of the thiosulfate had been decomposed. No darkening of the new test paper appeared even after 20 more days, suggesting that the active bacterial cells produced the sulfide.

In no case was there even slight darkening of the papers in the sterile flasks. Sulfur precipitation invariably accompanied growth of the organism on thiosulfate and there was an abundance of precipitate before a pronounced sulfide reaction was observed. It is likely that the sulfide originated from this sulfur.

The samples from the uninoculated solutions liberated no sulfide upon being heated. With similar treatment, the unfiltered inoculated solutions gave a slight test for sulfide after incubation for 3 to 4 days; a strong test was obtained after incubation for from 5 to 12 days. Later the test was weaker or disappeared. However, it seems unlikely that this test on the heated solutions indicates that sulfide was present in the culture media; the following evidence suggests that the sulfide was formed from the sulfur contained in the medium during the heating process. The following tests refer to solutions heated to detect sulfide.

One of the culture solutions which gave a strong sulfide test failed to give any blackening of the acetate paper after removal of the precipitate by filtration. Culture solution oxidized by iodine still gave a test for sulfide. A culture solution of pH 5.0, in which all thiosulfate had been decomposed, gave no test for sulfide; the same solution adjusted to pH 7.0 gave a positive test for sulfide; the test was still stronger after the solution was adjusted to pH 8.0. Neither uninoculated medium nor culture solution in which the thiosulfate had undergone decomposition gave a test for sulfide, but a mixture of the two gave a positive test. Uninoculated thiosulfate medium receiving a small amount of either rhombic or precipitated sulfur liberated sulfide upon being heated. Rhombic sulfur reacted the same as the sulfur precipitated by the bacteria in that there was little or no test for sulfide in a buffered solution at pH 5.4, a positive test at pH 7.0, and a strongly positive test at pH 8.0. These results show that the sulfide evolved from culture solutions which were being

heated originated from the sulfur through reaction with the solution, the reaction being favored by alkaline solutions, but still taking place in weakly acid solutions.

Formation of sulfide by T. thiooxidans.

Thiobacillus thiooxidans, the sulfur bacterium growing under very acid conditions (Waksman and Starkey, 1923, Starkey,

TABLE 6

Sulfide production by T. thiooxidans growing on elemental sulfur

INCUBATION	NaOH (0.07 N) USED FOR 5 CC. OF CULTURE*	NITROPRUSSIDE TEST		BLACKENING OF Pb-ACETATE PAPERS DURING INCUBATION		BLACKENING OF Pb-ACETATE PAPERS BY HEATED SOLUTIONS†	
		Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated
days	*cc.*						
4	4.8	−	−	−	−	±	±
5	5.0	−	−	−	−	±	±
6	6.9		−		−		±
7	6.6	−	−	−	−	±	±
8	8.8		−		−		±
10	9.1	−	−	−	±	±	±
11	10.1		−		±		±
12	13.8	−	−	−	±	±	±
15	18.6	−	−	−	+	±	±
18	21.6	−	−	−	+	±	±
20	25.2	−	−	−	+	±	±
23	26.6	−	−	−	++	±	±
25	26.7	−	−	−	++	±	±

* Titer of 5 cc. of uninoculated medium was 1.70 cc. NaOH. Reaction of uninoculated medium was pH 4.6. Reaction of inoculated medium was pH 1.6 at 4 days and below pH 1.2 at all later periods.

† Solutions were boiled gently for 5 minutes. The test was weak in all cases, no differences being noted between uninoculated and inoculated media.

1925), was studied for sulfide formation in medium II. Lead acetate papers were suspended above the culture solutions. Sulfide formation was very similar to that with *T. thioparus* (table 6). The acidity increased rapidly, due to the oxidation of the sulfur to sulfuric acid. No indication of sulfide was shown by the nitroprusside test on either the sterile or inoculated solutions. The lead acetate papers began to show slight darkening at the

edges in 10 days; the color became more intense and general as incubation continued, and was quite strong after 23 days. There was no coloration of the papers in the flasks of sterile medium.

The results clearly indicate that sulfide is produced by *T. thiooxidans*, that little is evolved before growth is well advanced, and that no appreciable amount persists in the solution medium. After 25 days, the darkened papers were replaced. Within one day there was slight coloration and this continued to darken, showing a strong test in 5 days. Sulfide apparently is formed continuously during the oxidation of sulfur to sulfuric acid by *T. thiooxidans*.

None of the solutions contained any detectable amount of reducing substances which reacted with iodine; one drop of the 0.01-normal iodine solution invariably gave a deep blue color to the starch indicator in the 5-cc. portions of the culture solutions. The tests for sulfide on the unfiltered heated solutions were very slight and were the same on both sterile and inoculated solutions. The elemental sulfur contained in the medium commonly reacted in the hot solutions to give a weak test for sulfide. No doubt this sulfide was produced during the test, but only in small amounts due to the relatively high acidity of the solutions.

Sulfide formation by other bacteria which decompose thiosulfate

Thiobacillus novellus, a facultative autotroph oxidizing thiosulfate to sulfate (Starkey, 1935b), produced no sulfide while growing on the thiosulfate medium for 20 days. Since no elemental sulfur was produced during this period it is not surprising that sulfide was not formed.

Other bacteria, referred to as cultures B, T, and K in previous communications (Starkey, 1934a and b, 1935a and b), oxidize thiosulfate to tetrathionate which breaks down into various other sulfur compounds by secondary reactions. Cultures of these bacteria gave rise to sulfide in some cases and not in others. Sulfur generally appears among the secondary decomposition products in the medium, and where there was evidence of sulfur precipitate, sulfide was formed. This emphasizes the fact that sulfide is produced by the various bacteria only when elemental sulfur is present in the medium.

Mechanism of sulfide formation

The following experiments were performed to discover the means of sulfide formation in the cultures.

Neither rhombic nor precipitated sulfur gave rise to sulfide in the sterile acid inorganic medium (medium II). In most cases, both rhombic and precipitated sulfur have been used in parallel experiments, since von Deines (1933a) claimed that precipitated sulfur obtained from various chemical reactions is a highly sulfured hydrogen polysulfide. In the present studies no qualitative differences have been noted between the two materials; precipitated sulfur is somewhat more reactive than the rhombic sulfur, probably due to its finer state of division.

In order to determine whether or not sulfide is formed from sulfur in the alkaline thiosulfate medium, the following three media were used in 100-cc. amounts in 250-cc. Erlenmeyer flasks; five flasks were employed in each case: (*a*) medium I, (*b*) medium I with about 0.5 gram of precipitated sulfur per flask, (*c*) medium I with about 0.5 gram of rhombic sulfur per flask. The sulfur was sterilized in flowing steam and added to the sterile solutions. The media were shaken to favor wetting of the sulfur. Since it did not become readily moistened and practically all remained on the surface, a second series was arranged like the first except that a small amount of lecithin (1 cc. of a sterile solution of approximately 0.25-per cent concentration) was added to each flask. A strip of lead acetate paper was suspended over each solution. In none of these sterile solutions was there any positive test for sulfide even after a period of 20 days. One flask which contained rhombic sulfur and lecithin became contaminated with *Aspergillus niger*. In this flask the test paper showed darkening, clearly indicative of sulfide formation. These results indicate that sulfur is not hydrogenated in the sterile culture solutions. However, sulfide was formed in similar solutions not only by the sulfur bacteria already mentioned, but also by *A. niger*.

In order to determine how general hydrogenation of elemental sulfur might be among microörganisms under the conditions of the preceding experiments, five cultures each of common bacteria, actinomycetes, and fungi were used. They were inoculated

into media prepared the same as (*a*)–(*c*) of the preceding experiment. No organic material was added. After inoculation with a small amount of cell material, a strip of test paper was sus-

TABLE 7

*Sulfide production by heterotrophic microörganisms in thiosulfate medium in presence or absence of elemental sulfur**

ORGANISM	PERIOD OF INCUBATION						
	2 days	5 days	6 days	8 days	10 days	14 days	20 days
Thiosulfate medium alone							
Act. californicus	0	0	0	0	0	0	0
Act. flavovirens	0	0	±	±	1	1	1
Act. griseus	0	1	1	1	1	1	2
Act. violaceus-ruber	0	1	1	1	1	1	2
Asp. flavus	0	0	0	±	1	1	1
Humicola sp	0	0	0	0	0	0	0
Rhizopus sp	0	1	2	2	2	2	3
Thiosulfate with rhombic sulfur							
Act. californicus	0	0	0	0	0	0	0
Act. flavovirens	0	0	0	0	0	0	0
Act. griseus	±	1	1	2	2	3	4
Asp. flavus	0	0	0	±	±	±	±
Humicola sp	0	0	0	0	±	±	±
Rhizopus sp	0	1	2	2	2	2	2
Thiosulfate with precipitated sulfur							
Act. californicus	0	0	±	1	1	1	1
Act. flavovirens	1	2	2	2	2	2	2
Act. griseus	1	1	2	2	2	3	4
Act. violaceus-ruber	1	2	2	2	4	4	6
Asp. flavus	0	0	±	1	1	2	2
Humicola sp	0	0	1	1	1	2	2
Rhizopus sp	1	2	2	3	3	4	6

* 0 = no test; ± = very slight; 1, 2, 3, 4, etc., indicate increasing intensity of dark coloration of the test papers.

pended above the solution in each flask. None of the uninoculated media gave a test for sulfide. None of the bacteria, including *Sarcina lutea*, *Bacterium radiobacter*, *Azotobacter chroococcum*,

Cellulomonas fima, and *Bacillus cereus* formed any sulfide. *Actinomyces pheochromogenus*, a species of *Trichoderma*, and a species of *Penicillium*, likewise gave no reaction. Those showing some indication of sulfide production are noted in table 7.

The nitroprusside test was negative on all solutions after incubation for 20 days. There was no indication of appreciable decomposition of thiosulfate on titration with iodine solution, and the pH was the same in the control and inoculated solutions (8.0).

In no case was there appreciable growth of the organisms since no organic substances were added to the medium; some fungal hyphae or small flocs of growth of the actinomycetes were detected by careful inspection. Even with this limited amount of cell material there was pronounced sulfide formation in some cases, particularly in the medium containing precipitated sulfur. The test papers became black in the flasks of this medium inoculated with *Rhizopus* sp. and *Actinomyces violaceus-ruber*. There was a strong test with *Actinomyces flavovirens* and *Actinomyces griseus* and weaker tests with three other orgnisms. The reaction was slower in developing and not so intense from the rhombic sulfur. Even in the absence of elemental sulfur some of the organisms produced sulfide, but in only one case (*Rhizopus* sp.) was there more than a weak test. Apparently some of these organisms are able to reduce thiosulfate to sulfide. Such reduction by bacteria was reported by Hölschewnikoff in 1889, and a short time later was noted by Petri and Maassen (1893) for numerous bacterial species. More recently it has been shown that this reduction can be effected by many bacteria (Beijerinck, 1900 and 1904b; Sasaki and Otsuka, 1912; Lederer, 1913; Tanner, 1917; Tarr, 1933), yeasts, and filamentous fungi (Kossowitz and Loew, 1912; Neuberg and Welde, 1915; Tanner, 1918; Armstrong, 1921; Korsakova, 1933, see also Bunker, 1936). These studies were made almost invariably in organic media supporting active growth of the organisms, providing conditions more favorable for reduction of thiosulfate than those used in the present experiments.

The formation of sulfide from elemental sulfur seems to be conditioned by the presence of appreciable amounts of cell material

in proximity to the sulfur; the tests have been most readily detected when the sulfur and cells are both on the surface. The following experiment was performed in order to establish this relationship more definitely. Five different media were used: (*a*) thiosulfate medium I, (*b*) medium I with rhombic sulfur, (*c*) medium I with precipitated sulfur, (*d*) medium I containing rhombic sulfur but no thiosulfate, (*e*) medium I with precipitated sulfur but no thiosulfate.

The cultures used included not only those which gave positive tests for sulfide in the previous tests, but also those which produced little or no sulfide. Considerable amounts of cell material were used. For the bacteria, the cells from a 4-day old culture on a nutrient agar slant were removed with a platinum blade and suspended in the solution before adding the sulfur. The fungi and actinomycetes were grown for 4 and 5 days respectively in 40-cc. portions of the following modified Krainsky medium in 125-cc. Erlenmeyer flasks: tap water 1000 cc., glucose 10 grams, asparagin 1 gram, K_2HPO_4 0.5 gram, $MgSO_4 \cdot 7H_2O$ 0.5 gram, $FeCl_3 \cdot 6H_2O$ 0.15 gram, $CaCl_2 \cdot 2H_2O$ 0.15 gram. After incubation, the medium was decanted, the cell material washed with two changes of sterile tap water, and transferred to the flasks of the media (*a*)–(*e*), all of the growth in one flask being used for each test. The weight of the cell substance of the fungus used in each case was equivalent to about 100 mgm. of oven-dry material; somewhat less cell material of the actinomycetes was used. Lead acetate papers were suspended above the solutions.

The cell material of the actinomycetes tended to sink in the medium in some cases (*Actinomyces flavovirens*, *Actinomyces pheochromogenus* and *Actinomyces californicus* on the rhombic sulfur) creating conditions rather unfavorable for rapid sulfide formation. Where the cell substance was in contact with the sulfur on the surface, a slight amount of sulfide was evolved within an hour; similar preparations where the cell material had settled, gave no evidence of sulfide formation in this short time.

None of the uninoculated solutions gave a test for sulfide. With the inoculated solutions, sulfide formation was very general and

TABLE 8

*Sulfide production by heavy inocula of heterotrophic microörganisms in inorganic media containing thiosulfate, or elemental sulfur, or both**

ORGANISM	PERIOD OF INCUBATION				
	1 day	3 days	5 days	7 days	12 days
Thiosulfate medium					
Sarc. lutea	0	0	0	±	±
Az. chroococcum	0	0	0	±	±
B. cereus	0	0	0	0	0
Act. flavovirens	1	4	5	6	8
Act. californicus	0	3	3	3	4
Act. pheochromogenus	0	0	0	0	0
Rhizopus sp.	0	0	±	±	1
Asp. flavus	0	0	0	0	0
Trichoderma sp.	0	0	0	0	0
Thiosulfate with rhombic sulfur					
Sarc. lutea	1	2	2	3	3
Az. chroococcum	0	1	1	2	3
B. cereus	0	0	0	0	±
Act. flavovirens	2	6	8	9	10
Act. californicus	0	4	5	7	8
Act. pheochromogenus	0	2	3	4	6
Rhizopus sp.	1	4	5	6	7
Asp. flavus	±	2	2	3	4
Trichoderma sp.	0	1	1	1	1
Thiosulfate with precipitated sulfur					
Sarc. lutea	1	4	5	6	7
Az. chroococcum	1	2	3	4	5
B. cereus	0	0	0	0	±
Act. flavovirens	3	6	8	10	14
Act. californicus	3	6	8	10	14
Act. pheochromogenus	0	2	3	4	5
Rhizopus sp.	2	4	6	8	10
Asp. flavus	±	2	3	4	5
Trichoderma sp.	1	2	2	2	3
Rhombic sulfur alone					
Sarc. lutea	±	4	5	6	7
Az. chroococcum	0	0	0	0	0
B. cereus	0	0	0	0	0
Act. flavovirens	0	4	8	10	12
Act. californicus	0	4	5	7	10
Act. pheochromogenus	0	±	1	2	3
Rhizopus sp.	2	4	5	7	9
Asp. flavus	±	2	2	3	4
Trichoderma sp.	0	0	1	1	1

TABLE 8—*Concluded*

ORGANISM	PERIOD OF INCUBATION				
	1 day	3 days	5 days	7 days	12 days
Precipitated sulfur alone					
Sarc. lutea	1	4	5	6	7
Az. chroococcum	1	1	1	1	2
B. cereus	0	1	1	1	2
Act. flavovirens	3	6	8	12	16
Act. californicus	4	5	8	9	14
Act. pheochromogenus	0	2	3	4	7
Rhizopus sp	2	4	8	9	10
Asp. flavus	±	2	3	4	6
Trichoderma sp	1	2	2	2	2

* 0 = no test; ± = very slight, 1, 2, 3, 4, etc., indicate increasing intensity of dark coloration of the test papers.

more rapid than in the previous experiment (table 8). It is clear that the presence of an abundance of cell material greatly favored hydrogenation of the sulfur. Two of the actinomycetes reduced thiosulfate to sulfide. Strong positive tests for sulfide were obtained with practically all of the actinomycetes and fungi and with some of the bacteria where elemental sulfur was present; stronger tests resulted from the finely divided precipitated sulfur than from the rhombic sulfur. It is probable that the sulfide originated principally from the elemental sulfur, since practically the same amount of sulfide was produced in the media with sulfur alone as in the media containing both sulfur and thiosulfate. The results show that there are quantitative differences in the production of sulfide by the various organisms.

The reaction would probably proceed more rapidly if the cells were mixed with the elemental sulfur in a moist state as a paste, but the object of the present investigation was to determine whether or not various heterotrophic organisms could produce sulfide under the conditions that led to its formation by *T. thioparus* and *T. thiooxidans*, and not to determine differences in the capacities of heterotrophic organisms to hydrogenate sulfur under the most favorable conditions.

None of the solutions gave a test for sulfide with the nitroprusside reagent, nor appreciable change in pH, nor evidence of disappearance of thiosulfate in those cases where it was added to the medium. The amounts of thiosulfate reduced to sulfide by the actinomycetes were therefore extremely slight.

DISCUSSION

These results with heterotrophic microörganisms increase the evidence that hydrogenation of elemental sulfur is effected by many microörganisms and various tissues. During the latter part of the nineteenth century, de Rey-Pailhade (1888a and b, 1898) noted that animal tissues, plant materials, and yeast cells effect this reduction. Heffter (1907) found this reducing capacity inherent in the sulfhydryl groups contained in the organic substances of the cells. He even attempted to measure the protein -SH groups by determining the amount of hydrogen sulfide produced from elemental sulfur through its reaction with these groups converting them to disulfide. More recently, Hopkins (1921) discovered that yeast and various plant and animal tissues contain a compound called glutathione; this was found to be a tripeptide composed of glycine, glutamic acid, and cysteine having a sulfhydryl group which reduces elemental sulfur ($G \cdot SH + HS \cdot G + S = G \cdot S \cdot S \cdot G + H_2S$) (Hopkins, 1929). Callow and Robinson (1925) obtained evidence of the general occurrence of glutathione in bacterial cells. Sluiter (1930) found that the hydrogenation of sulfur by glutathione as well as by tissues was, during a certain time, proportional to the quantity of sulfhydryl present, but many factors determine the number of active -SH groups in tissues at any one time (Guthrie and Wilcoxon, 1932; Mirsky and Anson, 1935-6).

There is much evidence that sulfur is reduced to sulfide by practically all tissues (McCallan and Wilcoxon, 1931). Less is known concerning the mechanism of sulfur reduction by microörganisms, but it has been reported that many of them hydrogenate sulfur.

Miquel in 1879 and Debraye and Legrain in 1890 found that sulfur could be reduced by bacteria. In 1904 Beijerinck noted this for some of the bacteria of the coli-aerogenes group and others

(1904b). Nineteen of 21 bacteria tested by Sasaki and Otsuka (1912) reduced sulfur to sulfide (see also Lederer, 1913 and Tarr, 1933). This reduction by yeast has been known since the studies of Dumas in 1874, and the interest developed by de Rey-Pailhade in what was believed to be a specific sulfur-reducing material in yeast and tissues (see Tanner, 1918, Hopkin, 1921 and Buchanan and Fulmer, 1930, page 201).

Therefore, the hydrogenation of sulfur as effected by *T. thioparus* and *T. thiooxidans* is a common transformation and should have been expected (Buchanan and Fulmer, 1930; Bunker, 1936). The fact that these organisms have this capacity may be interpreted as evidence that their cells contain glutathione or similar compounds containing sulfhydryl groups. It seems most likely that the sulfide produced by these bacteria has the same significance that it has with the numerous heterotrophic organisms which have been shown to effect the same transformation under the conditions used for the sulfur bacteria. In the case of the heterotrophic organisms it was necessary to add more than a small inoculum to obtain an active reaction since conditions in the inorganic media were not well suited for their growth. The sulfur bacteria were able to grow in such media and evolve detectable amounts of sulfide after sufficient cell material had been produced.

Quite recently, van Niel (1936) demonstrated similar reduction of elemental sulfur by purple bacteria. Gaffron (1934–5) believed the sulfide was formed from sulfate (see also Roelofsen, 1934 and 1935) but this did not prove to be the case. The fact that sulfur is hydrogenated by the purple bacteria is evidence that these organisms contain active -SH groups, as do most other microörganisms. Baas-Becking (1925) ascribed more significance to the glutathione and similar materials in the metabolism of the sulfur bacteria, but he assumed that they effected oxidation and not the reduction which has been commonly observed. He believed that hydrogen sulfide, the principal energy source of the higher sulfur bacteria, ionized to form HS- which diffused into the cells, and reacted with cystine or oxidized glutathione, resulting in the formation of elemental sulfur and cysteine or reduced

glutathione. Later results (van Niel, 1931) indicate that the sulfur bacteria are not confined to the use of ionized H_2S and that glutathione might be expected to cause reduction of elemental sulfur rather than oxidation of sulfide in the cells (van Niel, 1936).

One may be inclined to believe that the ability of the sulfur bacteria to utilize elemental sulfur is dependent upon their hydrogenation of the sulfur preceding its entrance into the cells as previously mentioned in this report. This would provide soluble sulfur material. Both *T. thiooxidans* and *T. thioparus* are able to oxidize elemental sulfur, *T. thiooxidans* producing the more rapid oxidation. However, it does not seem necessary to assume that the preliminary hydrogenation occurs.

Primarily, it is difficult to account for the production of sufficient organic matter having sulfhydryl groups to effect the hydrogenation of the sulfur outside of the cells. Presumably these organic compounds would be of use to the cells only in bringing about one reduction reaction. The sulfur would be the single source of energy for the organisms synthesizing these organic materials and all of the other cell constituents. However, the energy required to synthesize two molecules of cysteine would be greater than the energy the bacterium obtains from oxidation of one atom of H_2S to sulfate. It is generally considered that 2 -SH groups are concerned with the reduction of one sulfur atom. Therefore, if the sulfur is reduced before assimilation, some other mechanism than this one must be responsible for the reduction.

It has been shown by McCallan and Wilcoxon (1931), using fungus spores, that elemental sulfur exerts sufficient solution pressure to bring about the diffusion of sulfur into cells which are not even in contact with the solid sulfur particles (see also Sempio, 1932). "Sulphur in the vicinity of fungous spores, by reason of its vapor pressure, gives off sulphur vapor which diffuses into the spores. Here reduction takes place within the spores with hydrogen sulfide as a final product. The reaction is enzymatic in nature and is probably concerned with -SH compounds" (p. 35). If a similar transfer of sulfur takes place with

sulfur bacteria, a mechanism is available whereby the organisms can obtain elemental sulfur directly without requiring preliminary reduction or oxidation. Within the cells, the sulfur would then undergo oxidation characteristic of the specific organisms.

The results of McCallan and Wilcoxon also lead to the conclusion that, at least with the fungus spores, the sulfide is produced inside of the cells after entrance of the sulfur; therefore, evolution of sulfide follows its excretion from the cells. If this is the mechanism of the reaction with the sulfur bacteria, it cannot be assumed that the sulfur becomes available to the cells after preliminary hydrogenation; actually, sulfide production would be evidence that the cells excreted hydrogen sulfide after absorbing elemental sulfur.

Sulfide would be excreted by bacteria only if they were unable to oxidize it. It should be possible to demonstrate sulfide production by purple sulfur bacteria only when the cells are unable to effect their normal oxidations, such as in the absence of light; van Niel (1936) has shown that they reduce sulfur under this condition. With the colorless forms of the higher sulfur bacteria, which produce sulfur globules and do not require radiant energy, it should be possible to demonstrate sulfide formation after inhibiting the normal oxidative processes, as by excluding oxygen.

One might finally conclude that organisms which evolve sulfide under conditions favorable to their normal dehydrogenations, have either little or no capacity for dehydrogenating sulfide or are able to effect unusually active hydrogenation of sulfur; the former condition seems most likely to exist. It is probable therefore, that *T. thiooxidans* and *T. thioparus* are not able to utilize hydrogen sulfide as a source of energy to any appreciable extent under conditions which favor their oxidation of elemental sulfur and thiosulfate (Bunker, 1936).

None of the data gives any indication that the sulfur material produced by *T. thioparus* or the other sulfur bacteria is a polysulfide as conceived by von Deines (1933b), although it is possible that traces of sulfide may be present after having been produced through hydrogenation of the elemental sulfur itself. If the sul-

fide is of importance in the nutrition of the cells or if the sulfur material is a polysulfide, it should be possible to detect it consistently and in appreciable amounts. This is not the case. The sulfur material, as it occurs outside of the cells, has no reducing action on dilute iodine solution and has the characteristics of elemental sulfur.

SUMMARY

The studies were concerned with determining: (1) whether the precipitate, formed by *Thiobacillus thioparus* during oxidation of thiosulfate, is sulfur or a polysulfide; (2) whether or not elemental sulfur is reduced before being absorbed by sulfur bacteria such as *Thiobacillus thiooxidans*.

There was no ash residue after ignition of the material obtained by dissolving the precipitate in carbon bisulfide. The precipitate contained no substance which reacted with iodine solution. Neither sulfide nor sulfite was found in the thiosulfate medium in which *Thiobacillus thioparus* was growing. There was no indication that the precipitate contained sufficient sulfide to characterize it as a polysulfide.

Small amounts of sulfide were evolved by *Thiobacillus thioparus* and *Thiobacillus thiooxidans* growing in solution media. The sulfide is produced through hydrogenation of elemental sulfur.

Various heterotrophic bacteria, actinomycetes, and filamentous fungi likewise evolved sulfide from inorganic media containing elemental sulfur, considerable differences being apparent in the rates of sulfide production by the different organisms. Some of the actinomycetes and fungi produced sulfide from thiosulfate in strictly mineral media.

The hydrogenation of sulfur by the sulfur bacteria suggests the presence of active -SH groups in their cells; this reaction appears to have the same significance with the sulfur bacteria as with the heterotrophic microörganisms.

It is considered unlikely that elemental sulfur undergoes hydrogenation preceding its entrance into the cells of the sulfur bacteria which oxidize the sulfur to sulfate.

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