ON THE GROWTH AND RESPIRATION OF SULFUR-OXIDIZING BACTERIA.*

By SELMAN A. WAKSMAN and ROBERT L. STARKEY.

(*From the Department of Soil Chemistry and Bacteriology, New Jersey Agricultural Experiment Stations, New Brunswick.*)

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INTRODUCTION.

The respiration of sulfur-oxidizing bacteria is of particular interest, since this group of organisms belongs to the very few forms of life (autotrophic) which are able to obtain the carbon from the reduction of the CO_2 of the atmosphere and the necessary energy from the oxidation of inorganic substances, in this case sulfur compounds; in other words, it is able to assimilate carbon chemosynthetically, using inorganic sulfur and sulfur compounds as a source of energy. In the case of this group of organisms, accurate information can be obtained on the amount of chemical energy required in the process of building up complex organic substances from minerals and carbon dioxide. The simple composition of the nutrients will also help to throw light on the differentiation between the structural and functional energy required in metabolism as well as on other disputed questions of metabolism, such as the influence of concentration of nutrients, neutralization of waste products, and amount of total available energy utilized by the organism in connection with the carbon assimilation.

Respiration in the study of autotrophic bacteria is comparable with respiration of all the other forms of life in that it results in the liberation of energy necessary for the life activities of the organism. Respiration is commonly understood to mean the liberation of carbon dioxide as a result of the oxidation of organic matter by the organism, the addition of oxygen following a series of enzymatic reactions. In the case of sulfur oxidation, the energy is liberated as a result of direct combustion of the sulfur, as pointed out in 1887 by Winogradsky.

The recent investigations on the autotrophic bacteria brought out the fact that only very few organisms are strictly autotrophic; here belong the nitrifying bacteria, as pointed out by Winogradsky and others, particularly in the careful work of Meyerhof on the respiration and energy transformation of these organisms, and the sulfur organism under consideration. The methane, hydrogen, and iron bacteria are facultative autotrophic, or can exist also heterotrophically, as

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shown by Niklewski, Lieske (1912), and Beijerinck (1910). Of the sulfur bacteria, we know that at least some organisms, like the *Sulfomonas* (*Thiobacillus*) *denitrificans* of Beijerinck (1920) and the related form of Trautwein, can also exist heterotrophically. In other words, some forms are facultative autotrophic, or are capable of obtaining their energy both chemosynthetically and from organic matter. *Sulfomonas denitrificans* will oxidize sulfur compounds, using nitrate nitrogen, which results in the oxidation of the sulfur and the reduction of the nitrate (denitrification); about 1 Cal. is produced per gram of decomposed nitrate (Beijerinck (1920)).

The organism used in these experiments, *Sulfomonas* (*Thiobacillus*) *thiooxidans*, was isolated from sulfur floats (rock phosphate) soil composts, in which it is chiefly responsible for the oxidation of the elementary sulfur to sulfuric acid. It has been grown for nearly 2 years in inorganic media of simple composition, with elementary sulfur as the only source of energy, and it has not decreased in vitality and power of sulfur oxidation.

Sulfomonas thiooxidans will derive its energy from the oxidation of sulfides, thiosulfates, and primarily of elementary sulfur to sulfuric acid. It derives its carbon from carbon dioxide in the atmosphere, although it is not injured by the presence of organic matter and bicarbonates. The optimum reaction is distinctly acid, equivalent to pH 3.0 to 4.0. The presence of carbonates tending to make the medium alkaline are, therefore, injurious to the growth of the organism. Bicarbonates, particularly in the presence of sufficient buffering substances, like phosphates, may not be injurious. It has been generally assumed that the nitrifying bacteria derive their carbon from carbonates and bicarbonates. Meyerhof has demonstrated, however, that the presence of these substances is of importance, because they serve as buffers to keep the medium alkaline, since the optimum reaction for nitrifying bacteria lies at a pH of 8.0 to 9.0. When phosphates are substituted in place of carbonates, those bacteria are found to derive their carbon from carbon dioxide. The *Sulfomonas thiooxidans* having its optimum at a distinctly acid range will thrive very well in the complete absence of all traces of carbonates or bicarbonates. Like the nitrifying bacteria, it does not grow on organic media, unless sulfur is present as a source of energy, and is thus found to be unable to derive its energy from any organic materials. In this respect the facultative autotrophic *Sulfomonas denitrificans* studied by Beijerinck (1920) and Trautwein behaves differently since it is able to grow also on common organic media.

Winogradsky was the first to investigate the metabolism of the sulfur bacteria without, however, succeeding in isolating the organisms in pure culture. When a culture of *Beggiatoa* (thread-forming, colorless organism) is grown in a medium containing hydrogen sulfide, it is found to accumulate sulfur within its cells as a result of the oxidation of H_2S to H_2O and S. When left in the medium free from hydrogen sulfide, the sulfur granules present in the threads are found to disappear within 24 hours, presumably due to the oxidation of the sulfur to sulfuric acid; the medium, however, never became acid. This was explained by

the fact that the impure water was saturated with calcium carbonate, which neutralized the resulting acid. During the same period, the bacterial mass is almost doubled. Kruse calculated, from the data obtained by Winogradsky, that for the assimilation of 1 gm. of carbon, 8 to 19 gm. of sulfur have to be utilized. The reaction takes place as follows:

$$H_2S + O = H_2O + S$$
$$S + 3O + H_2O = H_2SO_4 + (142.3 \text{ Cal.})$$

Kruse suggested then that one atom of sulfur could liberate more energy than would be necessary for the building up of three atoms of carbon, or to synthesize aminopropionic acid from an equal weight of propionic acid and one atom of nitrogen (in the form of nitric acid).

As to the purple bacteria, it has been definitely demonstrated by Molisch that they are unable to assimilate carbon dioxide with a splitting of oxygen and that they thrive best in highly concentrated organic media.

Keil found that *Beggiatoa* and *Thiothrix* can exist in media entirely free from organic nitrogen, but are not injured by organic substances in not too high concentrations, with CO_2 as the only source of carbon; sufficient amounts of carbonates of alkali earths should be present to neutralize the acid formed from the oxidation of the sulfide.

The colorless sulfur bacterium isolated by Nathansohn and later described by Beijerinck as *Thiobacillus thioparus* (*Sulfomonas thioparus*) was found to be able to derive its carbon both from carbonate and from carbon dioxide of the atmosphere. This organism oxidizes thiosulfates and sulfide; the medium was not found to become acid in the absence of carbonates. This was explained by the fact that the thiosulfate is oxidized to sulfate and free sulfur and the sulfides to free sulfur.

$$Na_2S_2O_3 + O = Na_2SO_4 + S$$
$$H_2S + O = H_2O + S$$

This organism was found by Nathansohn to be able to obtain its carbon from carbonates. Trautwein, however, who studied a closely related organism which could use thiosulfate as the only source of energy, found that it obtained its carbon from carbon dioxide of the atmosphere or in solution, in the form of bicarbonates, but not from carbonates. This organism could also obtain its energy from organic compounds; in other words, it could exist both under autotrophic or heterotrophic conditions. The thiosulfate was oxidized to sulfate and higher sulfates, without an increase in the acidity of the medium and without sulfur separation. Beijerinck (1920) suggested that here we have an hereditary modification as a result of great changes in the nature of the food. Lieske (1912) found the denitrifying sulfur-oxidizing form capable of utilizing the carbonates of ammonium, calcium, magnesium, manganese, and sodium as sources of carbon, while free carbonic acid was not assimilated due to the high acidity of the medium. $Na_2S_2O_3$, $Na_2S_2O_6$, K_2S, H_2S, and finely divided sulfur can be used as sources of energy. For every gram of carbon assimilated, 100 gm. of $Na_2S_2O_3 \cdot 5H_2O$ are

oxidized. In the presence of an excess of nitrate the sulfur compounds were completely oxidized to sulfate. The sulfur was found to be first oxidized to lower acids, then to sulfuric acid.

EXPERIMENTAL.

Culture.—The organism used in the following investigations (*Sulfomonas thiooxidans*) is a minute, non-spore-forming, rod-shaped to oval, unicellular, colorless organism, measuring about 0.5 to 1 micron long and about 0.5 micron wide, described in detail elsewhere (Lipman, Waksman, and Joffe (1921), and Waksman and Joffe (1922, *b*)). It oxidizes elementary sulfur quantitatively to sulfuric acid; thiosulfates are completely oxidized to sulfates, without the secretion of elementary sulfur. It acts also, but to a lesser extent, on sulfides, oxidizing them to sulfates.

The medium of the following composition was used:

$(NH_4)_2SO_2$	0.2	gm.
$MgSO_4$	0.1–0.5	"
$FeSO_4$	0.01	"
$CaCl_2$	0.25	"
KH_2PO_4	3.0–5.0	"
Powdered sulfur	10.0	"
Distilled water	1,000.0	cc.

In the experiments on carbon assimilation, the water was distilled twice, the second time with glass containers and condensers only, in a manner similar to that used for preparing conductivity water. The sulfur is weighed out separately into the individual flasks, previously cleaned with potassium bichromate and sulfuric acid. The medium is placed, in 100 cc. and 300 cc. quantities, in 250 cc. and 1 liter Erlenmeyer flasks respectively, which are then plugged with cotton and sterilized in flowing steam, for 30 minutes, on 3 consecutive days, and all manipulations of the culture were carried out under sterile conditions. Sterility is not necessary with a well growing culture, since the medium will not allow any appreciable development of other microorganisms. Transfers were always made by means of a sterile platinum loop or sterile pipette, into fresh sterile flasks of medium or into the containers for analysis. A vigorously growing 7 day old culture was always used for inoculating the experimental flasks. The cultures were incubated at 26–28°C. for the desired length of time.

Methods of Analysis.—The reaction is determined both by the method of titration and by the hydrogen ion concentration. In titrating, 5 or 10 cc. of the culture were titrated with 0.1 N NaOH, using phenolphthalein as an indicator. Titer designates the amount of 0.1 N NaOH required to neutralize 1 cc. of the culture. The hydrogen ion concentration was determined by means of the Clark and Lubs series of indicators and checked carefully by an electrometric apparatus, consisting of a modification of the Hildebrand apparatus, in which the Clark vessel is used. However, at very high hydrogen ion concentrations (at pH less

than 1.0), which are readily obtained in the culture of the organism, the titer was found to be a more reliable index of the amount of acid formed, since both the colorimetric and electrometric methods for determining the H ion concentrations were found to be not sensitive enough with the apparatus available. The sulfates were determined by precipitating an aliquot filtered portion of the culture with $BaCl_2$, washing and weighing the $BaSO_4$; the residual sulfur was obtained by filtering the culture carefully through a weighed Gooch crucible, washing with a large amount of slightly acidulated water and drying to constant weight.

The total carbon of the culture was determined according to the following procedure. A wet combustion apparatus, similar to the one described by Gortner and to the one used by Meyerhof, was used (Fig. 1). A set of KOH bulbs, *A*, containing a 1:1 solution of

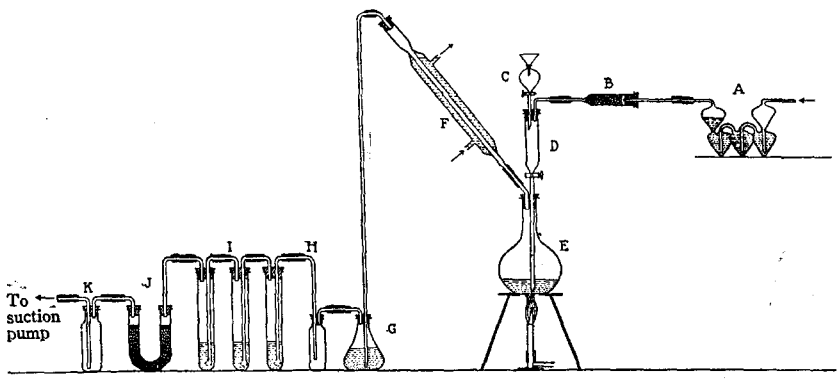

FIG. 1. Apparatus for carbon determinations.

KOH and a soda-lime tube, *B*, served to remove all CO_2 from the entering air. 100 cc. of the unfiltered mixed culture were introduced into a 500 cc. Pyrex flask, *E*, where the digestion took place. Concentrated sulfuric acid was added through the separatory funnel, *C*, into the wide mouthed separatory funnel, *D*, through which it entered into *E*. The condenser, *F*, led to a 250 cc. Erlenmeyer flask, *G*, containing a saturated solution of Ag_2SO_4 in 5 per cent H_2SO_4. This solution served to remove the chlorides from the gas. The bottle, *H*, is a trap to catch any of the standard alkali solution in case of back suction. The absorption of the CO_2 was carried out in *I*, consisting of a series of three large hard glass test-tubes of about 70 cc. capacity, each containing 25 cc. of standardized approximately

0.05 N $Ba(OH)_2$. The glass tubes through which the gas was passing were provided at the bottom with perforated bulbs similar to the Folin aeration tubes. *J* contained soda-lime to prevent any reversed diffusion of CO_2. *K* is a trap to catch any water from the water pump in case of back suction due to lowered water pressure.

The determinations were carried out as follows: A 100 cc. portion of the solution to be analyzed was placed in the combustion flask and 2.5 gm. $KMnO_4$ were added. After attaching the flask, suction was applied and 50 cc. of concentrated H_2SO_4 were very slowly admitted through the separatory funnel *D*. It took about half an hour to add this acid, as considerable heat was generated accompanied by a rather rapid evolution of gas. The stop-cock at *D* was then closed and the solution heated to gentle boiling with a small flame. After the gas had practically all been driven from the flask, as indicated by a backing up of the solutions, the stop-cock, *D*, was opened and the apparatus aerated without heat for about 30 minutes. A stream of air could be drawn rather rapidly through the apparatus. Heat was then again applied with the stop-cock closed, followed by further aeration; the two processes were then again repeated. The whole determination required about 3 hours. This period of time was found to be sufficient for the complete combustion of the carbon and the removal of the carbon dioxide. The $Ba(OH)_2$ was then titrated back with standard, approximately 0.05 N oxalic acid, with phenolphthalein as an indicator. The solutions were standardized daily. Two duplicate determinations were always made on the same culture.

The accuracy of the method was checked up by determining the carbon content of saccharose and urea, whereby 95 to 98 per cent of the theoretical amount of carbon was obtained.

Chemistry of Sulfur Oxidation.

By determining the sulfur and sulfates in the inoculated and uninoculated flasks, it is found that the sulfur is almost quantitatively transformed into sulfuric acid, as shown in Table I. In all cases, the amount of sulfur obtained in the form of sulfate was found to be greater than the loss in elementary sulfur unoxidized, due probably

to some inaccuracy in figuring the loss of moisture during the incubation period.

This would lead us to conclude that the sulfur is oxidized, by the microorganism in question, according to the following reaction, without any intermediary reactions taking place.

$$S + 3O + H_2O = H_2SO_4$$

When thiosulfate is used as a source of energy, it is found to be oxidized completely to sulfates, with a corresponding increase in acidity. By using 5 gm. of $Na_2S_2O_3 \cdot 5H_2O$ per liter and incubating the cultures for 30 days, the reaction of the culture changed from pH

TABLE I.

Oxidation of Elementary Sulfur to Sulfuric Acid by Sulfomonas thiooxidans.

Incubation.	Amount of culture in flask.	Control flask.		Inoculated flasks.		Elementary sulfur disappeared.	Increase in sulfate.
		Sulfur.	Sulfate.*	Sulfur.	Sulfate.		
days	*cc.*	*mg. of S*	*mg. of S*	*mg. of S*	*mg. of S*	*mg. of S*	*mg. of S*
15	100	1,001	86.4	788	302.1	213	215.7
30	100	992	90.5	735	354.0	257	263.5
15	300	3,002	112.2	2,496	633.0	506	520.8
30	300	2,997	126.5	1,974	1,168.0	1,023	1,041.5

* Mg. of soluble sulfates as sulfur in flask; averages of 3 flasks are given; the concentration of sulfates in the small flasks was greater, due to the fact that, in these, a medium containing 2 gm. $(NH_4)_2SO_4$ and 0.5 gm. $MgSO_4$ per liter was used.

5.0 to pH 1.8, the thiosulfate completely disappeared, and the sulfate increased from 12.60 mg. of S as sulfate in 100 cc. of medium to 142.73 mg.; in other words, all the thiosulfate was transformed into sulfate (the theoretical content of total sulfur in 100 cc. of medium is 141.6). The reaction should, therefore, be written as follows:

$$Na_2S_2O_3 + 4O + H_2O = Na_2SO_4 + H_2SO_4 \qquad (1)$$

It may be recalled, in this connection, that the sulfur organism of Nathansohn did not change the reaction to acid. He therefore suggested for his organism the following reaction:

$$3\,Na_2S_2O_3 + 5O = 2\,Na_2SO_4 + Na_2\,S_4O_6 \qquad (2)$$

The reaction did not account for the precipitation of sulfur by the organism from the thiosulfate. Beijerinck therefore suggested the following reaction:

$$Na_2S_2O_3 + O = Na_2SO_4 + S \qquad (3)$$

The organism of Trautwein oxidized thiosulfate without the separation of sulfur, as follows:

$$7\ Na_2S_2O_3 + 17O = 2\ Na_2SO_4 + Na_2S_4O_6 + 4\ Na_2S_2O_6 \quad (4)$$

The difference in the four reactions will account for the fact that different organisms were used by the various workers and that the organism under consideration (*Sulfomonas thiooxidans*) did not precipitate any sulfur and caused a definite increase in acidity; the organism of Nathansohn and Beijerinck (*Sulfomonas thioparus*) caused an intense precipitation of free sulfur and no increase in acidity; the organism of Trautwein (*Sulfomonas denitrificans* var.) caused no precipitation of sulfur and no acid formation.

Carbon Assimilation.

Qualitative.—Sterile 100 cc. portions of the regular medium in 250 cc. Erlenmeyer flasks were inoculated as usual and placed under bell jars, sealed around with a mixture of rubber and paraffin to prevent any leakage. The air aspirated through the jars was passed first through soda-lime tubes, then through a 50 per cent solution of KOH to free the air from carbon dioxide. Some flasks received 0.1 per cent $NaHCO_3$ and others 0.1 per cent dextrose. A duplicate series of flasks were placed outside of the apparatus on the incubator shelves. After 5 days incubation, the amount of sulfur oxidized in the various flasks, as shown by change in reaction, was determined. The results are presented in Table II.

It can be seen from the above experiments that the organism does not grow in a CO_2-free atmosphere and it cannot derive its carbon from dextrose. The slight increases in acidity are probably due to the acid introduced with the inoculum and perhaps imperfect evacuation of chamber the first day. In the presence of bicarbonate, the oxidation of sulfur is greatly reduced due to the unfavorable reaction, but there was a definite amount of growth and sulfur oxidation in

the CO_2-free atmosphere. This experiment was repeated again and it was definitely established that *Sulfomonas thiooxidans* can derive its carbon only from carbon dioxide of the atmosphere but not from organic materials; in the presence of carbon dioxide in solution, and in the form of bicarbonates, growth will also take place if the reaction is favorable for the activities of the organism.

Quantitative.—300 cc. portions of the medium and 3 gm. of sulfur were placed in a series of 1 liter flasks, sterilized and inoculated as usual. Some of the flasks were uninoculated and left as controls. At the end of various periods of incubation, one or two flasks were taken out for analysis. Two 100 cc. portions of the culture from each flask were used for duplicate carbon determinations; the rest of the

TABLE II.

Influence of CO_2 upon the Growth of Sulfomonas thiooxidans.

Control.	Ordinary atmosphere.						CO_2-free atmosphere.					
	Regular medium.		0.1 per cent $NaHCO_3$		0.1 per cent dextrose.		Regular medium.		0.1 per cent $NaHCO_3$		0.1 per cent dextrose.	
	pH	Titer.	pH	Titer.	pH	Titer.	pH	Titer.	pH	Titer.	pH	Titer.
Uninoculated.....	4.4	0.23	6.8	0.12	4.4	0.23	4.4	0.23	6.8	0.12	4.4	0.23
Inoculated...	1.4	1.05	2.8	0.36	1.5	0.92	3.8	0.25	6.0	0.20	3.3	0.25

culture was filtered through analytical filter paper; two 25 cc. portions of the filtrate were used for sulfate determinations, and the rest was used for titration and determination of the hydrogen ion concentration. The results are tabulated in Table III.

The course of sulfur oxidation and carbon assimilation is uninterrupted. Nearly one-third of the sulfur present in the medium is oxidized in 42 days, with the reduction of the hydrogen ion concentration of the medium to a pH of 1.0 or less and with the formation of titrable acidity equivalent to fourth-normal (0.25 N) acid. The initial titration of the control is due to the buffer content of the medium (5 gm. KH_2PO_4 per liter). The ratio of the sulfur oxidized to the carbon assimilated varies from 28.0 at the beginning of the active growth period to 34.22, falling down to 31.25, at the end of the experiment, with an average of 31.82 parts of sulfur oxidized for

TABLE III.

Course of Sulfur Oxidation and Carbon Assimilation.

Age of culture.	Total carbon, as mg. of C in 100 cc.				Total sulfate, as mg. of S in 100 cc.				$\frac{S^*}{C}$	pH	Titer 1 cc. = cc. of 0.1 N NaOH.
	1	2	Average.	Blank subtracted.	1	2	Average.	Blank subtracted.			
					Uninoculated.						
days											
0	2.318	1.885	2.102		9.51	9.67	9.59			4.0	0.40
13	2.349	1.913	2.131		11.19	11.27	11.23			3.9	0.41
43	2.167	2.087	2.127		11.60	11.96	11.78			4.0	0.42
Average			2.120				10.87			4.0	0.41
					Inoculated.						
5	4.725	4.680	4.703	2.583	83.272	84.320	83.296	72.426	28.04	2.0	0.90
9	5.201	5.201	5.201	3.081	111.214	111.274	111.244	100.374	32.58	1.5	1.04
12	6.202	6.754	6.478	4.358	160.016	160.016	160.016	149.146	34.22	1.3	1.37
19	8.917	9.606	9.262	7.142	253.150	253.202	253.176	242.306	33.94	1.2	2.02
21	10.164	9.405	9.785	7.665	252.062	252.098	252.080	241.210	31.47	1.2	2.00
27	11.156	11.857	11.507	9.387	303.842	303.788	303.815	292.945	31.21	1.2(?)†	2.20
42	11.528	12.389	11.959	9.839	318.376	318.404	318.390	307.520	31.25	1.0(?)†	2.50
Average									31.82		

$\frac{*S}{C}$ = ratio of sulfur oxidized to carbon assimilated.

† Determination inaccurate due to high acidity.

every gram of carbon assimilated from the carbon dioxide of the atmosphere. The somewhat low initial ratio may be due to the fact that in the early period of growth, when carbon assimilation is most active, the energy obtained is utilized most economically.

It is interesting to note in this connection that the $\frac{N}{C}$ ratio for nitrite-forming bacteria (Meyerhof) was found to be 35, while for nitrate bacteria 135, but in view of the fact that the heat liberated in the second process is about one-fourth of that liberated in the first,

TABLE IV.

Assimilation of Carbon by Sulfomonas thiooxidans with Thiosulfate as a Source of Energy.

	Total carbon, mg. of C in 100 cc.		Total sulfate, mg. of S in 100 cc.		$\frac{S}{C}$	pH	Titer.
	Determinations.	Average.	Determinations.	Average.			
Control.	1.663		11.37				
	1.998		12.85				
	1.705	1.789	16.70				
			14.55	13.87		4.9	0.14
Inoculated.	3.577		130.98				
	3.284		130.49				
	3.965		135.38				
	3.764	3.648	135.87	133.18		1.85	0.53
Control subtracted.		1.859		119.31	64.2		

the utilization of energy, in both processes, for chemosynthetic purposes, is identical.

With $Na_2S_2O_3$ as a source of energy, Lieske (1912) found that 10.9 mg. of carbon were assimilated by *Sulfomonas denitrificans* for every gram of thiosulfate oxidized; in other words, the oxidation of 405 mg. of sulfur in the form of thiosulfate will allow an assimilation of 10.9 mg. of carbon chemosynthetically, with $\frac{S}{C} = 37.2$. For the study of the utilization of thiosulfate as a source of energy by *Sulfomonas thiooxidans*, 5 gm. of $Na_2S_2O_3 \cdot 5H_2O$ were used, in place of the 10 gm. of sulfur, per liter of medium. The flasks were incubated for 16 to 20 days. The results are recorded in Table IV.

The theoretical amount of sulfur as thiosulfate in the medium is 129.0 mg. in 100 cc.; 119.31 mg. were found in the form of sulfate. The higher sulfur-carbon ratio is due in this case to the fact that there is less energy liberated from the oxidation of one unit of sulfur in the form of thiosulfate than in the form of elementary sulfur.

Heat Formation and Sulfur Oxidation.—As pointed out above, the oxidation of sulfur by *Sulfomonas thiooxidans* takes place according to the following reaction:

$$S + 30 + \underset{68.5}{H_2O} = \underset{210.8}{H_2SO_4} + 142.3 \text{ Cal.}^{1}$$

In other words, from the oxidation of 1 mol or 32 gm. of sulfur, 142.3 Cal. will be liberated. It takes 113 Cal. to transform 1 mol of CO_2 into dextrose; or to assimilate 12 gm. of carbon, 113 Cal. are required. But in the process of assimilation of 12 gm. of carbon, 31.82 times as much sulfur has to be oxidized, or $31.82 \times 12 = 381.84$ gm. This quantity of sulfur will liberate $\frac{381.84}{32} \times 142.3 = 1{,}698$ Cal. Of this total heat liberated, only 113 Cal., or 6.65 per cent, are utilized for the assimilation of carbon.

The percentage of energy made available from the oxidation of sulfur and utilized by the organism for chemosynthetic purposes, *i.e.* carbon assimilation from the CO_2 of the atmosphere, is found to be much higher than in the case of higher plants, which are variably estimated to utilize 0.5 to 3 per cent of the energy of rays of the sun. The much more favorable utilization of the energy for the chemosynthetic carbon assimilation than for the photosynthetic has also been pointed out for nitrifying bacteria by Meyerhof, who found that both the nitrite and nitrate formers utilize about 5 per cent of the energy liberated for the chemosynthetic assimilation of carbon.

The calorific quotient of oxygen is found, in the sulfur oxidation, to be $\frac{141.5}{3 \times 16} = 2.95$; or for every milligram of oxygen consumed in the reaction, 2.95 cal. of heat are liberated. In this case it approaches the calorific quotient of oxygen in the combustion of organic substances which is 3.0 to 3.5 cal. for 1 mg. of oxygen and is much higher than

[1] Data are taken from Landolt-Börnstein tables, 4th edition.

that obtained in nitrite and nitrate formation, which is only about 1.35 to 1.75 cal.

Concentration of Sulfates and Sulfur Oxidation.—Various investigators (Meyerhof) called attention to the fact that the accumulation of the oxidation product by nitrite and nitrate bacteria injures growth of the organisms and respiration. Trautwein could not demonstrate any injurious effect of sulfate upon the growth of sulfur-oxidizing bacteria; even where as much as 5.0 per cent Na_2SO_4 has been used, no injurious influence of the oxidation product was found. NaCl,

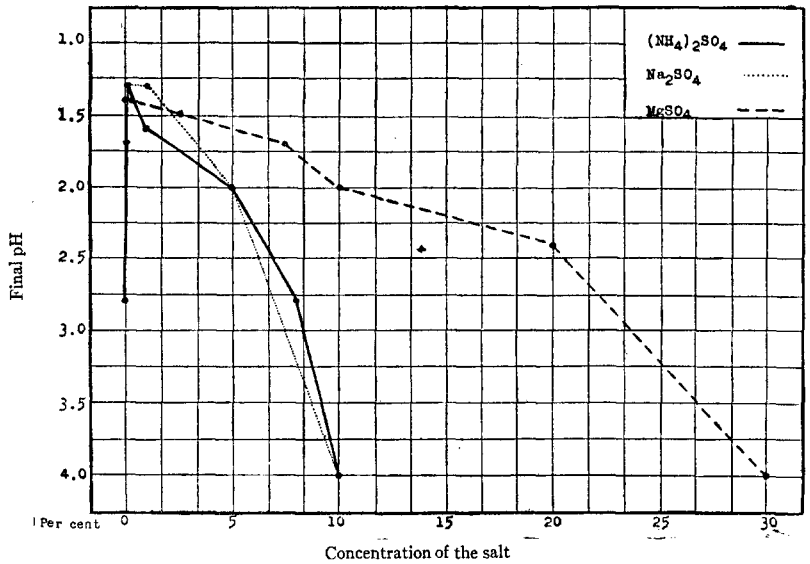

FIG. 2. Effect of concentration of Na, (NH_4), and Mg sulfates on sulfur oxidation by *Sulfomonas thiooxidans*.

however, had an injurious influence, which was explained by the influence of the Cl-ion.

Three different sulfates, $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$ (the influence of sulfuric acid is reported later), have been tested as to their influence on sulfur oxidation by *Sulfomonas thiooxidans*, and in no case could the injurious influence be ascribed to the sulfate-ion, growth coming to a standstill only with very high concentrations. Where an influence has been found, it was due more to the influence of the cation and not to the oxidation product, as seen from Fig. 2. Calcium sulfate was found to be entirely uninjurious to the growth of the organism;

TABLE V.

Influence of Salt Concentration upon Sulfur Oxidation by Sulfomonas thiooxidans.

Concentration.	0		0.01		0.1		1		2.5		5		10		20	
	pH	S*	pH	S	pH	S	pH	S	pH	S	pH	S	pH	S	pH	S
per cent																
KH_2PO_4	1.0	147.17			1.2	181.15			2.8	49.63	4.0		4.0	0		
$MgSO_4$	1.2	282.8			1.2		1.2	271.8			1.6	205.5	1.3	280.9	1.9	41.1
$(NH_4)_2SO_4$	2.8		1.7		1.3		1.6				2.0					
Na_2SO_4	1.2	336.4					1.2	300.8			1.4	112.4	2.0	35.2		
NH_4Cl					1.0	249.9			1.4	114.5			4.0	0	4.0	
NaCl	1.2	260.3					1.2	277.3			1.6	75.6	4.0	0	4.0	
$MgCl_2$	1.2	252.0					1.0	310.6			1.2	162.7	1.8	34.4	4.0	
$NaNO_3$			1.3	112.4	1.3	110.4	2.6	10.3	3.8	0	3.8	0				
$Ca(NO_3)_2$			1.3	118.9	1.3	102.1	2.6	12.4	3.6	0.45	3.8	0				
$KAl(SO_4)_2$			1.2		1.2				1.4							

* S = milligrams of sulfur oxidized to sulfate in 100 cc. of medium; pH of control medium = 4.0; cultures incubated 7 days.

in view of the fact that this salt is soluble only in small amounts, large amounts of it can be added without appreciable injury to the culture.

To compare the influence of sulfates and other anions as well as cations upon the oxidation of sulfur, a series of experiments was carried out, and the results are summarized in Tables V and VI. The results indicate that, among the anions, the sulfate and chloride are uninjurious in low concentrations and become injurious only in concentrations of 0.2 to 0.5 molar, the nitrate being most toxic. The phosphates seem to become toxic in lower concentrations than

TABLE VI.

Salt Concentrations Which Limit Growth of Sulfomonas thiooxidans.

	Molar Concentration.	
	No growth.	Growth.
H_2SO_4	1.00	0.50
$MgSO_4$	1.20	0.80
$(NH_4)_2SO_4$	0.80	0.60
Na_2SO_4	0.75	0.60
$MgCl_2$	1.00	0.50
NH_4Cl	1.00	0.50
$NaCl$	0.75	0.50
$CaCl_2$	0.75	0.50
$NaNO_3$	0.20	0.15
KNO_3	0.15	0.10
$Ca(NO_3)_2$	0.20	0.10
KH_2PO_4	0.50	0.30

the sulfates and the chlorides, but to a much smaller extent than the nitrate. The injurious action of the nitrate is exerted both on the growth of the organism, as indicated by the smaller amount of carbon assimilated in proportion to the sulfur oxidized, and on the respiration of the organism, as will be shown later.

Concentration of Phosphates and Sulfur Oxidation.—In the study of sulfur oxidation, a buffer has to be employed in the medium, otherwise the reaction will rapidly become acid. The phosphates form an excellent buffering agent for the study of the activities of this organism, particularly because they are of inorganic nature and can be adjusted on the acid side of neutrality. The concentration of

phosphates on the sulfur oxidation has also an important practical application in the composting of sulfur, rock phosphate, and soil, whereby the sulfuric acid formed from the oxidation of sulfur by the organism acts upon the rock (tri-calcium) phosphate converting it into mono-calcium phosphate. The influence of phosphates is found to become injurious in concentrations of 0.3 M, as shown in Fig. 3.

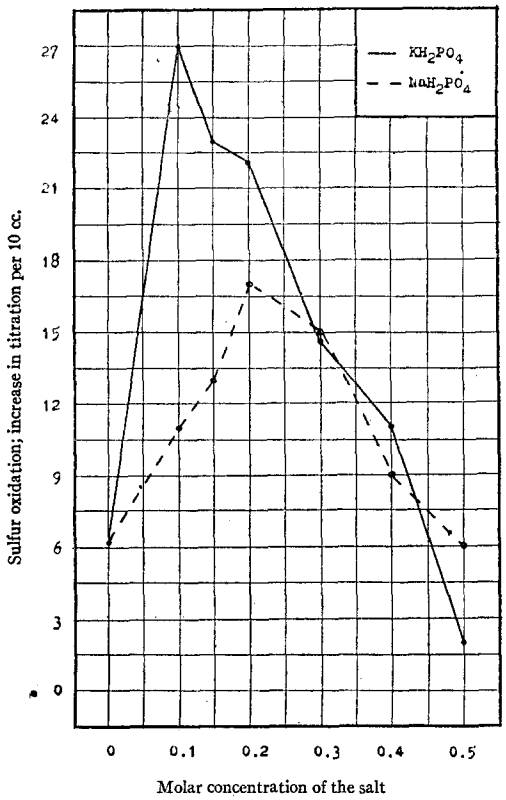

FIG. 3. Influence of phosphate concentration upon sulfur oxidation.

It is interesting to note that there is (Table VII) a gradual acclimatization of the organism to the concentration of the salt, with a prolonged period of incubation.

Influence of Sulfur Concentration upon Sulfur Oxidation.—With most cells it is very difficult to study the relation between concentration of nutrient and velocity of transformation due to the fact that the cells may be able to use reserve substances, or due to the difficult

differentiation between the amount of nutrient used for energy and that used for structural metabolism. The simpler the chemical composition of the nutrient the easier is the subject for investigation, particularly in the case of organisms where structural metabolism is distinct from energy utilization and where there are none or mere traces of the reserve materials, as in the case of nitrifying and sulfur-oxidizing bacteria.

Various amounts of sulfur were added to 100 cc. portions of medium, then sterilized as usual. At the end of 10 days incubation, the

TABLE VII.

Influence of Concentration of Phosphates on the Oxidation of Sulfur by Sulfomonas thiooxidans.

Molar concentration.	KH_2PO_4				NaH_2PO_4			
	6 days.	9 days.	15 days.		6 days.	9 days.	15 days.	
	pH*	pH	pH	Titer.†	pH	pH	pH	Titer.
None.	1.2	1.2−	1.2−	0.62				
0.10	2.4	1.55	1.2	2.7	2.4	2.0	1.7	1.1
0.15	3.0	2.0	1.3	2.3	2.4	1.9	1.7	1.3
0.20	3.0	2.0	1.4	2.2	2.5	1.9	1.65	1.7
0.30	3.3	2.45	2.0	1.4	2.9	2.25	1.9	1.5
0.40	3.9	3.2	2.4	1.1	3.9	3.6	2.4	0.9
0.50	4.2	4.2	3.6	0.2	4.2	3.4	2.6	0.6

* pH of control media is about 4.2.

† Titer = increase in titratable acidity over control, in terms of cc. 0.1 N NaOH for 1 cc. of culture.

amount of sulfur oxidized was determined and the results are presented in Table VIII and Fig. 4.

The curve (Fig. 4) obtained for the correlation between sulfur concentration and oxidation velocity is exactly the same as that found by Harden, for the correlation between sugar concentration and fermentation velocity by yeasts, and by Meyerhof, for the relation between nitrite concentration (in small amounts) and nitrate formation.

Meyerhof obtained the maximum velocity with 0.1 per cent $NaNO_2$, due to the fact that increasing concentrations of the nitrite become toxic to the organism. In the case of *Sulfomonas thiooxidans*, there is no injurious effect from the increase of the sulfur oxidation

TABLE VIII.

Relation between Sulfur Concentration and Oxidation Velocity.

Amount of sulfur per 100 cc. of medium.	Sulfur oxidized to sulfate.	Increase in titer.
gm.	*mg. of S*	
0.025	19.91	0.12
0.100	65.70	0.41
0.500	189.67	1.20
1.000	210.49	1.34
2.000	264.93	1.70
5.000	390.55	2.51
10.000	413.41	2.68
25.000	504.93	3.25
50.000	502.93	3.28

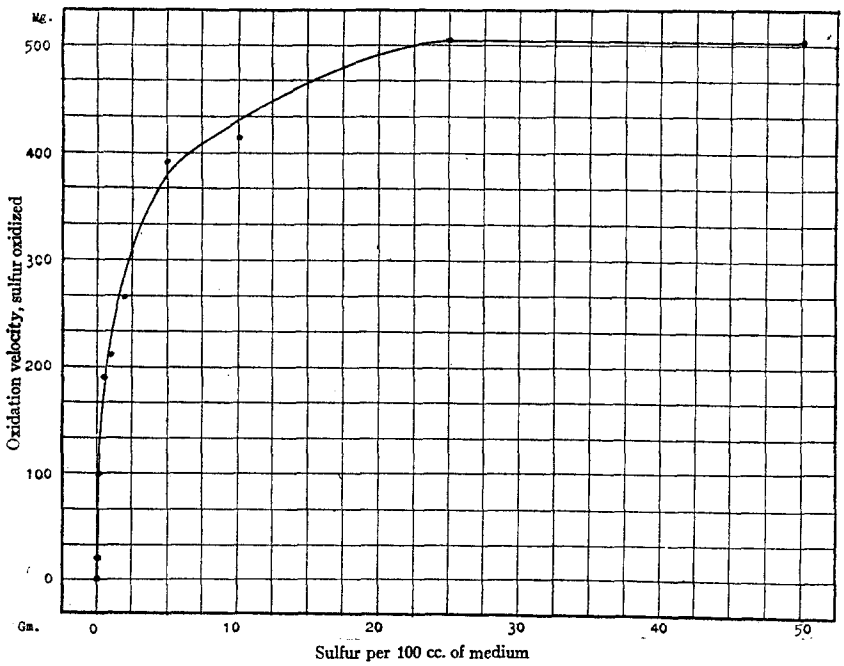

FIG. 4. Sulfur concentration and oxidation velocity.

even above the maximum which is about 25 per cent. It is important to note that the same interrelationship exists between the respiratory power of an organism and source of energy present in solution and that of an organism and source of energy existing in a solid phase. The very large surface exposed by the powdered sulfur (about 100 mesh) to the action of the organism seems to make the conditions similar.

TABLE IX.

Influence of Dextrose and Peptone on Sulfur Oxidation by Sulfomonas thiooxidans.

Dextrose.	pH	Sulfate as S in 100 cc.
per cent		
Control.	4.0	11.20
0	1.4	176.22
0.02	1.4	170.97
0.20	1.4	164.40
1.00	1.4	194.54
5.00	1.4	179.20
10.00	2.8	24.00
20.00	3.8	12.30
Peptone.	**pH**	**Sulfate as S in 100 cc.**
per cent		
0.01	1.4	188.70
0.1	2.0	83.60
1.0	2.8	19.40
2.5	4.0	11.20

Influence of Dextrose and Peptone on Sulfur Oxidation.—Winogradsky and Meyerhof pointed out that glucose will injure growth of nitrifying bacteria in 0.001 molar solution, but respiration is not affected by 0.2 molar concentration and is only injured 40 per cent in 0.6 molar (22 per cent). To amino compounds, respiration and growth are equally sensitive.

By adding various amounts of dextrose and peptone to 100 cc. quantities of media, sterilizing and inoculating in the regular manner, the following results were obtained after 7 and 14 days incubation, as shown in Table IX.

The organism is not injured in its activities by dextrose up to 1 per cent concentration. At 5 per cent, a decided injurious effect sets in. However, it takes nearly 20 per cent concentration of dextrose to stop growth and sulfur oxidation. Peptone injures the sulfur-oxidizing power of the organism even in small amounts. The uninjurious and even slightly stimulating effect of dextrose upon the respiration of the organism and the toxic effect of peptone and amino compounds has been pointed out elsewhere (Waksman and Starkey).

TABLE X.

Influence of Dextrose on the Carbon Assimilation by Sulfomonas thiooxidans.

	Dextrose.		
	Control.	Inoculated.	
		Flask 1.	Flask 2.
C in 100 cc., *mg*	22.94 24.60	25.61 25.77	29.45 28.32
Average	23.77	25.69	28.88
Increase over control		1.92	5.11
Sulfate as S in 100 cc., *mg*	10.26 9.88	87.93 85.51	200.79 203.64
Average	10.07	86.72	202.22
Increase over control		76.65	192.15
$\frac{S}{C}$		39.9	37.6
pH	3.6	1.7	1.2
Titer	0.16	0.67	1.41

To learn whether the presence of dextrose in the medium leads to a diminution in the carbon assimilation from carbon dioxide in the atmosphere, the organism was grown in the presence of small amounts (0.066 per cent) of dextrose for 10 and 16 days. The preparation of culture and cultivation of organism were followed by the procedure previously outlined. The results are given in Table X.

The S:C ratio, in the presence of dextrose, is thus found to be 38.8, or somewhat higher than the normal ratio, which is only 31.82. This

is not due to the assimilation of carbon from the dextrose, but to a natural variability of the growth of the organism or to a disturbed change in the metabolism of the organism. Any substance, whether of an organic or an inorganic nature, stimulating or injuring growth, effects the sulfur-carbon ratio, as will be further shown in the case of the nitrate. We can thus again confirm the fact that dextrose is not used either as a source of carbon or as a source of energy by the organism. This is true also of other carbohydrates and proteins, as found in the case of peptones.

Influence of Nitrates on Sulfur Oxidation.—It has been found in various studies carried out in this laboratory[2] that nitrates exert a depressive action upon sulfur oxidation, both in soil and in solution. As shown in Table VI, a concentration of 0.15 to 0.20 molar of the various nitrates is sufficient to repress completely the growth of *Sulfomonas thiooxidans*. This depressive action of the nitrate is the same when ammonium sulfate is present in the medium, indicating that the nitrate is directly toxic to the organism and not merely that it is an unfavorable nitrogen source. This action is exerted both on the growth and the respiration of the organism. By determining the sulfur-carbon ratio in the presence of nitrate, it is found to be more than twice as large as the normal ratio, indicating a distinct injurious effect upon the amount of carbon assimilated for a certain quantity of sulfur oxidized. By adding the nitrates to a fully grown culture and determining their influence on the respiration of the organism, by a method described elsewhere (Waksman and Starkey), it was found that 0.025 molar concentration of the various nitrates will repress respiration to about 25 per cent of normal, 0.05 molar to about 10 per cent, and 0.1 molar will repress respiration completely.

Influence of Reaction upon the Activities of the Sulfur-Oxidizing Organism.—Attention has been called to the fact elsewhere that the optimum reaction for the activities of *Sulfomonas thiooxidans* is at pH of 2.0 to 4.0 with limiting reactions at 6.5 and 0.6. However, this is true of the culture kept on artificial culture media, of a distinctly acid reaction, for a number of generations.

When freshly isolated, particularly from a neutral or slightly alkaline soil where sulfur oxidation has taken place, it may grow readily in

[2] Particularly in the work of Allison (to be published).

neutral or slightly alkaline reactions, although when isolated from acid soils, it seems to have as narrow a range of reaction.

The question of influence of reaction, as expressed in terms of hydrogen ion concentration as well as total concentration of acid, was studied both by adding different quantities of sulfuric acid to the regular medium (Table XI, Fig. 5) and by making up a series of media of various buffer contents. Molar concentrations of H_3PO_4, KH_2PO_4,

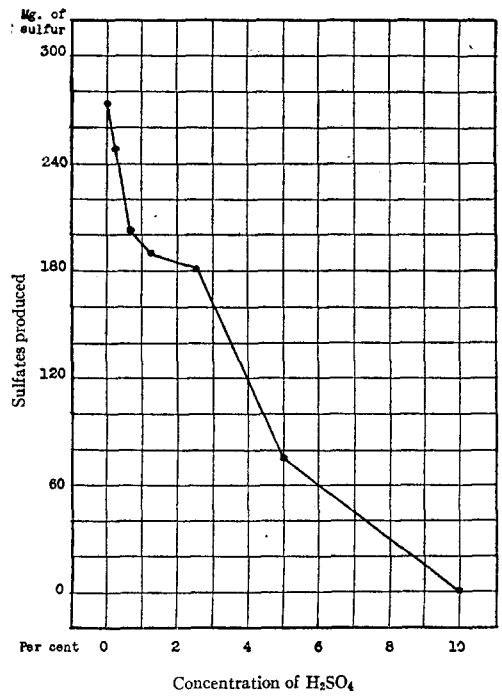

FIG. 5. Influence of concentration of H_2SO_4 on the growth of *Sulfomonas thiooxidans*.

K_2HPO_4, and NaOH were used, and 25 cc. of the proper buffer mixture were added to 475 cc. of the regular medium (free from phosphate) to give the range of reactions (Fig. 6).

The large concentrations of sulfuric acid that the organism can stand without injury are of interest. The optimum growth takes place when no acid is originally added to the medium (Table XI). This is in accord with the law of mass action. The organism still

grows when the medium contains 0.5 molar concentration of sulfuric acid, equivalent to nearly 5 per cent concentration of the acid. The growth in 0.25 molar concentration is almost near the optimum and, in many instances, no better sulfur oxidation is obtained even in normal media. The concentrations of sulfuric acid that the or-

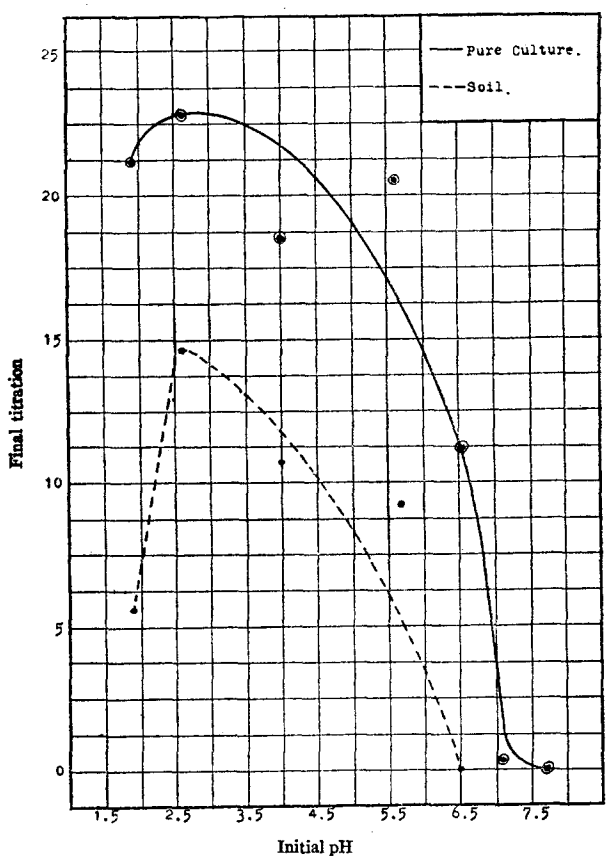

FIG. 6. Correlation between the hydrogen ion concentration of the medium and the growth of *Sulfomonas thiooxidans*.

ganism can stand without being destroyed are above 0.5 molar. In some cases, particularly when 10 to 25 parts of sulfur are added to 100 cc. of medium, sufficient acid is produced from the oxidation of sulfur to be equivalent to 1.5 normal alkali; the organism remains alive and capable of further oxidation even in these acid concentrations.

Growth and Respiration.—It was pointed out elsewhere (Waksman and Joffe (1922, *a*)) that the course of sulfur oxidation by *Sulfomonas thiooxidans* obeys the law of autocatalysis. When a culture is taken at the maximum period of its growth, and filtered free from sulfur, it can be used very readily for studies of respiration, in a manner similar to those carried out by Meyerhof for nitrifying bacteria. This method is usually carried out as follows: The organism is grown for 3 to 5 days, on the regular medium, in large 1 liter flasks, till a prominent turbidity is produced indicating active development. The culture is then filtered through paper free from sulfur. 50 cc. portions of the culture are then added to 1 gm. portions of sulfur in small flasks and incubated

TABLE XI.

*Influence of Concentration of H_2SO_4 upon the Oxidation of Sulfur by Sulfomonas thiooxidans.**

Approximate molar concentration of H_2SO_4.	Titer per 1 cc. 0.1 N alkali.			Sulfate oxidized as sulfur in 100 cc.		
	Control.	Inoculated.	Increase.	Control.	Inoculated.	Increase.
0	0.58	2.70	2.12	13.2	288.2	275.0
0.025	1.07	2.89	1.82	100.3	349.6	249.3
0.062	1.80	3.29	1.49	242.7	443.9	201.2
0.125	3.03	4.45	1.42	474.8	665.0	190.2
0.250	5.89	6.85	0.96	844.3	1,026.8	182.5
0.500	10.18	10.75	0.57	1,559.8	1,635.3	75.5
1.000	20.03	19.98	−0.05	3,134.3	3,075.0	−59.3

* Period of incubation 12 days.

for 12 to 24 hours. The difference in titration or sulfate content is a reliable index of the amount of sulfur oxidized in this period of time. By adding to the culture various stimulants or depressive agents, we can readily study their influence upon the respiration of the organism as expressed in the amount of sulfur oxidized in the brief period of time. We can also study by this method the influence of gas pressure upon respiration as well as the influence of other treatments which in any way modify respiration. The results obtained by this method indicate that by increasing the CO_2 pressure in the atmosphere, respiration is greatly stimulated, while reducing the CO_2 pressure or using an inert atmosphere, respiration is largely repressed.

SUMMARY.

1. It is shown that *Sulfomonas thiooxidans* oxidizes elementary sulfur completely to sulfuric acid. Sodium thiosulfate is oxidized by this organism completely to sulfate. *Sulfomonas thiooxidans* differs, in this respect, from various other sulfur-oxidizing bacilli which either produce elementary sulfur, from the thiosulfate, or convert it into sulfates and persulfates.

2. The organism derives its carbon from the CO_2 of the atmosphere, but is incapable of deriving the carbon from carbonates or organic matter.

3. The S:C, or ratio between the amount of sulfur oxidized to sulfate and amount of carbon assimilated chemosynthetically from the CO_2 of the atmosphere, is, with elementary sulfur as a source of energy, 31.8, and with thiosulfate 64.2. The higher ratio in the case of the thiosulfate is due to the smaller amount of energy liberated in the oxidation of sulfur compound than in the elementary form.

4. Of the total energy made available in the oxidation of the sulfur to sulfuric acid, only 6.65 per cent is used by the organism for the reduction of atmospheric CO_2 and assimilation of carbon.

5. Sulfates do not exert any injurious effect upon sulfur oxidation by *Sulfomonas thiooxidans*. Any effect obtained is due to the cation rather than the sulfate radical. Nitrates exert a distinctly injurious action both on the growth and respiration of the organism.

6. There is a definite correlation between the amount of sulfur present and velocity of oxidation, very similar to that found in the growth of yeasts and nitrifying bacteria. Oxidation reaches a maximum with about 25 gm. of sulfur added to 100 cc. of medium. However, larger amounts of sulfur have no injurious effect.

7. Dextrose does not exert any appreciable injurious effect in concentrations less than 5 per cent. The injurious effect of peptone sets in at 0.1 per cent concentration and brings sulfur oxidation almost to a standstill in 1 per cent concentration. Dextrose does not exert any appreciable influence upon sulfur oxidation and carbon assimilation from the carbon dioxide of the atmosphere.

8. *Sulfomonas thiooxidans* can withstand large concentrations of sulfuric acid. The oxidation of sulfur is affected only to a small

extent even by 0.25 molar initial concentration of the acid. In 0.5 molar solutions, the injurious effect becomes marked. The organism may produce as much as 1.5 molar acid, without being destroyed.

9. Growth is at an optimum at a hydrogen ion concentration equivalent to pH 2.0 to 5.5, dropping down rapidly on the alkaline side, but not to such an extent on the acid, particularly when a pure culture is employed.

10. Respiration of the sulfur-oxidizing bacteria can be studied by using the filtrate of a vigorously growing culture, to which a definite amount of sulfur is added, and incubating for 12 to 24 hours.

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