CULTIVATION OF ORGANISMS CONCERNED IN THE OXIDATION OF THIOSULFATE

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Although numerous investigations deal with the isolation and description of bacteria which decompose thiosulfate and other incompletely oxidized sulfur materials, considerable confusion still exists concerning certain aspects of the nutrition of these bacteria and the significance of the sulfur materials in their development. The present report includes studies on the influence of environmental conditions upon the development of some bacteria which up to this point at least would be classified as species of the genus *Thiobacillus*, Beij. The cultures used in these studies have been previously described in some detail (Starkey, 1934a). They may be briefly characterized as follows.

They are all Gram-negative, non-sporulating, small, rod-shaped bacteria. *Th. novellus* increases the acidity of mineral media containing thiosulfate and develops a uniform turbidity of the solution. This is a facultative autotrophic organism. It was described as a new species in the preceding report (Starkey, 1934a).

Culture B is closely related to the bacteria described by Trautwein (1921; 1924) and called *Th. trautweinii*, Bergey. It was fortunate that two of Trautwein's three cultures were available in this laboratory, having been supplied by their discoverer over ten years ago. Since that time they have been regularly transferred on nutrient agar. They show the same general characteristics originally ascribed to them. Their original identification as cultures T and K have been retained in this report. Culture B, isolated in this laboratory and T and K grow well on most of

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the common laboratory media but make little visible growth in the mineral medium containing thiosulfate. As a result of their development in this solution, thiosulfate disappears and the reaction becomes more alkaline.

Culture C fails to grow on organic media in the absence of thiosulfate or sulfur. In the mineral medium, thiosulfate is decomposed with the precipitation of considerable sulfur, and the development of uniform turbidity throughout the solution. The reaction becomes increasingly more acid as growth progresses. This culture shows many features suggesting its identity with *Th. thioparus*, Beij. and is called by this name in the following pages.

INFLUENCE OF REACTION

The medium commonly used in these studies was prepared of the following materials and distributed in 100 cc. portions in 250 cc. Erlenmeyer flasks:

	gm.
Tap water	1,000
$MgSO_4 \cdot 7H_2O$	0.1
$CaCl_2 \cdot 2H_2O$	0.1
$MnSO_4 \cdot 2H_2O$	0.02
$FeCl_3 \cdot 6H_2O$	0.02
$(NH_4)_2SO_4$	0.1
$Na_2S_2O_3 \cdot 5H_2O_4$	10.0
K_3PO_4, K_2HPO_4, or KH_2PO_4 or mixtures of these as stated for each experiment	2.0

The ammonium sulfate and sodium thiosulfate were sterilized separately. In this experiment, mixtures of phosphates were used to create a range of reactions from pH 9.0 to 4.8. Media at each reaction were inoculated with pure cultures of the five bacteria. Periodically the media were examined for extent of decomposition of thiosulfate and change in reaction. Residual thiosulfate was determined by titration with 0.01 N iodine solution. Reaction was measured colorimetrically.

Some of these results are shown in figure 1. In the upper portion of the figure is recorded the extent of decomposition of the thiosulfate and in the lower portion the pH numbers of the same cultures. It is apparent that culture B and Trautwein's cultures

T and K react qualitatively alike. Decomposition developed from pH 9.0 to 6.0 in all cases and also at pH 5.5 with cultures B and K. Maximum development took place in media which were initially acid—pH 5.5 or 6.0. In the early stages, maximum oxidation was found at pH 7.2 for B and 6.5 for K. The pH rose rapidly, reaching at least 9.8 with cultures T and K in two days in all cases where the initial reaction was above 6.5. No determinations were made covering reactions above 9.8. A slower increase is noted with culture B which made less growth at this

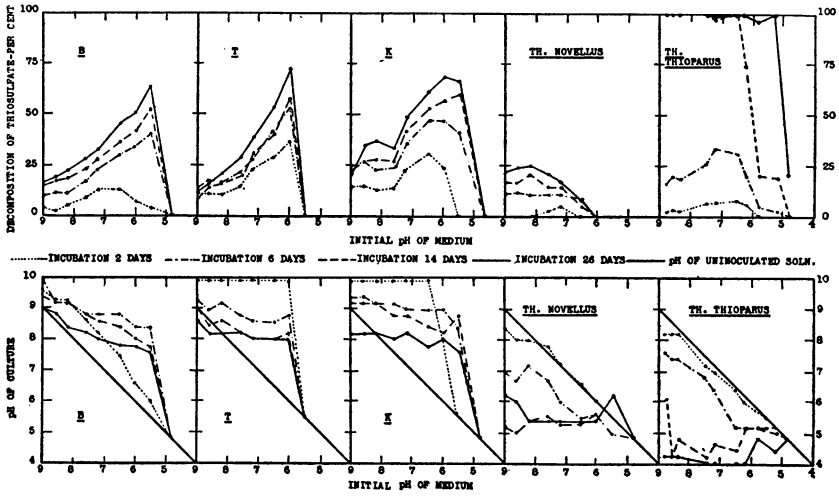

FIG. 1. DECOMPOSITION OF THIOSULFATE AND CHANGES IN REACTION BROUGHT ABOUT BY BACTERIA DEVELOPING IN MEDIA DIFFERING IN INITIAL REACTION

period of two days. After the initial rapid increase, the reaction shows a tendency to decline as the cultures age. In some cases the reaction had dropped to that of the uninoculated controls at twenty-six days. The initial increase in pH is believed to be due to transformation effected by the bacteria; the subsequent drop is undoubtedly caused by secondary decomposition of the initial products as will be brought out in a later communication.

Trautwein (1921) noted practically the same decomposition of thiosulfate between pH 6.9 and 9.2 with best oxidation at pH 7.9 to 8.5. He stated that the culture solutions became alkaline

during development of the bacteria except at high initial pH where the pH dropped in eight days.

Th. novellus is most active at alkaline reactions and makes slight development where the reaction is initially acid. Maximum oxidation occurred in media initially between pH 8.0 and 9.0. No growth was observed where the initial reaction was below pH 6.0. Even at the point of maximum development only about one-third as much thiosulfate was oxidized in twenty-six days as by cultures B, T or K. However, the change in reaction was extreme, but in the opposite direction from the preceding cultures. The acidity gradually increased to about pH 5.0 to 5.5 which seemed to be unfavorable for further growth. The halophilic bacterium of Saslawsky (1927) and Saslawsky and Harzstein (1930) oxidized thiosulfate completely to sulfate rendering the medium acid. In some respects this organism behaves similarly to *Th. novellus*. Guittonneau's culture also oxidized thiosulfate to sulfate (1925; 1927; 1928).

Th. thioparus initially grew best in media adjusted close to neutrality but, after twenty-six days, decomposed practically all of the thiosulfate in media initially having reactions from pH 5.2 to 8.8. The rapid increase in acidity delayed development in the solutions which were originally acid. This is much the most active of the cultures under investigation (excepting *Th. thiooxidans*) in several respects: rapidity of growth, wide range of pH over which development occurs, extreme acidity produced by decomposition of thiosulfate. The acidity increased rapidly to between pH 4.0 and 5.0. The medium of initial pH 6.5 changed to pH 3.4; this is shown only as pH 4.0 on the graph.

The medium supporting growth of the culture studied by Nathansohn (1902) remained alkaline and did not change in reaction in ten days. Since polythionates were detected in the solutions, it is possible he was not using a pure culture. Issatschenko and Salimowskaja (1929) and Lange-Posdeeva (1930) worked with cultures showing characteristics similar to those of *Th. thioparus*; they state that the reaction becomes acid during growth. Products of oxidation similar to these produced by *Th. thioparus* were formed by a culture studied by Salimowskaja (1931) but

the pH of the medium increased during growth, as from an initial pH of 4.4 to pH 7.6.

In contrast with these cultures, *Th. thiooxidans* develops best in strongly acid media growing most rapidly between pH 2.0 and 3.0 but developing from below pH 1.0 up to 6.5 or 7.0 (Waksman and Starkey, 1923; Starkey, 1925). There appears to be little available information on reaction changes brought about by *Th. denitrificans* but, from the transformation ascribed to it, there is likelihood of an increase in acidity (Lieske, 1912; Tjulpanova-Mossevitch, 1930).

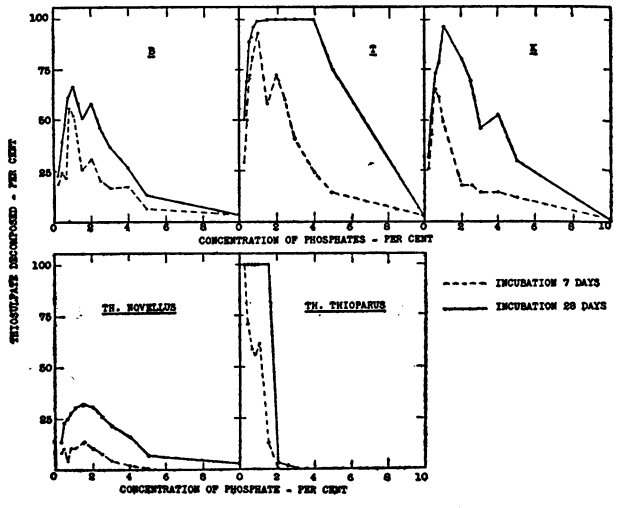

FIG. 2. INFLUENCE OF PHOSPHATE CONCENTRATION UPON DECOMPOSITION OF THIOSULFATE BY VARIOUS BACTERIA

CONTROL OF CHANGE IN REACTION

Media were prepared the same as for the preceding experiment with the exception of the phosphates. Since *Th. novellus* and *Th. thioparus* developed better in alkaline media, K_2HPO_4 was the only phosphate used, creating a pH of 7.8 irrespective of the phosphate concentration. For cultures B, T and K equal portions of K_2HPO_4 and KH_2PO_4 were used in the media buffering the solutions at pH 6.6. The phosphates were used in various concentrations from 0.2 to 10.0 per cent. The extent of decomposition of thiosulfate by the various cultures is shown in figure 2.

With cultures B, T and K there was an increase in pH; with *Th. novellus* and *Th. thioparus* the pH dropped. At the low phosphate concentration, inhibitive reactions were quickly reached; at high phosphate concentrations, the influence of the salt concentration was the factor determining activity. The curves are probably resultants of the effects of these two factors.

Buffering the media quite strongly to prevent extreme changes in reaction favored development in all cases. The concentration of phosphate salt commonly used in the media for many of the early experiments in these investigations was 0.2 per cent. It is apparent that this is not the optimum concentration for any of the cultures except *Th. thioparus* and even this organism decomposed all of the thiosulfate in 28 days in concentrations up to 1.5 per cent. *Th. thioparus* was least tolerant to high phosphate concentrations making very limited or no growth above 1.5 per cent. Concentrations of 1 to 2 per cent gave best results with *Th. novellus* but oxidation was quite meagre compared to the optimum development of the other organisms. Concentrations of 0.8 to 2 per cent seemed most favorable for cultures B, T and K and oxidation continued even at 5 per cent. Culture T was particularly active over a wide range of concentrations.

Another experiment was conducted in which the reactions of the media were periodically adjusted. The medium was the same as that initially described but contained 0.2 per cent K_2HPO_4 creating a reaction of pH 7.8. Part of the cultures were not altered during incubation. Other solutions were neutralized after the determinations for residual thiosulfate on the fifteen- and twenty-six-day periods using determined amounts of sterile 0.1 N NaOH for *Th. novellus* and 0.1 N H_3PO_4 for cultures B, T and K. Table 1 indicates that oxidation was favored in all cases by neutralization of the media. The products of oxidation of thiosulfate which are most inhibitive to further development of the organisms appear to be the OH-ions for cultures B, T and K and the H-ions for *Th. novellus*.

When bacterium B, T or K is cultivated with *Th. novellus* in a medium having a reaction close to neutrality, oxidation of thiosulfate is more extensive than when any of these organisms is

cultured alone. This is probably due to the fact that *Th. novellus* tends to increase the acidity of the medium and B, T and K to decrease it during development. Since *Th. thioparus* grows very rapidly, over a wide range of reaction, oxidation of thiosulfate is not appreciably modified by the presence of any of the other cul-

TABLE 1

Decomposition of thiosulfate by cultures in media periodically neutralized

CULTURE	NEUTRALIZED (+) OR UNNEUTRALIZED (−)	THIOSULFATE DECOMPOSED		
		15 days	26 days	39 days
		per cent	*per cent*	*per cent*
B_1	−	10.9	18.1	23.1
	+	10.9	19.8	29.6
B_2	−	8.2	9.7	13.0
	+	8.8	13.5	22.7
T_1	−	20.3	30.6	37.6
	+	22.1	37.1	52.0
T_2	−	29.5	48.5	58.9
	+	28.4	52.4	67.3
K_1	−	23.1	35.5	43.9
	+	23.7	41.5	57.1
K_2	−	22.8	35.3	42.9
	+	24.9	46.1	61.5
Th. novellus (1)	−	4.0	7.0	8.7
	+	3.8	13.8	24.8
Th. novellus (2)	−	10.2	12.3	13.2
	+	13.5	29.1	41.3

tures. The same applies to *Th. thiooxidans* except in special cases. When the initial reaction is close to the critical maximum pH (between 6.5 to 7.0), cultures B, T or K in association tend to make the reaction alkaline before growth of *Th. thiooxidans* becomes established. In some cases of such associations, growth of *Th. thiooxidans* is prevented in media that would be suitable

TABLE 2

*Composition of media used for culturing sulfur bacteria**

Medium Constituents	No. 1	No. 2	No. 3	No. 4	Nathansohn† (1902)	Beijerinck† (1903 a and b)	Beijerinck‡ (1904 a and b)	Jacobsen† (1912; 1914)	Lieske‡ (1912)	Trautwein§ (1921)	Salimovskaja‖ (1930)
$Na_2S_2O_3 \cdot 5H_2O$	10.0	10.0	10.0	10.0	5.0	5.0	5.0	5.0	5.0	2.0	10.0
K_2HPO_4	2.0	4.0	6.0	2.0		0.2	0.2	0.5	0.2		3.0
KH_2PO_4		4.0									
$Na_2HPO_4 \cdot 12H_2O$					5.0					0.2	
$CaCl_2$	0.1	0.1	0.1	0.1					Trace		0.2
$MgSO_4 \cdot 7H_2O$	0.1	0.1	0.1	0.1							
$MgCl_2 \cdot 6H_2O$					2.5	2.5	0.1	0.2	0.1	0.1	0.5
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1							
NH_4Cl						0.1		0.5		0.1	2.0
KNO_3					0.1		0.5		5.0		
$CaCO_3$ (or $MgCO_3$)				In excess (5.0)	In excess (5.0)		In excess (20.0)	In excess (20.0)			
Na_2CO_3							0.1				
$NaHCO_3$						1.0			1.0	1.0	
$FeCl_3 \cdot 6H_2O$	0.02	0.02	0.02	0.02				Trace	Trace		
$MnSO_4 \cdot 2H_2O$	0.02	0.02	0.02	0.02							

* On basis of grams per 1,000 cc. Distilled water was used for all except Nos. 1, 2, 3, 4, in which tap water was used. These four are original media.

† For *Th. thioparus.*

‡ For *Th. denitrificans.*

§ For *Th. trautweinii.*

‖ For an organism similar to *Th. thioparus.*

for development of the pure culture. On the other hand, *Th. novellus* or *Th. thioparus* may favor growth of *Th. thiooxidans* if they occur together in media that, initially, are slightly too alkaline for the last organism. This is due to the fact that both *Th. novellus* and *Th. thioparus* increase the acidity of the medium during growth. It was noted by Waksman and associates that sulfur and thiosulfate are oxidized more rapidly in solutions and soils by mixed cultures of sulfur bacteria than by the separated pure cultures (Waksman, 1922 a and b; Waksman, Wark, Joffe and Starkey, 1923).

DEVELOPMENT UPON VARIOUS MINERAL MEDIA

In table 2 is given a list of the media used in this study; some of these have been previously recommended for culturing members of the genus *Thiobacillus*. Numbers 1, 2, 3 and 4 are first described in this report. The calcium carbonate, ammonium sulfate and thiosulfate were sterilized separately in the autoclave. The sodium carbonate and bicarbonate were sterilized by passage through a Seitz filter. Almost all of the organisms developed in the media.

The media most suitable for development of each culture, judging from oxidation of thiosulfate at earlier periods and at the twenty-six day period are underlined in table 3. For cultures B, T and K, medium 2 seemed the best although Nathansohn's solution was quite suitable. *Th. novellus* made the best growth on Jacobsen's medium although medium 3 which was prepared especially for its cultivation was nearly as satisfactory at the twenty-six-day period; at earlier periods, medium 3 was superior to the others. *Th. thioparus* makes remarkably fine growth on a great variety of media. Observations at an earlier period (six days) indicated that medium 2 and the one of Trautwein were particularly favorable for rapid development.

INFLUENCE OF SOME MEDIUM CONSTITUENTS

Cultures B, T, K and *Th. novellus* became relatively inactive after continuous cultivation in thiosulfate solution. They were cultivated in sterilized soil to which sodium thiosulfate had been

added but development in soil failed to alter their activity significantly.

With the expectation that some substances not commonly incorporated in the medium might favorably affect development of

TABLE 3

Decomposition of thiosulfate in various mineral media

MEDIUM	CONTROL	B	T	K	TH. NOVELLUS	TH. THIOPARUS
Thiosulfate decomposed, per cent*						
No. 1		4.5	23.4	22.5	13.4	100.0
No. 2		14.9	68.5	72.5	15.0	100.0
No. 3		2.5	22.2	21.7	31.0	100.0
No. 4		2.0	13.2	23.9	26.1	100.0
Nathansohn		12.9	56.9	52.6	20.0	31.9
Beijerinck†		0	4.1	9.5	0	2.3
Beijerinck‡		9.1	29.3	35.8	12.9	100.0
Jacobsen		6.3	41.4	40.5	36.3	100.0
Lieske		0	3.5	20.4	0	100.0
Trautwein		0	5.7	49.4	0	100.0
Salimowskaja		8.4	31.7	43.7	21.0	100.0
Reaction (pH)						
No. 1	7.8	8.0	8.8	8.6	5.4	4.4
No. 2	6.6	7.0	7.8	7.8	5.8	4.8
No. 3	8.0	8.0	8.8	8.6	6.4	6.8
No. 4	>9.8	>9.8	>9.8	>9.8	8.6	7.8
Nathansohn	7.4	7.6	8.2	8.0	7.0	7.2
Beijerinck†	>9.8	>9.8	>9.8	>9.8	>9.8	>9.8
Beijerinck‡	8.8	8.8	9.2	9.0	8.2	8.0
Jacobsen	8.4	8.6	8.4	8.4	8.0	8.0
Lieske	>9.8	>9.8	>9.8	>9.8	>9.8	6.0
Trautwein	9.6	>9.8	>9.8	>9.8	>9.8	4.0
Salimowskaja	7.4	7.6	8.0	8.2	5.8	4.4

* Incubation period 26 days.

† For *Th. thioparus*.

‡ For *Th. denitrificans*.

the cultures, a series of media were prepared containing, in addition to the constituents of medium 1 in table 2, bicarbonate (0.1 per cent), a small amount of an iron lignoprotein complex prepared by Waksman and Iyer (1932 a and b) or $NaNO_3$ (0.1 per

cent). None of these substances alone or in combination increased growth significantly. In an additional experiment, certain organic substances were utilized (table 4). The yeast infusion was prepared according to the directions of Fred and Waksman (1928).

It is apparent from table 4 that organic substances greatly favor oxidation of thiosulfate by cultures B, T and K and exert no significant favorable or depressing effect on *Th. novellus*. However, growth of *Th. novellus*, as judged from the turbidity of the culture, was far more extensive on media 2–4 than on 1, but apparently decomposition of thiosulfate bears no close relation-

TABLE 4

Influence of some organic materials upon decomposition of thiosulfate

MEDIUM NUMBER	SUBSTANCE ADDED	THIOSULFATE DECOMPOSED*			
		Culture B	Culture T	Culture K	Th. novellus
		per cent	*per cent*	*per cent*	*per cent*
1†		5.9	14.6	14.7	4.1
2	No. 1 + 0.01 per cent asparagine	20.9	27.8	28.6	5.6
3	No. 1 + 0.01 per cent peptone	12.7	26.0	29.3	3.9
4	No. 1 + 5 per cent yeast infusion	24.0	33.3	47.5	4.2

* Incubation period, eleven days.

† Same as medium 1, table 2.

ship to amount of growth in the presence of organic materials. Cultures B, T and K also made extensive growth in the organic media and decomposition of thiosulfate also increased, but probably not in proportion to the increase in abundance of cells.

INFLUENCE OF ORGANIC SUBSTANCES

In view of the apparent favorable effect of the organic substances on oxidation of thiosulfate by some of the cultures, a more extensive experiment was performed making use of the five different cultures and both carbohydrates and nitrogenous compounds. Three groups of media were prepared: (1) The first group included medium 1 (table 2) for *Th. thipoarus*, medium 2 (table 2) for cultures B, T and K, and medium 3 (table 2) for

Th. novellus. (2) This group received, in addition to the substances in group one, certain organic compounds in concentrations of 0.01, 0.1 or 0.5 per cent as indicated in figure 3. (3) Media of group three received no thiosulfate but in other respects were identical with the media of group 2. The organic materials, thiosulfate and ammonium sulfate were sterilized separately.

In figure 3 the white columns indicate the amount of thiosulfate decomposed in five days, the black columns show the change in twenty-two days. *Th. thioparus* made no growth in any of the

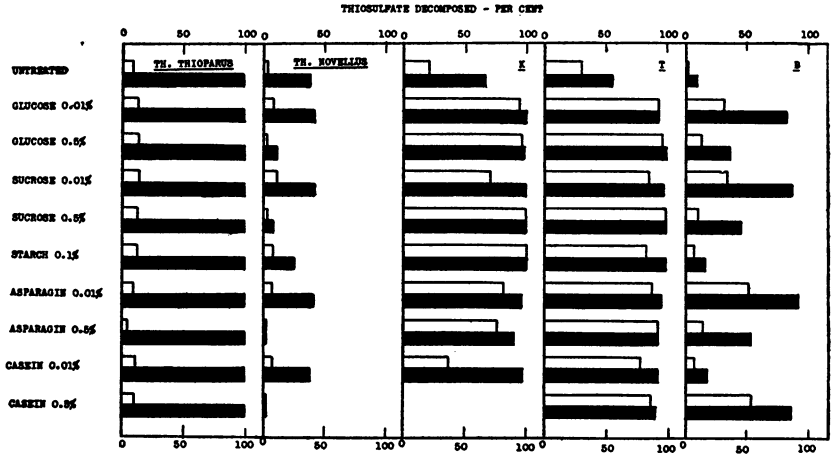

FIG. 3. DECOMPOSITION OF THIOSULFATE BY VARIOUS BACTERIA DEVELOPING IN MEDIA CONTAINING SOME ORGANIC MATERIALS

Incubation period five days for unshaded columns; twenty-two days for black columns

solutions lacking thiosulfate as would be expected with a strict autotroph. The presence of organic materials appeared to exert no influence upon decomposition of thiosulfate. All of the thiosulfate had disappeared in all cases at twenty-two days as shown by the figure. Other determinations not recorded indicated that complete decomposition had been accomplished in all cases at thirteen days. It would thus appear that this organism is quite tolerant to appreciable concentrations of organic materials without being affected by them. Similar results were obtained for

pure cultures of *Nitrosomonas monocella* and *Nitrobacter agilis* by Nelson (1931), and for *Th. thiooxidans* by Waksman and Starkey (1923) and Starkey (1925).

In the initial studies with *Th. thioparus*, Nathansohn found that organic materials were not utilized, and that they had no injurious effects on the organism (1902). Beijerinck stated that no other source of carbon could replace carbonic acid (1904 a and b). Klein and associates (1923; 1926) presumed that they were using a similar organism but it developed upon organic substances. The culture of Salimowskaja grew in nutrient solution in the presence of thiosulfate (1931). The bacteria of Issatschenko and Salimowskaja (1928; 1929) were believed to benefit from organic matter since they appeared to develop better with nitrogen supplied as peptone or asparagine than in the ammonium or nitrate form.

Referring again to figure 3, it is apparent that *Th. novellus* did not decompose thiosulfate to any greater extent in the presence of the organic materials than in their absence. In fact, at the 0.5 per cent concentrations of the substances, oxidation was almost completely inhibited. This is particularly interesting in view of the fact that growth was much more extensive at the higher concentrations of organic materials and was always greater in the presence of the lower amounts of organic matter than in the strictly mineral medium. Growth appeared to be equally extensive in the media of series 3, which contained organic substances but lacked thiosulfate, as in the comparable media of series 2. In dealing with this culture which is a facultative autotroph it is interesting to note that, in the presence of two available different sources of energy, one may support extensive development while the other is attacked less rapidly than where it occurs alone. This recalls the frequently noted sparing effects of carbohydrates upon the decomposition of proteins or protein derivatives when both are supplied in the same solution to certain bacteria (Waksman, 1932). It seems probable that in view of the fact that much more extensive growth occurs on organic than on thiosulfate media, the organic materials are metabolized more readily, and, in the presence of both substances, support the development of

practically all of the cells. The culture of Guittonneau (1925) also grew upon both thiosulfate and organic compounds.

Decomposition of thiosulfate by cultures B, T and K was greatly increased in the media containing organic substances (fig. 3). Both T and K made extensive growth in the media containing organic matter both where thiosulfate was present and where none was added. Heavier growth was apparent in the cultures containing the higher concentrations of organic matter. Culture B reacted similarly except that there was practically no growth in the presence of starch or 0.1 per cent casein. Although oxidation of thiosulfate is greater where the bacterial cells are more numerous in the media with organic matter, there is not an absolute correlation between growth and oxidation since more growth of culture B occurred in the media containing the higher concentrations of glucose, sucrose and asparagine. However, there was less decomposition of thiosulfate at the higher concentration. Trautwein was also able to cultivate his cultures on a great variety of organic media (1921; 1924).

On the basis of their reactions to organic materials, the organisms thus fall into 3 groups: (1) The strict autotroph, *Th. thioparus*, (2) the facultative autotroph, *Th. novellus*, (3) a special group of organisms represented by cultures B, T and K.

In regard to the tolerance of *Th. thioparus* for organic substances it may be of interest to note that this bacterium persisted for at least ten days on nutrient agar which contains little or no apparent energy-yielding substance to the cells; no growth was apparent during this period but typical development occurred upon inoculation into the thiosulfate medium.

The continuous cultivation of *Th. novellus* and bacteria B, T and K upon nutrient agar as compared with culture upon thiosulfate agar, for a considerable period of time, does not appreciably alter their behavior. Slants of these cultures were compared by inoculation into the mineral thiosulfate medium after monthly transfer upon the agar media for about one year. The differences were slightly in favor of the cultures grown upon nutrient agar, but this can be explained by the fact that greater numbers of cells were inoculated from the nutrient agar slants since growth was more extensive upon this medium.

Trautwein reported that his cultures did not lose their capacity to attack thiosulfate by growth for 9 months on nutrient agar (1921). Lange-Posdeeva was able to carry an organism, similar to *Th. thioparus*, on thiosulfate agar for three years without its losing the capacity to decompose thiosulfate in solution media (1930). The purple sulfur bacteria, although facultatively autotrophic, do not readily lose their autotrophic habit. Van Niel found that, after transferring them for four months, every three weeks, on both Na_2S and yeast extract media, the cultures from the organic medium developed as well as the Na_2S strains when again transferred to the Na_2S medium (1931). During his early studies with *Th. denitrificans*, Beijerinck believed that two organisms existed in his denitrifying solutions, one heterotrophic, the other autotrophic (1904 a and b). At a later date (1920) he was convinced that the two organisms were originally alike but that the autotrophic function of *Th. denitrificans* becomes permanently lost by development upon organic media. Similar statements were made by him about the nitrifying bacteria (1914). The criticisms of Winogradsky (1922) and Bonazzi (1923) concerning the stability of the autotrophic characteristic of the nitrifying bacteria seem equally applicable to the sulfur bacteria. The available evidence justifies the conclusion that the autotrophic function is very stable and not readily lost; the bacteria retain their individual characteristics in the presence of organic matter and after exposure to a great variety of other cultural environments.

AVAILABILITY OF VARIOUS SOURCES OF NITROGEN

In this experiment the media used were the same as numbers 1, 2, and 3 in table 2 except for the nitrogen source, with No. 1 for *Th. thioparus*, No. 2 for cultures B, T, K, *Ps. fluorescens* and *Achrom. hartlebii*, and No. 3 for *Th. novellus*. The amounts of the various nitrogenous substances used are indicated in figure 4. It was discovered during experiments reported elsewhere (Starkey, 1934 b) that *Ps. fluorescens* and *Achrom. stutzeri* attack thiosulfate in much the same manner as cultures B, T and K, which explains their use in this study.

After twenty-six days, the culture solutions were tested to

determine the amounts of thiosulfate lost by decomposition, for changes in pH and ammonia content and for nitrite. It is evident that *Th. novellus* decomposed relatively small quantities of thiosulfate in any of the media, not oxidizing over 17 per cent in any case. Practically equal oxidation occurred in media to which no nitrogen was added as in the presence of the nitrogenous materials, suggesting that in most cases, nitrogen was probably not the factor limiting activity. Growth was greatly favored in all

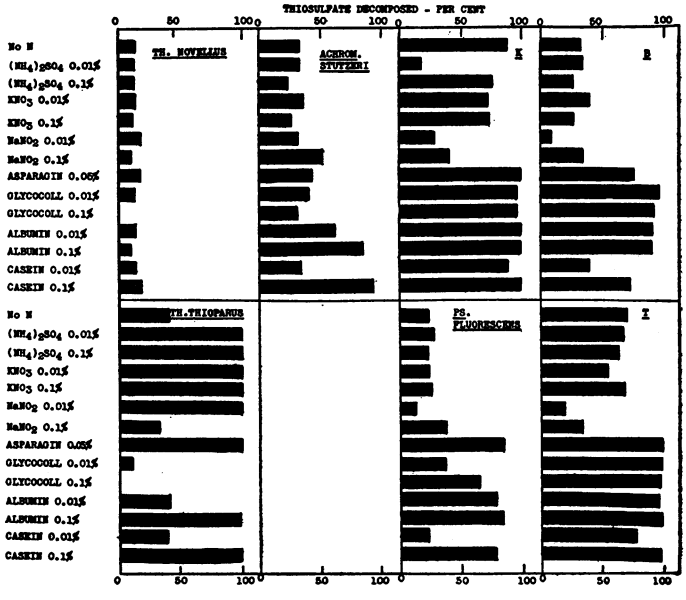

FIG. 4. DECOMPOSITION OF THIOSULFATE BY BACTERIA IN MEDIA CONTAINING VARIOUS NITROGENOUS SUBSTANCES

of the media receiving the organic substances although oxidation of thiosulfate was not increased. Since growth and ammonia production were favored by the organic materials these substances are undoubtedly available sources of nitrogen. Nitrate was not reduced to nitrite by this culture.

Th. thioparus showed marked response to the various nitrogenous materials and decomposed all of the thiosulfate in the media which were favorable to growth. All three inorganic

sources of nitrogen, ammonia, nitrate and nitrite can be utilized, but the last is toxic in concentration of 0.1 per cent. Nitrate was reduced with the accumulation of considerable nitrite. Glycocoll was quite toxic and there was practically no oxidation of thiosulfate nor evidence of growth at either concentration. The data in the figure suggest that *Th. thioparus* utilized nitrogen from asparagine, albumin and casein. This was unexpected in view of the fact that in none of the previous observations has there been any suggestion that this bacterium could attack organic compounds. It is still believed that the organic nitrogenous materials were not decomposed. No ammonia was formed in the media containing the asparagine, albumin or casein which could be said to be due to bacterial attack and there was no evidence that growth of the culture was greater in the presence of the organic materials than of the inorganic compounds. With the low concentrations of albumin and casein, oxidation of thiosulfate was practically the same as in the absence of nitrogen although the amount of nitrogen present should have been quite ample to supply the need of the bacterium if it had been available. It is believed that at the higher concentrations, 0.05 per cent asparagine and 0.1 per cent albumin and casein, sufficient ammonia was split from the organic compounds during sterilization under steam pressure to satisfy the requirements of growth for complete decomposition of thiosulfate. Ammonia was recovered in small amounts from the controls after sterilization. The conclusions of Issatschenko and Salimowskaja (1928; 1929) that an organism similar to *Th. thioparus* was able to utilize nitrogen in organic combination from peptone and asparagine as well as from NH_4^+ or NO_3^- may also need qualification for a similar reason. *Th. thiooxidans* requires ammoniacal nitrogen and cannot use nitrate which is toxic (Starkey, 1925).

Nitrite appears to be somewhat toxic to culture B, and more so to T and K. It is not believed that the development of cultures B, T, K, *Ps. fluorescens* and *Achrom. stutzeri* indicate utilization or lack of availability of the inorganic compounds since actual *growth* was very limited in all cases. Even nitrite was not toxic for *Ps. fluorescens* or *Achrom. stutzeri*. In the presence of

organic materials, oxidation of thiosulfate was increased in practically all cases where there was evidence of increased growth of the organisms. Ammonia was formed from glycocoll by cultures B, T, K and *Ps. fluorescens* and from albumin and casein by *Achrom. stutzeri*. Nitrate was reduced to nitrite by culture K. All of these cultures grew very well upon asparagine and glycocoll.

TABLE 5

Oxidation of elemental sulfur

CULTURE	SULFATE-S IN 100 CC. MGM.		TOTAL S IN 100 CC. MGM.	
	14 days	34 days	14 days	34 days
Medium 1				
Control	2.9	2.4	3.2	2.0
B	2.9	2.3	4.0	2.3
T	2.9	2.3	3.2	2.9
K		2.9	3.2	2.6
Th. novellus	4.0	2.9	3.4	2.6
Th. thioparus	13.2	17.2	11.2	17.5
Medium 2				
Control	4.3	3.0	4.9	4.3
B	4.3	3.2	4.3	4.6
T	4.4	3.2	4.0	4.9
K	4.3	3.2	4.6	4.3
Th. novellus	4.6	4.0	3.4	4.6
Th. thioparus	29.3	42.9	29.5	39.5

OXIDATION OF SOME INORGANIC SULFUR MATERIALS

The two media prepared for these studies had the same composition as the medium initially described in this report with the following exceptions. Elemental sulfur (2 per cent) was substituted for thiosulfate. Medium 1 received 7.5 grams K_2HPO_4 per liter. Medium 2 received 2 grams KH_2PO_4 and 2 grams $CaCO_3$ per liter. As indicated in table 5, *Th. thioparus* was the only culture which oxidized sulfur in the mineral media, development being much more active in the presence of $CaCO_3$. Sulfate was the only product of oxidation detected. However, the oxidation of sulfur by this bacterium is very slow compared to its oxidation of thio-

sulfate. No sulfate or other product of oxidation was found in the media inoculated with the other bacteria.

In other experiments, these cultures were inoculated into media containing sodium dithionate (0.05 and 0.5 per cent), sodium sulfite (0.05 and 0.5 per cent) and sodium sulfide (0.05 per cent). All of the sulfite became oxidized to sulfate in a few days in all of the inoculated and uninoculated media and none of the change could be ascribed to bacterial action. Sulfite is so unstable in contact with air that it appears unlikely that organisms of the type under consideration could utilize it as a source of energy. None of the cultures seemed able to attack sulfide in the concentration used. Dithionate appeared to be slowly oxidized by *Th. thioparus* but not by the other cultures. This oxidation was accompanied by the appearance of sulfate and an increase in acidity.

The results of studies by various investigators using several different organisms indicate that all of the sulfur bacteria of the genus *Thiobacillus* are able to utilize thiosulfate. The green sulfur bacteria utilize only sulfide, but the purple bacteria use thiosulfate, sulfite, elemental sulfur and sulfide (Van Niel, 1931; Van Niel and Muller, 1931). Previous investigators are also in general agreement that thiosulfate is more readily oxidized than other sulfur materials (Nathansohn, 1902; Beijerinck, 1904a and b; Lange-Posdeeva, 1930; Lieske, 1912; Gehring, 1915; Guittonneau, 1928; Salimowskaja, 1931). This does not appear to be the case with *Th. thiooxidans* which rapidly oxidizes both sulfur and thiosulfate (Waksman and Starkey, 1923; Starkey, 1925a and b). The culture of Rountree reacts similarly (1933) but cannot develop under the extreme acidity tolerated by *Th. thiooxidans*. In several reports it is stated that organisms resembling *Th. thioparus* are able to oxidize sulfur (Issatschenko and Salimowskaja, 1928 and 1929; Lange-Posdeeva, 1930; Saslawsky, 1927). The most significant results are those of Jacobsen, (1912) who observed that the bacterium could also oxidize sulfide (1914) although it was very sensitive to this substance (see also Nathansohn, 1902; Beijerinck, 1904a and b). *Th. denitrificans* oxidizes sulfur (Beijerinck, 1904a and b; Lieske, 1912; Gehring, 1915; Tjulpanova-

Mossevitch, 1930) and was found to make poor or fair development upon sulfide, sulfite (Beijerinck, 1904a) and dithionate (Lieske, 1912; Gehring, 1915) Salimowskaja's culture grew only on thiosulfate (1931). Trautwein claimed that sulfide and elemental sulfur were oxidized by his bacteria (1921). Due to inadequate control in many of these studies, the actual diversity of sulfur materials suitable as sources of energy for some of the species of *Thiobacillus* still remains uncertain.

SUMMARY

Results are reported of studies concerned with the cultivation of several bacteria generally classified as members of the genus *Thiobacillus*, Beij. These bacteria include an organism which is strictly autotrophic and appears to be much the same as *Th. thioparus*, a facultative autotrophic bacterium *Th. novellus* recently described as a new species, and 3 cultures (B, T, K) differing from these. The first two bacteria increase the acidity during growth in thiosulfate media; cultures B, T and K decrease the acidity. Development of *Th. thioparus* and *Th. novellus* is favored in media which are initially alkaline in reaction; B, T and K develop best in media initially slightly acid and well buffered. Buffering the media strongly, also favored oxidation of thiosulfate by *Th. novellus* but did not appreciably affect *Th. thioparus*. Similar favorable effects on oxidation resulted from periodically adjusting the reaction of solutions supporting development of the cultures. Associations of pairs of cultures, one of which increases the acidity and the other decreases the acidity, frequently effect more rapid oxidation of thiosulfate than the pure cultures alone.

Various mineral media previously suggested for study of species of *Thiobacillus* were used with the pure cultures. The media devised by the author were, in most cases best suited for the cultures but many media were favorable for *Th. thioparus*. Several substances added to the solution media failed to favor oxidation of thiosulfate by *Th. thioparus* and *Th. novellus*. Organic materials greatly increased both growth and decomposition of thiosulfate by B, T and K. Although growth of *Th.*

novellus increased in the presence of organic materials, the decomposition of thiosulfate was not increased and was frequently decreased. Oxidation of thiosulfate by *Th. thioparus* did not increase in the presence of organic materials and this culture failed to grow on organic substances. *Th. thioparus* was not affected by as much as 0.1 or 0.5 per cent concentration of some organic materials. Continuous cultivation of *Th. novellus* and cultures B, T and K on nutrient agar for a year did not alter their ability to attack thiosulfate when they were again introduced to the solution medium.

Th. novellus and cultures B, T and K use a variety of organic materials as sources of nitrogen. *Th. thioparus* probably is able to use only inorganic nitrogen; nitrite, nitrate and ammoniacal nitrogen are used. Nitrate is reduced to nitrite.

Of the various sources of sulfur used, thiosulfate is the only one which is rapidly oxidized. Elemental sulfur is slowly oxidized to sulfate by *Th. thioparus* but not by B, T, K or *Th. novellus*. Sulfite is oxidized so rapidly by chemical action that it seems unlikely that these bacteria are concerned in its oxidation. No oxidation of sulfide by the bacteria was observed. Dithionate appeared to be slowly oxidized to sulfate by *Th. thioparus*.

REFERENCES

BEIJERINCK, M. W. 1904a Centbl. Bakt. (II), **11**, 592–599. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann and Obernetter, München, **14**, 242–248, 1921.

BEIJERINCK, M. W. 1904b Arch. des Sci. Exactes et Naturelles, Haarlem, Ser. 2, **9**, 131–157. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann and Obernetter, München, **4**, 192–211, 1921.

BEIJERINCK, M. W. 1914 Folia Microbiologica, year 3; 91–113. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann and Obernetter, München, **5**, 178–193, 1922.

BEIJERINCK, M. W. 1920 Proc. of Section of Sciences, Kon. Akad. v. Wetenschappen, Amsterdam, **22**, 899–908. Also in Verzamelde Geschriften van M. W. Beijerinck, Bruckmann and Obernetter, München, **5**, 281–288, 1922.

BONAZZI, A. 1923 Jour. Bact., **8**, 343–363.

FRED, E. B., AND WAKSMAN, S. A. 1928 Laboratory Manual of General Microbiology. McGraw-Hill Book Company, Inc.

GEHRING, A. 1915 Centbl. Bakt. (II), **42**, 402–438.

GUITTONNEAU, G. 1925 Compt. Rend. Acad. Sci. (Paris), **181**, 261–262.

GUITTONNEAU, G. 1927 Compt. Rend. Acad. Sci. (Paris), **184**, 45–46.
GUITTONNEAU, G. 1928 Proc. of the First Intern. Cong. of Soil Sci., Washington, D. C., **3**, 274–284.
ISSATSCHENKO, B. L., AND SALIMOWSKAJA, A. G. 1928 Izyiestia Gosud. Gidrobiol. Instituta (Proc. of the Gov't Hydrobiol. Inst.), No. 21, 61–73.
ISSATSCHENKO, B. L., AND SALIMOWSKAJA, A. G. 1929 Proc. of the Intern. Congr. of Plant Sci., Ithaca, N. Y., **1**, 221–224.
JACOBSEN, H. C. 1912 Folia Microbiologica, **1**, 487–496.
JACOBSEN, H. C. 1914 Folia Microbiologica, **3**, 155–162.
KLEIN, G., AND LIMBERGER, A. 1923 Biochem. Zeit., **143**, 473–483.
KLEIN, G., AND SVOLBA, F. 1926 Zeit. f. Botanik, **19**, 65–100.
LANGE-POSDEEVA, I. P. 1930 Arch. d. Sci. Biol. (Russian), **30**, 189–201.
LIESKE, R. 1912 Ber. deutsche botan. Gesell., **30**, 12–22.
NATHANSOHN, A. 1902 Mitt. a. d. Zool. Sta. z. Neapel, **15**, 655–680.
NELSON, D. H. 1931 Centbl. Bakt. (II), **83**, 280–311.
ROUNTREE, P. M. 1933 Australian Jour. of Exp. Biol. and Med. Sci., **11**, 209–217.
SALIMOWSKAJA, A. G. 1931 Leningrad Microbiol. Lab. of Hydrobiol Inst., Mém. de l'Inst. Hydrol. (Russian), **4**, 37–48.
SASLAWSKY, A. S. 1927 Centbl. Bakt. (II), **72**, 236–242.
SASLAWSKY, A. S., AND HARZSTEIN, N. 1930 Centbl. Bakt (II), **80**, 165–169.
STARKEY, R. L. 1925a Jour. Bact., **10**, 135–163.
STARKEY, R. L. 1925b Jour. Bact., **10**, 165–195.
STARKEY, R. L. 1934a Soil Science. (In Press.)
STARKEY, R. L. 1934b Jour. Bact. (In Press.)
TJULPANOVA-MOSSEVITCH, M. W. 1930 Arch. d. Sci. Biol. (Russian), **30**, 203–214.
TRAUTWEIN, K. 1921 Centbl. Bakt. (II), **53**, 513–548.
TRAUTWEIN, K. 1924 Centbl. Bakt. (II), **61**, 1–5.
VAN NIEL, C. B. 1931 Arch. f. Mikrob., **3**, 1–112.
VAN NIEL, C. B., AND MULLER, F. M. 1931 Extrait du recueil d. travaux botan., **28**, 245–274.
WAKSMAN, S. A. 1922a Jour. Bact., **7**, 609–616.
WAKSMAN, S. A. 1922b Soil Sci., **13**, 329–336.
WAKSMAN, S. A. 1932 Principles of Soil Microbiology. The Williams & Wilkins Company, Baltimore, 2nd ed. Chap. 17.
WAKSMAN, S. A., AND IYER, K. R. N. 1932a Soil Sci., **34**, 43–69.
WAKSMAN, S. A. AND IYER, K. R. N. 1932b Soil Sci., **34**, 71–79.
WAKSMAN, S. A., AND STARKEY, R. L. 1923 Jour. Gen. Physiol., **5**, 283–310.
WAKSMAN, S. A., WARK, C. H., JOFFE, J. S., AND STARKEY, R. L. 1923 Jour. Agr. Res., **24**, 297–305.
WINOGRADSKY, S. 1922 Compt. Rend. Acad. Sci. (Paris), **175**, 301–304.