

OXIDATION OF SULPHUR BY MICROORGANISMS IN BLACK ALKALI SOILS¹

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The accumulation of sodium carbonate in the soil (black alkali soil) brings about a condition in which the soil practically has to be abandoned as far as utilization for the growth of plants is concerned. Irrigation, whereby the carbonates are washed out from the soil, brings about a temporary and unsatisfactory relief. If the sodium carbonate could be converted into sodium sulphate (white alkali), much more satisfactory results could be obtained, since, as pointed out recently by Vinson and associates (13),² white alkali is readily and completely leached out from soil, while black alkali resists leaching. The use of sulphuric acid on alkali soil has been suggested by C. B. Lipman and Sharp (6); this acid was found to exert a favorable influence upon the soil by neutralizing the carbonate and improving the physical condition of the soil through flocculating the colloids. However, the sudden introduction of large quantities of acid into the soil may have an injurious influence upon the soil microflora. Elementary sulphur would prove, in this respect, of greater benefit, since, not only would the injury to the soil microflora be less but the addition of small quantities of sulphur even may stimulate bacterial activities.

J. G. Lipman (7) was the first to suggest the use of sulphur for alkali soil, in order that the acid formed from the oxidation of sulphur may reduce the alkalinity of the soil and transform the sodium carbonate into sodium sulphate. O'Gara (9), when applying sulphur and sulphuric acid to soils, observed that there was a reduction of the carbonate and an increase in the sulphate content of the soil and a decided increase in the crop yield. Hibbard (3) demonstrated that by adding sulphur to alkali soil the alkalinity is neutralized, this effect being of great value in the reclamation of alkali land. Rudolfs (10) found on adding elemental sulphur, at the rate of 1,000 pounds per acre, that the reaction of the soil was reduced from P_H between 9.6 and 9.8 to P_H 9.3; with 2,000 pounds per acre, to P_H 9.2; with 3,000, to P_H 8.9; and with 3,500, to P_H 8.2. However, if black alkali soil is first leached, the use of 3,000 pounds of sulphur per acre will change the reaction of the soil from P_H 9.2 to P_H 7.7.

Although some of the sulphur may be oxidized in the soil without the intervention of life, as pointed out by Kappen and Quensell (5) and others, it is primarily as a result of activities of certain microorganisms that the rapid oxidation of the elemental sulphur takes place in both

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² Reference is made by number (italic) to "Literature cited," p. 305.

acid and alkaline soils. Two organisms have been isolated, which are responsible for this process. These two organisms are different in nature, in both their morphological and physiological characters: (1) *Thiobacillus thiooxidans*, isolated at this station from sulphur-soil composts and described in detail elsewhere (15), was found to be very active in oxidizing sulphur practically quantitatively in acid soils; and (2) *Thiobacillus B* was isolated from alkaline soils on which the sulphur had been actively oxidized. The organism resembles in general morphological and physiological characteristics *T. thioparus*, studied by Nathanson (8) Beijerinck (1), and others, and occurs commonly in the soil. It grows readily in alkaline media having a reaction equivalent to P_H 9.8 and oxidizes readily thiosulphate with an abundant formation of elementary sulphur. A detailed description of methods for the study of this organism and its relation to the oxidation of sulphur in the soil are published elsewhere (14). No definite proof can, however, be given that *T. B* is absolutely pure and not contaminated with *T. thiooxidans* or a related strain.

In the experiment reported below, a black alkali soil from the University of California Ranch near Fresno, Calif., was used. Dr. Hoagland, of the University of California, who kindly supplied the soil, stated that "the physical condition of the soil was bad, the soil was extremely alkaline, and no ordinary growth of plants is supported." The reaction of the soil was about P_H 9.6 to 9.8.

The soil was placed in 100-gm. portions in tumblers and the proper amount of sulphur was added and thoroughly mixed with the soil. Fifty mgm. of sulphur in 100 gm. of soil is equivalent to 1,000 pounds of sulphur per 1 acre of soil (2,000,000 pounds per acre basis). The proper quantity of water was added and the tumblers were incubated at 25° to 28° C. in the dark.

The reaction of the soil was determined by shaking thoroughly 5 gm. of soil with 10 cc. of water, centrifuging, then determining the hydrogen-ion concentration (P_H value) colorimetrically and in some cases electrometrically. The sulphates were determined by shaking the soil with distilled water (2) in a shaking machine for 2 to 6 hours, then filtering clear and precipitating as barium sulphate. The carbonates and bicarbonates were determined by the method outlined by Schreiner and Failyer (11).

Tables I and II show the course of reaction resulting from the oxidation of sulphur in alkali soil and the chemical transformations that have taken place as a result of these changes.

TABLE I.—Course of sulphur oxidation in alkali soil

Period of incubation.	No sulphur added.	50 mgm. sulphur per 100 gm. soil.	100 mgm. sulphur per 100 gm. soil.	200 mgm. sulphur per 100 gm. soil.	500 mgm. sulphur per 100 gm. soil.	1,000 mgm. sulphur per 100 gm. soil.
	P_H	P_H	P_H	P_H	P_H	P_H
20 days.....	9.6	9.6	9.1	9.0	8.9	8.7
42 days.....	9.6	9.4	9.0	8.5	7.1	6.7
62 days.....	9.6	9.4	8.5	7.8	7.6	7.0
86 days.....	9.6	9.3	8.6	8.0	7.2	6.8
105 days.....	9.6	9.1	7.9	7.2	6.0	4.4
167 days.....	9.6	7.9	7.4	6.7	5.7	3.5

TABLE II.—Transformation of a black alkali soil into a white alkali¹

	No sulphur added.		50 mgm. sulphur per 100 gm. soil.		100 mgm. sulphur per 100 gm. soil.		200 mgm. sulphur per 100 gm. soil.		500 mgm. sulphur per 100 gm. soil.		1,000 mgm. sulphur per 100 gm. soil.	
	Individual tumblers.	Average.	Individual tumblers.	Average.	Individual tumblers.	Average.	Individual tumblers.	Average.	Individual tumblers.	Average.	Individual tumblers.	Average.
pH	9.6	9.6	{ 7.8 8.0 7.9 77.6	7.9	{ 7.6 7.1 7.4 95.5	7.4	{ 6.4 7.0 6.6 158.0	6.7	{ 5.5 5.5 6.2 229.3	5.7	{ 2.8 3.0 4.6 636.3	3.5
Sulphates (milligrams of S in 100 gm. of soil)	{ 26 26 29.5	27.2	{ 72.4 77.5	75.8	{ 95.5 95.5 93.6	94.9	{ 172.8 162.4	164.4	{ 225.5 188.6	211.1	{ 436.7 270.0	447.7
Percentage of sulphur oxidized				97.2		67.7		67.6		36.8		42.05
Carbonates (milligrams of CO ₂ in 100 gm. of soil) ..	{ 47.2 51.2 49.2	49.2	{ 7.9 5.9 5.9	6.6		0		0		0		0
Bicarbonates (milligrams of HCO ₃ in 100 gm. of soil)	{ 182 178 172	177.3	{ 102 104 104	103.3	{ 70 72 74	72.0	{ 38 32 34	34.7	{ 20 15 24	20.0	{ 0 0 10	3.3

¹ Cultures incubated 167 days at 25° to 27° C.

The results brought out in Tables I and II point out definitely that not only is elemental sulphur oxidized readily to sulphuric acid, which results in the neutralization of the carbonates of the soil, but the reaction of the soil can be brought down to any desired point, depending entirely upon the quantity of sulphur added and the length of time during which the sulphur is allowed to be in contact with the soil.

The next experiment deals with the oxidation of sulphur by pure and crude cultures of the sulphur-oxidizing bacteria in alkaline soil under sterile conditions. The soil was placed in 100-gm. portions in 250 cc. Erlenmeyer flasks; 100, 200, and 500 mgm. portions of sulphur were added to the soil portions, which were then thoroughly mixed, then the optimum amount of water was added and the flasks were sterilized for 1½ hours at 15 pounds pressure. The flasks were then inoculated by means of a sterile pipette, with 2 drops of the vigorously growing cultures and incubated at 25° to 28° C. Alkaline soils in which active sulphur oxidation had taken place were used as the crude alkaline compost. The results are given in Table III.

TABLE III.—Oxidation of sulphur in alkaline soil by different bacteria

Quantity of sulphur used.	Type of culture.	Incubated 14 days.	Incubated 30 days.			Incubated 60 days.			
		pH	pH	Carbonates in 100 gm. of soil (mgm. of CO ₂).	Bicarbonates in 100 gm. of soil (mgm. of HCO ₃).	pH	Carbonates in 100 gm. of soil (mgm. of CO ₂).	Bicarbonates in 100 gm. of soil (mgm. of HCO ₃).	Sulphates in 100 gm. of soil (mgm. of S).
None	Control	9.6	9.6	49.0	159	9.6	52.5	143	30.1
100 mgm.	Crude alkaline compost.	7.7	7.5	0	50.0	7.7	0	43.5	115.5
200 mgm.	do	7.4	6.3	0	31.0	5.6	0	8.5	201.0
500 mgm.	do	7.0	4.9	0	15.0	4.7	0	6.5	254.8
100 mgm.	<i>Thiobacillus thiooxidans</i> .	9.2	8.8	8.0	142.0	8.6	0	86.0	109.4
200 mgm.	do	8.6	9.0	14.7	127.0	7.5	0	53.0	145.3
500 mgm.	do	8.6	9.0	5.8	118.0	7.4	0	45.0	160.7
100 mgm.	<i>Thiobacillus B.</i>	8.8	9.0	29.4	132.0	8.8	0	80.0	78.2
200 mgm.	do	8.2	8.0	18.5	138.0	8.0	0	55.5	118.4
500 mgm.	do	8.0	7.8	15.6	136.0	7.6	0	48.0	123.3

The crude culture proved to be most efficient in oxidizing sulphur in alkaline soil to such an extent that the reaction has been reduced, where sufficient sulphur has been used, from most alkaline to distinctly acid. This was accompanied by a complete disappearance of the carbonates, an almost complete disappearance of the bicarbonates, and an increase in the quantity of sulphates. With 100 mgm. of sulphur per 100 gm. of soil, equivalent to 1 ton of sulphur per acre (on the basis of the upper $6\frac{2}{3}$ inches of soil), 85 per cent of the sulphur has been oxidized by the crude culture to sulphates within 60 days, and the reaction reduced from P_H 9.6 to P_H 7.7. When the quantity of sulphur was doubled, practically the same percentage of sulphur (85.5) was oxidized in 60 days and the P_H changed to 5.6. Where a large excess of sulphur was used, or 500 mgm. per 100 gm. of soil, only 44 per cent of the sulphur was oxidized to sulphate, and the reaction of the soil changed to P_H 4.7, which would already prove injurious to certain crops because of the excess acidity. The pure cultures did not prove quite as effective as the crude culture. This may be due to the fact that the oxidation of sulphur in alkaline soil is carried out not by one organism but by several organisms taking part in the process, as is shown by the experiments reported in Table IV, where a mixture of the two organisms is used. The acid soil used in these experiments is a Sassafras sandy loam, slightly acid in reaction. These experiments were carried out both under sterile and nonsterile conditions in tumblers.

TABLE IV.—Oxidation of sulphur in acid and alkaline soils by pure and crude cultures of sulphur-oxidizing bacteria

Soil type.	Sterilization of soil. ¹	Organism.	Incubated 21 days.	Incubated 48 days.
			P_H	P_H
Acid; 100 gm. of soil + 100 mgm. of sulphur.	+	Control	6.0	5.8
	-	do	5.2	4.4
	+	Crude, acid compost	5.7	3.8
	-	do	4.8	3.8
	+	Crude, alkaline compost	5.4	5.0
	-	do	5.4	4.0
	+	<i>Thiobacillus thiooxidans</i>	5.6	5.0
	-	do	5.0	3.8
	+	<i>Thiobacillus B</i>	5.8	5.4
	-	do	5.4	4.0
	+	<i>Thiobacillus thiooxidans</i> + <i>Thiobacillus B</i>	5.4	3.4
	-	do	4.8	3.6
	+	Control	9.6	9.2
	-	do	8.8	8.2
Alkali; 100 gm. of soil + 200 mgm. of sulphur.	+	Crude, acid compost	9.0	8.2
	-	do	9.0	8.4
	+	Crude, alkali compost	7.5	7.1
	-	do	7.6	7.3
	+	<i>Thiobacillus thiooxidans</i>	8.8	8.6
	-	do	8.2	8.0
	+	<i>Thiobacillus B</i>	8.2	7.8
	-	do	8.0	7.6
	+	<i>Thiobacillus thiooxidans</i> + <i>Thiobacillus B</i>	7.8	7.6
	-	do	7.6	7.4

¹ + indicates that the flasks containing soil and sulphur were sterilized, at 15 pounds pressure, for 1½ hours; - = soil unsterilized, in tumblers.

In the acid soil, oxidation of sulphur took place in the unsterilized containers whether inoculated or uninoculated, more so in the inoculated cultures, particularly where the *Thiobacillus thiooxidans* was introduced. This is due to the fact that the sulphur added, in nonsterile condition, probably has been inoculated previously from the laboratory air. However, when the soil and the sulphur are previously sterilized, oxidation takes place only to a very unappreciable extent, unless the proper sulphur-oxidizing organisms are introduced. In the alkaline soil, the most efficient results were obtained from inoculation with the crude alkaline culture, or alkaline soil, in which sulphur oxidation had taken place previously. The pure cultures, particularly the *Thiobacillus B* culture, which was almost inactive in the acid (sterilized) soil, proved more efficacious than the *T. thiooxidans*; but a mixture of the two cultures proved nearly as efficacious as the crude alkaline composts.

The fact that the change of the soil carbonates to sulphates would also favor plant growth, which is found to be so in actual field results, was demonstrated in these experiments by the increase in the number of bacteria developing in the soil. The soils used in the experiment reported in Tables I and II were at the end of the experiment air dried, then again moistened and, after 5 to 6 days, the bacterial numbers were determined by the ordinary plate method. The alkali soil, to which no sulphur had been added, contained 320,000 bacteria per gram of soil; where 50 mgm. of sulphur had been added per 100 gm. of soil and the reaction changed to P_H 7.9, there were 665,000 bacteria per gram; 100 mgm. of sulphur, reaction P_H 7.4, 875,000; 200 mgm. of sulphur, reaction P_H 6.7, 1,275,000; 500 mgm. of sulphur, reaction P_H 5.7, 3,650,000; 1,000 mgm. of sulphur, reaction P_H 3.5, no bacteria; only a few occasional fungi. In this case, the soil reaction has been made so acid as to completely kill off the bacteria. This danger, however, could hardly be expected in the field, because no such large quantities of sulphur (1 per cent) would ever be employed.

In the following experiment, the reaction of the soil was adjusted by the addition of sulphuric acid and sodium carbonate. The following amounts of acid and alkali were used to adjust the reaction of the soil:

	P_H .
Untreated soil.....	5.6
5 cc. $N/2$ H_2SO_4 per 100 gm. of soil.....	5.3
0.5 cc. $M/2$ Na_2CO_3 per 100 gm. of soil.....	5.8
1.0 cc. $M/2$ Na_2CO_3 per 100 gm. of soil.....	6.0
3.0 cc. $M/2$ Na_2CO_3 per 100 gm. of soil.....	6.2
7.0 cc. $M/2$ Na_2CO_3 per 100 gm. of soil.....	6.4
20.0 cc. $M/2$ Na_2CO_3 per 100 gm. of soil.....	8.4

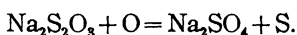
One-gm. portions of powdered sulphur were added to each 100-gm. portion of soil in Erlenmeyer flasks; the flasks were well shaken, plugged with cotton, sterilized at 15 pounds pressure for $1\frac{1}{2}$ hours, then inoculated with pure cultures of the organisms and incubated at $25^\circ C$. At the end of definite intervals of time the moisture content was brought up by the addition of sterile distilled water. The results are presented in Table V.

TABLE V.—Oxidation of sulphur by pure cultures of bacteria at various reactions

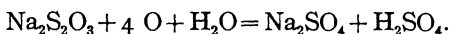
Type of culture.	P _H value.				Soluble sulphates (sulphur) in 100 gm. of soil after 50 days' incubation.	
	Initial reaction of soil.	16 days after.	30 days after.	50 days after.		
Control.....	5.2	5.2	5.3	5.4	Mgm. 19.2	
	5.6	5.6	5.6	5.6	13.7	
	5.8	5.8	5.8	5.7	11.0	
	6.0	6.0	5.8	5.8	13.7	
	6.2	6.2	6.2	6.2	13.7	
	8.4	8.4	8.2	8.2	21.9	
	5.2	5.2	3.4	2.8	315.1	
	5.6	5.4	3.0	2.8	314.6	
	5.8	5.6	5.5	5.5	20.6(?)	
	6.0	5.8	3.0	2.6	358.0	
<i>Thiobacillus thiooxidans</i>	6.2	5.4	2.8	2.8	380.0	
	6.6	6.6	6.6	3.0	304.4	
	8.4	8.4	8.2	8.0	27.4	
	5.2	5.3	5.2	5.4	39.7	
	5.6	5.3	5.2	5.2	20.6	
	5.8	5.5	5.5	5.4	21.9	
	6.0	6.0	5.5	5.5	15.2	
	6.2	6.0	5.8	5.4	19.2	
	6.6	6.6	6.6	6.4	24.7	
	8.4	8.2	8.0	7.8	
<i>Thiobacillus B</i>	5.2	5.2	3.4	2.8	302.5	
	5.6	3.1	2.8	2.6	287.7	
	5.8	2.8	2.8	2.6	311.1	
	6.0	2.8	2.6	2.6	308.3	
	6.2	2.8	2.8	2.6	367.0	
	6.6	3.1	2.8	2.8	274.0	
	8.4	8.2	8.0	7.4	
	<i>T. thiooxidans</i> + <i>T. B</i>	5.2	5.2	5.3	5.4
		5.6	5.6	5.6	5.6
		5.8	5.8	5.8	5.7
6.0		6.0	5.8	5.8	
6.2		6.2	6.2	6.2	
8.4		8.4	8.2	8.2	
5.2		5.2	3.4	2.8	
5.6		5.4	3.0	2.8	
5.8		5.6	5.5	5.5	
6.0		5.8	3.0	2.6	

Both the reaction and the sulphur content of the control indicate that no sulphur or only traces of it were oxidized under sterile conditions. However, in the presence of the sulphur-oxidizing bacteria, the oxidation of the sulphur took place very rapidly, with the transformation of large quantities of sulphur to sulphuric acid. When the reaction of the soil was acid, the oxidation of the sulphur in 50 days was carried on entirely by *Thiobacillus thiooxidans*. *Thiobacillus B* oxidized only very small quantities of sulphur; but, in the presence of both organisms, the speed of the reaction was hastened, as indicated by the measurements of the P_H values obtained in 16 days. This may be explained by either of two assumptions: (1) If we suppose a so-called "lag phase" in bacterial development, *Thiobacillus B* produces substances which appreciably shorten the "lag phase" of *T. thiooxidans*; (2) *Thiobacillus B* may oxidize the sulphur not directly to sulphates but to other compounds of sulphur, which are then rapidly oxidized to sulphates by *T. thiooxidans*. This is made clear in the study of the pure cultures of *Thiobacillus B*, with sodium thiosulphate as a source of energy, when a large part of the thiosulphate is transformed to persulphates, a part to elementary sulphur, and only a part to sulphates. This preparatory function of the *Thiobacillus B* may explain a number of hitherto unexplained difficulties observed in the study of sulphur oxidation by microorganisms in the soil. Of these we need mention only a few.

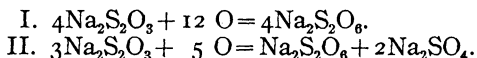
Nathanson (8), who first studied the *Thiobacillus* group, of which *Thiobacillus B* (not *T. thiooxidans*) is a representative, stated that thiosulphate is oxidized to sulphate and tetrathionate, with the production of free sulphur; however, on sodium sulphid agar plates, when free sulphur is formed abundantly in a nonbiological way, this sulphur is oxidized by the bacteria. The reaction proposed by Nathanson ($3\text{Na}_2\text{S}_2\text{O}_3 + 5\text{O} = \text{Na}_2\text{S}_4\text{O}_6 + 2\text{Na}_2\text{SO}_4$) could hardly be justified, particularly in view of the fact that the sulphur was supposed to be produced in a purely nonbiological way, by the interaction of the tetrathionate with the remaining thiosulphate, and was not supposed to play any part in the reaction. Beijerinck (1), who identified Nathanson's organism with his *T. thioparus*, then suggested the following reaction:



But Jacobsen (4), who worked in Beijerinck's laboratory, found that the same organism oxidized elemental sulphur quantitatively to sulphate, which would again hardly justify Beijerinck's formula, if the organism was the same. Trautwein (12), however, obtained the separation of elemental sulphur from thiosulphate only in crude cultures of the organisms; but, in pure culture, no separation of elemental sulphur was found. At the same time a large part of the thiosulphate was transformed to sulphate. At first he thought the following reaction to be justified:



In view of the fact that the reaction of the medium did not turn acid and that a large quantity of persulphate was formed, he finally concluded that the reaction takes place as follows:



In the investigations carried on in this laboratory with pure cultures of *Thiobacillus B*, it was found that this organism transforms thiosulphate into persulphate, sulphate, and elemental sulphur, with only a limited production of acid (P_{H} changed in culture solution from 9.8 to 6.4 or 7.0). When *T. thiooxidans* is added to the culture solution, the elemental sulphur separated by the *Thiobacillus B* is rapidly oxidized to sulphuric acid and the final reaction may go down from P_{H} 9.8 to P_{H} 1.2 as a result of the action of the two organisms. The culture solution used in this case consists of 5 gm. sodium thiosulphate, 1 gm. sodium bicarbonate, 0.2 gm. dipotassium phosphate, 0.1 gm. magnesium chlorid, 0.1 gm. ammonium chlorid, 0.25 gm. calcium chlorid, and 1,000 cc. of tap water.

These discrepancies in the type of reaction that takes place when thiosulphate is acted upon by sulphur-oxidizing bacteria can be explained by the fact that different workers used different forms of a closely related group of organisms; in some cases the culture used possibly contained not one organism but a mixture of two or more organisms. *Thiobacillus B*, isolated in this laboratory and closely related, in its morphology and physiology, to *T. thioparus* and allied forms studied by Nathanson (8), Beijerinck (1), Trautwein (12), and others (4), was possibly contaminated in some cases by *T. thiooxidans*, which oxidizes actively elemental sulphur and is described by Waksman and Joffe (15). *T. thioparus* will act upon sulphur compounds under distinctly alkaline conditions, as in

culture media containing soluble carbonates or bicarbonates or black alkali soil; the elemental sulphur will be oxidized only comparatively slowly, but, in the presence of *T. thiooxidans* this oxidation will be rapidly hastened and the reaction quickly changed to any degree of acidity, depending merely on the quantity of sulphur used.

The possibility of the participation of other organisms oxidizing sulphur or sulphur compounds in the soil, or in any way influencing the process, in addition to the two mentioned, is not excluded. Neither is the possibility excluded that the oxidation of sulphur in alkaline soil is carried on chiefly by a strain of *Thiobacillus thiooxidans* which has adapted itself to alkaline conditions. While *T. thiooxidans* grows vigorously on artificial media and does not deteriorate with age, *Thiobacillus B* rapidly deteriorates under cultural conditions and the very slow oxidation of sulphur by this organism, particularly in the latter part of the work, may be due to this phenomenon.

SUMMARY

(1) The use of sulphur in the presence of the proper sulphur-oxidizing bacteria will result in the transformation of black alkali soil to white alkali soil.

(2) The final reaction of the soil depends on the quantity of sulphur used and the length of time which the sulphur is allowed to be in contact with the soil.

(3) The oxidation of sulphur in black alkali soil is probably carried on by the agency of more than one sulphur-oxidizing bacterium.

(4) In the presence of two bacteria, one of which can act upon sulphur under distinctly alkaline conditions while the other rapidly oxidizes sulphur under acid conditions, the speed of the reaction is greatly hastened.

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