II.—BIOLOGICAL OXIDATION OF SULPHUR. PART III. A Sulphur-Oxidising Organism From Activated Sludge.

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A review of recent literature on the isolation and study of organisms connected with the oxidation of sulphur and its compounds shows that the special organisms responsible for the process differ widely according to their source and the conditions of their normal activity. It has thus been shown that the reaction of media on which they are cultivated influences their behaviour to a marked extent, and that organisms isolated from sea-water, mud and sulphur-soil composts respectively exhibit specific differences in their morphological and physiological characters. These facts suggested that it might be possible to isolate from various other sources organisms which are able to bring about the oxidation of sulphur but differ in character from those previously described.

By composting sulphur, rock phosphate and soil it was found that the sulphur was rapidly oxidised to sulphuric acid which acted upon the tricalcium phosphate producing di- and monocalcium phosphates. In absence of a neutralising agent considerable quantities of acid accumulated in the compost, and by inoculating a suitable culture medium with such material, Waksman and Joffe (*J. Bact.*, 1922, **7**, 239) isolated a minute organism capable of rapidly oxidising elemental sulphur. Brown (*J. Amer. Soc. Agron.*, 1923, **15**, 350) succeeded in isolating a motile organism from activated sludge by inoculating a suitable liquid medium with fresh sludge, the organism being able rapidly to oxidise sulphur and produce acid. These observations of previous workers suggested the possibility of isolating a specific organism from suspensions of sulphur in activated sludge aerated until a high acidity developed.

Isolation of the Organism.

In the foregoing paper it was shown that suspensions of sulphur in activated sludge, aerated for over a month, developed an acidity of P_H 2·1 and eliminated practically all the contaminating organisms, leaving the sulphur-oxidisers as almost the sole survivors. If the

mixture is then directly inoculated into the sterilised liquid medium of Waksman and Joffe (*loc. cit.*) and incubated at 28–30°, the growing organisms render the culture medium uniformly cloudy within a week when sulphur is present as the chief source of energy. By repeated sub-culturing in the liquid medium pure cultures of sulphur-oxidising organisms were obtained. In presence of a neutralising agent such as tricalcium phosphate, there was a characteristic formation of gypsum crystals which appeared concurrently with the development of cloudiness. The P_H of the medium remained stationary at about 2·4 with the conversion of the tricalcium into monocalcium phosphate.

EXPERIMENTAL.

The methods and media employed were mainly those of Waksman and Joffe (*J. Bact.*, 1922, **7**, 606) with certain modifications. The liquid medium contained (in grams per 1000 c.c. of distilled water):—$(NH_4)_2SO_4$, 0·2; $MgSO_4$, 0·1; $FeSO_4$, 0·01; H_2KPO_4, 5·0; $CaCl_2$, 0·25; Sulphur, 10·0. The solutions were prepared in bulk and measured in 500 c.c. portions into 850 c.c. conical flasks plugged with cotton wool and sterilised by steaming for 30 minutes on three consecutive days. The inoculations were made from a seven-day old culture and the flasks incubated at 28–30°; the P_H of the medium was 4·6.

Washed agar medium.—The agar was prepared by washing the fibres in distilled water for several days and drying at 60° (*J. Bact.*, 1920, **5**, 591); a 2·5 per cent. solution was filtered clear, tubed in 10 c.c. portions and sterilised in an autoclave at 15 lbs. The following solutions were prepared (in grams per 100 c.c.) and sterilised:—(1) H_2KPO_4, 3·0; (2) NH_4Cl, 0·1; $MgCl_2$, 0·1 and $CaCl_2$, 0·25; (3) $Na_2S_2O_3 \cdot 5H_2O$, 5·0.

The agar was melted, cooled to 40° and plates made by placing 1 c.c. each of solutions 1, 2 and 3 in sterile petri-dishes, adding the desired inoculum and then the melted agar. The solutions were ten times the usual strength and were used in the preparation of agar or silica gel media.

Silicic acid gel.—The method of Beijerinck as described by Gibbs (*Soil Sci.*, 1919, **8**, 439) combined with the modification suggested by Waksman and Carey (*J. Bact.*, 1926, **12**, 90) gave satisfactory results. From a normal solution of hydrochloric acid, 5 c.c. portions were placed in a series of test tubes and varying amounts of an equivalent solution of sodium silicate added. The contents of the tubes were immediately mixed and poured into dishes, and the mixture where the gel formation took place within two to five minutes was chosen. It was found that

5 c.c. of silicate solution gave the best results. To 15 c.c. of normal hydrochloric acid 15 c.c. of 8 per cent. silicate solution was added and the mixture immediately shaken and poured into petri-dishes which rested on a level surface until the gel was well formed. They were then placed in wash-basins, dialysed in running tap-water until free from chlorides and then transferred to sterile vessels containing boiled distilled water, this being replaced several times. After draining, they were flamed to sterilise the surface of the gel, on to which was poured the sterile concentrated nutrient solution; this was allowed to diffuse through the gel for about 10 minutes, the excess was poured off and the surface of the gel once again flamed to ensure sterility. The medium was then inoculated with a drop of the culture spread evenly over the plate which was then allowed to remain in the incubator at 28–30°. The character of the growth was very satisfactory for the study of colonies.

Morphology.—The organism when grown on the synthetic medium described above, consisted of short rods with rounded ends, usually occurring in pairs. Spore formation was absent. The cells were motile in the earlier stages and remained so during over a month. By adopting the new method of gram-staining (Burke, *J. Bact.*, 1922, **7**, 178) the organism was found to be gram negative. Deposition of sulphur did not occur within or without the cells (Figure I).

In the course of the morphological study of the organism the presence of long filamentous forms were occasionally observed. At first they were believed to be due to contamination, but a careful study of hanging drop preparations showed that they were only involution forms of the sulphur-oxidising organism, appearing in old cultures or when conditions became unfavourable. The involution forms varied in length sometimes reaching 100 μ with numerous forms intermediate in size (Figure II). They were non-motile and differed in physiological activity from the normal, developing most quickly when the temperature was too high for normal growth, i.e., between 35° and 37°; if the temperature is again lowered, they disappear and the organism regains its normal characteristics.

Agar plates.—Plates of washed agar were prepared as previously described, inoculated from one of the pure cultures on liquid medium and incubated at 28–30°. In seven days, colonies began to appear; they were small, but visible to the naked eye as an opaque, creamy colony. Examination under the microscope revealed a central nucleus with a creamy, uniform margin (Figure III).

Silicic acid gel plates.—The colonies obtained by using silicic acid gel were more satisfactory than those on washed agar, which they

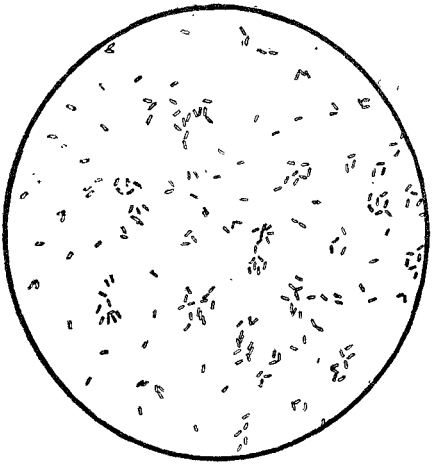


Fig. 1

Normal Forms

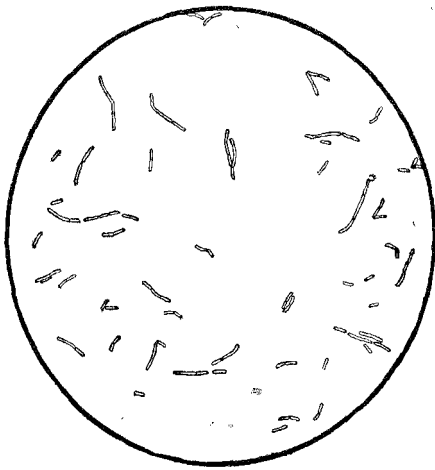


Fig. II
Involution - Forms.

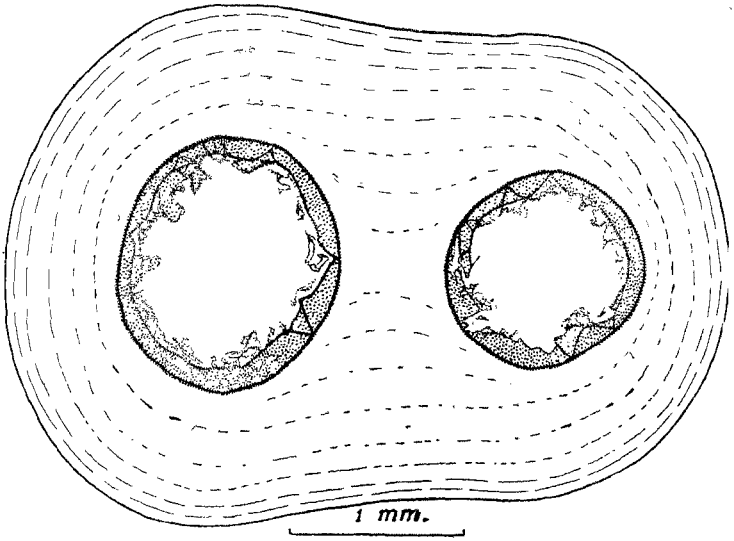
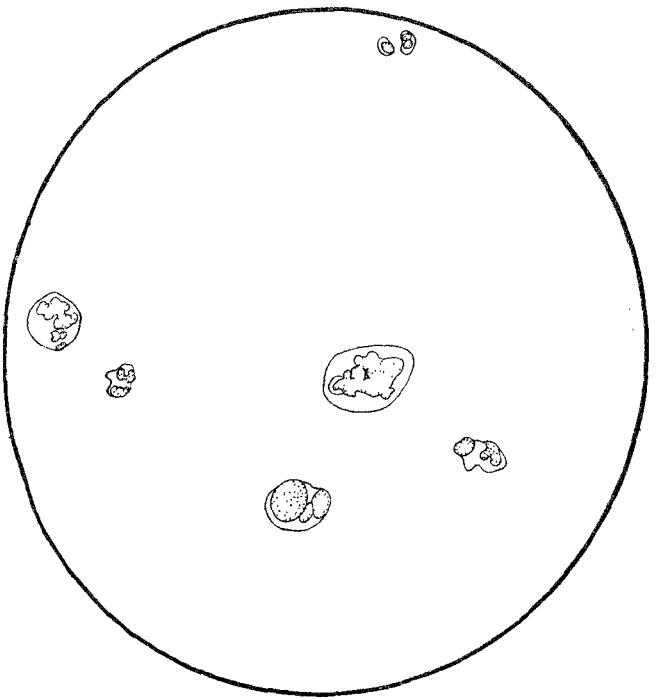


Fig. III
Colonies on agar.



Colonies on Silica Jelly

Fig. IV

resembled but were somewhat more dense. They began to appear in two days and developed rapidly all over the plate attaining their maximum development in 7 to 10 days, increasing in density from the margin to the centre, the former being wavy in appearance under the microscope. The organisms exhibited the same characteristics as those grown on liquid medium, but appeared slightly protuberant due to the change in the environment (Figure IV).

Gypsum blocks.—These were prepared as usual, and having been sterilised by heating in an electric muffle furnace at a temperature of 400° for half an hour, were transferred to petri-dishes containing 50 c.c. of a liquid in which thiosulphate was the source of sulphur. They were inoculated on the surface from a pure culture grown in liquid medium and incubated at 28–30°. Pale brown colonies developed, in size equal to those on agar plates. By repeated sub-culturing in liquid medium the organism could be kept alive and active for any length of time. It was often noticed that there was a decided increase in their activity.

Physiology.—Besides deriving all its carbon from the carbon dioxide of the atmosphere, the organism was able also to utilise carbohydrates and glycerol, but not carbonates which even in small amounts were toxic because in their presence the medium remained alkaline throughout, a condition unfavourable to the growth of the organism. Sulphur and thiosulphate were the important sources of energy to the organism. The amount of sulphur oxidised and acid produced were greater in presence of carbohydrates than in their absence, indicating the beneficial influence of such compounds. The organism grew equally well whether the medium contained elementary sulphur or thiosulphate, the cloudiness appearing in either case within a week's time. Sulphides and sulphites were not utilised. Mere traces of inorganic salts in addition to phosphates were sufficient for growth. In absence of nitrogen the organism did not normally thrive and the presence of ammonium salts such as the sulphate assisted its growth. In presence of mannitol, however, the organism seemed able to make good growth even in absence of ammonium salts, suggesting that it might be able to assimilate atmospheric nitrogen. This aspect of the question is being further investigated.

Influence of various inorganic and organic compounds on the amount of sulphur oxidised.—A detailed study has been made of the influence of various inorganic and organic compounds on the oxidation of sulphur by a pure culture of the organism. The medium was distributed in 500 c.c. portions in 850 c.c. flasks containing 5 gms. of sulphur flowers and 0·1 per cent. of salt under test, the flasks being sterilised in steam in the usual way. The organic compounds were

sterilised separately and then added to the sterile medium. The flasks were all inoculated with 1 c.c. of a 1-in-30 dilution of a 7-day old culture of the organism and incubated at 28–30°, 50 c.c. portions being withdrawn from time to time with sterile pipettes for analysis. The P_H was determined by the colorimetric method using the colour standards of Medalia (*J. Bact.*, 1920, **7**, 589) and the total acidity estimated by titrating 5 c.c. of the culture medium against N/50 sodium hydroxide using phenolphthalein as indicator. The water-soluble sulphates were determined gravimetrically.

The amounts of sulphur oxidised under various treatments as compared with sulphur alone are shown in Figures V and VI. The amount of sulphur oxidised per 100 c. c. in the control was 65 mgms. in 40 days, whereas in the presence of ammonium sulphate, aluminium sulphate and sodium silicate over the same period the amounts were 438·0, 307·0, and 278·0 mgms. respectively. In presence of organic compounds such as lactose, mannitol and glycerol the amounts oxidised were 256·0, 223·0 and 185·2 mgms. respectively. From this it will be observed that while such organic compounds had beneficial influence on sulphur-oxidation they were less effective than inorganic salts.

The general behaviour of the cultures under these different treatments requires some explanation. Although there was considerable difference in the initial reaction of all the various media, the fall in P_H was quite rapid in all and reached 1·2 within 40 days, there being a progressive development of titratable acidity in all the cultures. The behaviour of sodium silicate was the most remarkable. Initial P_H was 6·6 and the organism did not show any activity for over 10 days being able, presumably, to acclimatise itself to the new conditions; it eventually began to grow rapidly, P_H being reduced to the level of other cultures in 30 days, and the amount of sulphur oxidised comparing favourably with the rest of the series. This indicates that in the silicate medium the P_H limits are from 6·6 to 1·4, far higher than those observed by Joffe (*loc. cit.*) for his organism. With aluminium sulphate, manganous sulphate, ammonium sulphate and tricalcium phosphate, the P_H was favourable from the beginning, and the organisms increased rapidly and oxidised more sulphur. The effect of ferrous sulphate was not markedly favourable and was inferior to that of the other salts studied. Throughout the series accumulation of free acid rose proportionately with the amounts of sulphur oxidised as indicated by the titration figures shown in Tables I and II. The P_H values of the series are shown in Tables III and IV.

In presence of organic compounds it was observed that the series uniformly started with P_H 5·0, the fall therefore measuring the influence

Fig. V

Influence of inorganic salts

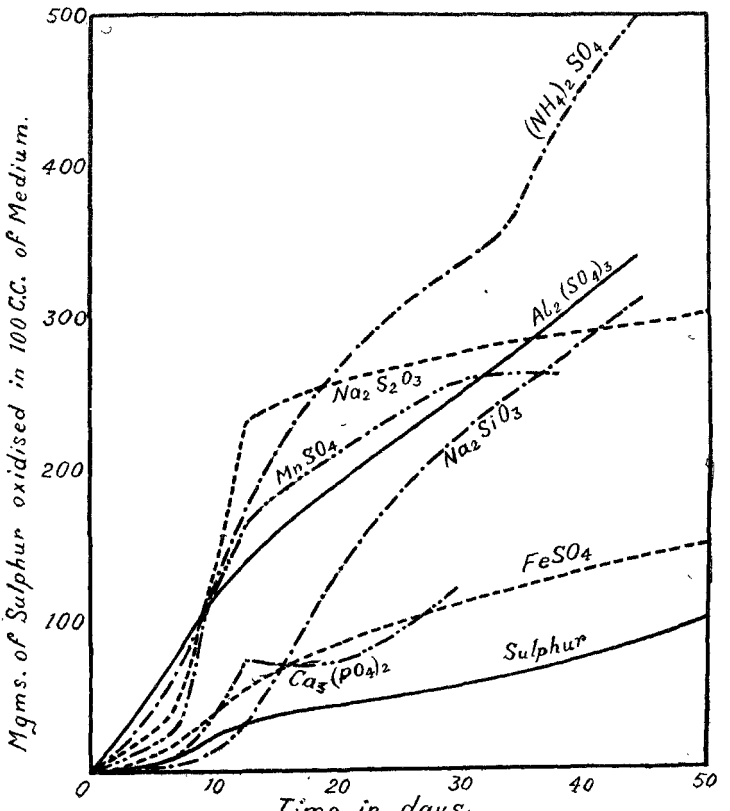


Fig. VI

Influence of organic Compounds.

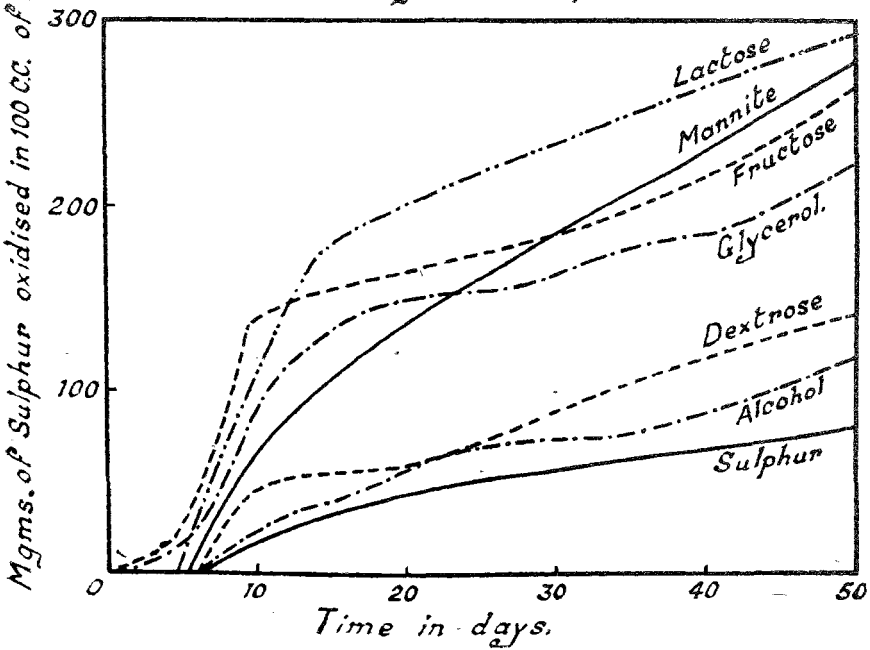


TABLE I.

INORGANIC SALTS SERIES.

Titratable Acidity in c.c. of N/50 Sodium Hydroxide for 5 c.c. of Medium.

	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·5	5·7	9·6	12·3	15·9	...	22·1
Sodium thiosulphate only ...	5·4	5·5	20·3	22·2	27·5	...	30·8
With aluminium sulphate ...	6·4	17·5	28·2	34·5	45·1	54·1	60·3
,, manganous sulphate ...	6·5	12·6	31·5	39·9	45·5	...	...
,, ammonium sulphate ...	6·7	14·4	32·2	41·9	57·5	72·5	83·4
,, ferrous sulphate ...	6·1	8·9	14·0	18·1	22·0	26·1	31·3
,, calcium phosphate ...	5·5	7·0	16·8	22·7	24·3	25·4	26·4
,, sodium silicate ...	3·5	3·5	6·1	20·8	32·5	44·3	51·1

TABLE II.

ORGANIC COMPOUNDS SERIES.

Titratable Acidity in c.c. of N/50 Sodium Hydroxide for 5 c.c. of Medium.

	Time in days						
	Start	7	12	20	32	40	50
Sulphur only	5·5	5·7	9·6	12·3	15·9	...	22·1
With dextrose	5·4	5·3	16·5	18·1	20·2	22·4	26·9
,, glycerol	5·4	5·7	17·4	27·3	30·4	33·4	40·2
,, alcohol	5·3	5·9	10·5	14·2	16·9	19·0	23·4
,, mannitol	5·2	5·3	14·5	23·7	34·8	39·0	49·4
,, lactose	5·3	5·7	17·2	33·7	40·6	44·3	51·0
,, fructose	5·3	7·9	23·7	29·3	32·8	37·1	47·0

TABLE III.

Inorganic Salt Series ; Change in P_H.

	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·1	3·8	2·4	2·2	1·9	1·7	1·7
Sodium thiosulphate	5·0	4·6	1·8	1·8	1·8	1·7	1·7
Sulphur + aluminium sulphate	2·8	1·9	1·7	1·5	1·2	1·2	1·2
,, + manganous sulphate	4·5	2·2	1·5	1·4	1·3	1·3	1·2
,, + ammonium sulphate	4·7	2·3	1·6	1·3	1·2	1·2	1·2
,, + ferrous sulphate	3·2	2·5	2·0	1·8	1·7	1·7	1·7
,, + calcium phosphate	4·9	3·0	2·8	2·5	2·4	2·4	2·4
,, + sodium silicate	6·6	6·6	3·5	1·7	1·6	1·3	1·3

TABLE IV.

Organic Compounds Series ; Change in P_H.

	Time in days						
	Start	7	12	20	30	40	50
Sulphur only	5·0	3·8	2·4	2·2	1·9	1·7	1·7
With dextrose	5·0	3·3	1·9	1·9	1·7	1·7	1·7
,, glycerol	5·0	4·4	1·8	1·7	1·6	1·6	1·3
,, alcohol	5·0	3.4	2·3	2·1	1·9	1·8	1·7
,, mannitol	5·0	3·8	1·9	1·7	1·6	1·4	1·2
,, lactose	5·0	3·7	1·8	1·6	1·4	1·3	1·2
,, fructose	5·0	2·6	1·7	1·6	1·6	1·6	1·4

of the substances added. All the sugars disappeared rapidly, lactose and mannitol seeming to be the carbohydrates with maximum stimulating action, followed by glycerol and fructose. Joffe found dextrose to be more favourable for his cultures. Gas was not evolved, but the

characteristic cloudiness observed in the medium was even more marked in these cultures. Sulphur-oxidation was not as vigorous as in the inorganic media.

The stimulating action of such inorganic salts on biological processes has been noticed previously, and their influence on nitrification and ammonification studied by Brown and Munges (*Soil Sci.*, 1916, **3**, 67) and others. It has been argued that the salts of aluminium undergo hydrolysis producing a strongly acid medium. With initial P_H 2·8 it would be expected that the sulphur-oxidising organisms, being able to tolerate such high acidity, would thrive; while others, less adaptable organisms such as nitrifiers and ammonifiers, would not. Consequently it was not surprising to find that aluminium salt showed a distinctly stimulating effect. On the other hand, the superiority of ammonium sulphate to aluminium sulphate could be traced to the favourable effect of easily available nitrogen, readily utilised by the organisms for their development and activity. In the case of sodium silicate, however, the remarkable effects are most probably attributable to the increased surface presented by the colloidal silicic acid, and not to the reaction of the medium which approached neutrality.

Influence of organic compounds.—It has been mentioned above that carbohydrates were favourable to the bacterial oxidation of sulphur; this was observed also by Waksman and Joffe (*loc. cit.*) who attributed the stimulation to their utilisation by the organisms. Starkey (*J. Bact.*, 1925, **10**, 192) found that dextrose disappeared from the media with growth of the organisms during incubation and a definite correlation existed between its disappearance and the amount of acid produced. Hence he also attributed the stimulation to the assimilation of carbon from dextrose.

It is therefore desirable to ascertain whether the easily available carbon compounds would serve as a source of carbon for the organisms in absence of atmospheric carbon dioxide. Sterile 100 c.c. portions of the synthetic medium in 250 c.c. Erlenmeyer flasks, containing the various organic compounds in the same concentration as before, were inoculated with a pure culture of the organism and placed under bell-jars covered with black paper and sealed with tap-grease. The jars containing the cultures were aspirated with air passed through 100 per cent. caustic potash and soda-lime tubes. Similar guard-tubes were also provided on the other side of the bell-jars close to the pump by which air was drawn into the system. Care was taken to renew the solution as frequently as possible during aeration; a corresponding series was run under normal conditions over the same period. The cultures were analysed after one month.

In comparing the activity of the respective series, the amount of sulphur oxidised in the sulphur series without the organic compound was taken as unity ; the ratios are given in Table V.

TABLE V.

Substance added	Ratios of sulphur oxidised	
	Ordinary air	Air free from CO_2
Sulphur only ...	1·00	1·00
,, + glucose ...	1·60	0·56
,, + lactose ...	2·95	2·27
,, + mannitol ...	4·42	3·06
,, + glycerol ...	3·42	1·75

It will be seen that in absence of carbon dioxide the organisms did not utilise glucose, while the other organic compounds stimulated the oxidation of sulphur, but not to such an extent as under normal conditions. The figures support the view that this activation has been effected by the assimilation of readily available carbon from the organic compounds studied. Under normal conditions the acceleration was proportionately greater as the carbon could be obtained from the air and from the organic compounds. This observation confirmed the view expressed by Starkey (*loc. cit.*) that in the presence of organic compounds a general stimulation is noticeable, due to utilisation of carbon from the substances supplied.

Influence of aeration.—Ranganathan and Norris (*J. Indian Inst. Sci.*, 1927, **10A**, 114) have observed that the supply of air to a culture containing ammonium salts in presence of nitrifying organisms hastens the nitrification process leading to the accumulation of nitrates. Hence it was thought that a continuous supply of air to a culture of sulphur-oxidising organisms would accelerate oxidation of sulphur. A culture of the organism incubated at 28–30° was aerated, and periodical samples withdrawn under sterile conditions to determine titratable acidity, P_H and sulphur oxidised. Similar determinations were conducted with an unaerated sample. The results are recorded in Table VI, indicating a general fall by 50 per cent. in the amount of sulphur oxidised in the aerated culture as compared with the unaerated. Even though there had been a slight fall in P_H with rise in titratable acidity, these did not compare favourably with the unaerated series, confirming the observation that additional air disturbing the

culture adversely affects the normal growth and development of the organisms, with considerable diminution in their activity.

TABLE VI.

Rate of sulphur oxidised with and without aeration.

Analysis after	Mgms. of sulphur in 100 c.c.		Diminution in sulphur oxidised, mgms.	P_H		Titratable acidity in c.c. N/50 NaOH per c.c. medium	
	Aerated	Unaerated		Aerated	Unaerated	Aerated	Unaerated
Control ...	8·78	8·78	..	4·8	4·8	1·10	1·10
7 days ...	9·32	9·90	0·58	3·8	3·8	1·16	1·24
12 ,, ...	19·23	32·40	13·17	2·7	2·4	1·46	1·92
20 ,, ...	31·59	50·50	18·91	2·4	2·2	1·79	2·46
30 ,, ...	41·20	70·80	29·60	2·3	1·9	2·08	3·20

Influence of Light.—The effect of exposing micro-organisms to direct sunlight, diffused light, polarised light and in darkness has been recorded (Giltner, *Microbiology*), it being found that direct sunlight is for the most part fatal to their growth. Whilst diffused light exerts a beneficial influence on some fungi, most organisms develop well in darkness and function normally. From a study of the influence of diffused light and of darkness on the amount of sulphur oxidised, it was found that after an incubation period of 45 days the amounts of sulphur oxidised were 174 and 368 mgms. respectively in 100 c.c. of medium. The results indicated that, although diffused light did not completely retard growth and activity, darkness was more favourable to them. This observation agrees with those recorded with many soil organisms preferring darkness to light, and as the sulphur-oxidisers are commonly present in all soils, their behaviour appears quite normal.

Influence of Charcoals.—The important effect of surface phenomena in biological reactions has been well recognised, and chemical changes produced by organisms mostly occur at surfaces. The relative acceleration in activity has also been shown to depend on the dispersion of the substrate, finer division leading to greater activity. In sulphur-oxidation it was found by Starkey (*J. Bact.*, 1925, **10**, 154) that different forms of sulphur varying only in their fineness of distribution in the liquid phase affected markedly the rate of oxidation. In the case under investigation, it was found that in any hanging drop culture

examined under a microscope with a few particles of sulphur, the organisms clung to the surface of the particles which gradually disappeared in the course of a few minutes, suggesting that the reaction occurred at the surface of contact of the organisms and the substrate; hence the finer it is, the more rapid will be the conversion of sulphur to sulphuric acid. Any process enlarging the surface exposed should lead to increased oxidation by the organisms.

It has been known that charcoals are definite catalysts to oxidation, possessing enormous surface and high power of adsorption; hence it was thought that their introduction into cultures might lead to an increased rate of oxidation. Moreover, an interesting observation has been recorded by Hutchinson (*Sci. Rep. Agri. Res. Inst. Pusa*, 1924–25, 38) that addition of charcoal in small quantities to a sulphur-phosphate compost enhanced the rate of phosphate solubilisation. No reason has been assigned to the acceleration, but it is quite probable that this might have resulted in the increased oxidation of sulphur with consequent production of sulphuric acid, this in turn hastening the production of soluble phosphate. This solitary and remarkable observation suggested a detailed inquiry into the general behaviour of charcoals, both vegetable and animal, to explain the mechanism of acceleration.

At this stage, it is enough to investigate whether there was any acceleration in oxidation of sulphur due to the introduction of various charcoals in the least quantity possible as in the experiments cited above. In the experiments to be reported hereunder only 0·05 gm. of the respective dry charcoals has been employed. To 100 c.c. of liquid medium contained in 250 c.c. conical flasks was added 0·05 gm. of the charcoals, sterilisation being effected by flowing steam for 30 minutes in 3 days. Inoculation with one c.c. of the pure culture followed and incubation at 28–30°, periodical analysis being made for titratable acidity, P_H and amount of sulphur oxidised. These are given in Tables VII, VIII and IX. It will be seen from the tables that there has been an all round acceleration in the amount of sulphur oxidised with all the charcoals irrespective of their source, the stimulating influence being at a maximum in the norit series. The nature and mechanism of this activation will be discussed in a later paper. The ratio of increased oxidation varied from 1·5 to 3·0 when compared with the sulphur series without charcoal. With the fall in P_H there was a corresponding increase in titratable acidity, which might be due to various factors. These may be the increase in the amount of surface introduced by the charcoals, their electro-kinetic behaviour, or the displacement of bases from the charcoals liberating some stimulating bases like aluminium or manganese, which have been previously shown to accelerate the oxidation of sulphur. Alternatives are general

stimulation of growth or adsorption of gases from the air which might be readily utilised by the organisms for their cell respiration and development with the consequent increase in activity. Other physical factors such as surface tension and viscosity, which numerous workers have shown to be effective in modifying profoundly the behaviour of micro-organisms, might also account for the observed stimulation. These possibilities are being investigated.

TABLE VII.

Amount of sulphur oxidised in mgms. per 100 c.c. of medium.

No.	——	Time in days					
		Start	10	20	30	40	50
1	Control	6·04	6·04	7·42	7·69	8·24	8·24
2	Inoculated	7·69	29·12	56·31	66·92	77·18	87·35
3	,, + Animal charcoal ...	7·69	10·16	35 98	79·38	84·87	97·24
4	,, + Norit	7 69	63·46	104·65	130·47	159·90	209·6
5	,, + Coconut charcoal..	6·59	32·14	53·02	90·93	102·18	121·7
6	,, + Wood charcoal ...	7·14	12·64	50·82	89 08	104·11	172·8
7	,, + Polycarbon ...	7·14	10·16	65·67	83·78	103·9	139·0

TABLE VIII.

Titratable acidity in c.c. of N/50 Sodium Hydroxide per c.c. of medium.

No.	——	Time in Days					
		Start	10	20	30	40	50
1	Control	1·88	1·88	1·88	1·88	1·88	1·88
2	Inoculated	1·88	2 48	3·33	3·76	4·08	4·46
3	,, + Animal charcoal ..	1·80	1·88	2 63	4·04	4·20	4·62
4	,, + Norit	1·84	3·51	4·78	5 64	6·24	8·03
5	,, + Coconut charcoal ...	1·84	2·54	3·16	4·22	4·83	5·34
6	,, + Wood charcoal ...	1·80	2 01	3·10	4·20	4·61	6·92
7	,, + Polycarbon ...	1·84	1·88	3·57	4·39	4·62	5·96

TABLE IX.

Change in P_H.

No.	——	Time in Days					
		Start	10	20	30	40	50
1	Control	4·6	4·6	4·6	4·6	4·6	4·6
2	Inoculated	3·8	3·0	2·8	2·6	2·6	2·2
3	,, + Animal charcoal ...	4·4	4·4	3·2	2·8	2·6	2·0
4	,, + Norit	4·2	2·4	2·0	1·6	1·4	1·4
5	,, + Coconut charcoal ...	4·2	3·0	2·8	2·4	2·4	1·8
6	,, + Wood charcoal ...	4·4	4·2	2·8	2·4	2·4	1·6
7	,, + Polycarbon ...	4·3	4·2	2·5	2·2	2·2	1·6

Sulphur-Carbon ratio of the sulphur-oxidising organism.—The general morphological characters of an organism do not satisfactorily settle the question of its identity, for most of the observations are purely qualitative; but they may be used with advantage provided definite quantitative data are revealed by its physiological behaviour. In the study of similar autotrophic oxidising organisms it has been found that a correlation existed between the amounts of carbon assimilated and of substrate oxidised. This relationship has been ascertained in the case of nitrifying organisms by Meyerhof (*Arch. ges. Physiol.*, 1916, **164**, 353) and of the sulphur-oxidising organism isolated from soil-sulphur compost by Waksman and Starkey (*J. Gen. Physiol.*, 1923, **5**, 289). Thus the sulphur-carbon ratio of the organism isolated from activated sludge should decide whether this is identical with the one so well studied by the foregoing authors; accordingly, the question has been studied with the normal forms of organisms at 28° and with the involution forms at 35° to ascertain definitely the variations due to alteration in the structure of the organisms.

The usual liquid medium of Waksman and Joffe was employed, but conductivity water was used in its preparation. The sulphur was weighed into the individual flasks of 850 c.c. capacity which had been cleaned previously with potassium dichromate-sulphuric acid mixture. The medium (300 c.c.) was placed in the respective flasks, plugged with cotton wool and sterilised in flowing steam for 30 minutes on three consecutive days, all manipulations being conducted under strictly sterile conditions; one c.c. from a seven-day culture of actively growing organism was used for inoculating each flask. Some flasks were not inoculated and thus served as controls.

Methods of analysis.—Acidity in 5 c.c. of the filtered medium was titrated with N/10 sodium hydroxide using phenolphthalein as indicator. The hydrogen-ion concentration was determined colorimetrically using the colour standards of Medalia (*loc. cit.*) with the indicator series of Clark and Lubs. Sulphate was estimated gravimetrically as barium sulphate. Total solids were determined by evaporating the filtered culture in the desiccator over sulphuric acid in vacuum and weighing the residue. The total carbon of the culture was estimated by means of the wet combustion apparatus resembling that of Waksman and Starkey (*J. Gen. Physiol.*, 1923, **5**, 289), the details of estimation being strictly observed. The accuracy of the method was checked with pure sucrose which gave nearly 98 per cent. of the theoretical yield. Carbon was estimated in the control and inoculated samples at the end of various incubation periods, using 100 c.c. of unfiltered medium; the balance was filtered and used for other determinations. The results are tabulated in Tables X and XI.

In presence of the normal forms, sulphur-oxidation and carbon-assimilation progressed rapidly; with increase in the carbon assimilated there was a corresponding rise in the amount of sulphur oxidised. In the course of 45 days 36 per cent. of the sulphur was oxidised with a fall in the hydrogen-ion concentration to less than P_H 1·4. The ratio of the sulphur oxidised to carbon assimilated varied from 22·4 at the beginning of active growth to 46·95, falling to 40·85 at the end of the experiment, with an average of 40·75 parts of sulphur oxidised for every gram of carbon assimilated as carbon dioxide from the atmosphere. During the early stages the ratio was small indicating the economic utilisation of energy at the active growth phase, but with multiplication of organisms there was an increased intake of carbon with a proportionate rise in the sulphur oxidised, the sulphur-carbon ratio of the organism isolated by Joffe being 31·8 (*loc. cit.*). As the organisms developed under normal conditions of temperature and medium composition, the sulphur-carbon ratio of 40·75 seems to be quite characteristic of the activated sludge organism; but it has been observed by Starkey (*J. Bact.*, 1925, **10**, 160) that with initial concentrations of 1 and 5 per cent. sulphuric acid the ratios were 36·6 and 43·0 respectively, with the organisms having 31·8 as the ratio. Hence under favourable conditions the organisms might have a high sulphur-carbon ratio. Such conditions being absent with the observations herein recorded, we are justified in classifying this as a distinct species of sulphur-oxidising organism.

In presence of involution forms working at a temperature (35°) most suited to their development, the amount of carbon assimilated was very small, and the sulphur oxidised was also low. If the same

TABLE X.

The Sulphur-Carbon Ratio with Normal forms.

Days	Mgms. of Carbon in 100 c.c. of medium.			Mgms. of carbon assimilated	Mgms. of sulphur as SO_4 in 100 c.c. of medium.			Mgms. of sulphur oxidised	Ratio of Sulphur to Carbon	P_H	Titer N/10 NaOH per c.c. of medium.	Total solids: mgms. in 100 c.c.
	1	2	Average		1	2	Average					
0 Control	2·59	1·78	2·18	...	7·69	8·24	7·96	...	...	4·6	0·39	570
26 ,, ...	2·46	2·04	2·25	...	7·69	8·24	7·96	...	...	4·6	0·38	580
42 ,, ...	2·49	2·01	2·25	...	7·69	7·69	7·69	...	...	4·6	0·38	570
Average ...			2·23				7·87					
6 Inoculated	4·24	3·71	3·98	1·75	46·70	47·25	46·98	39·11	22·35	3·0	0·63	710
12 ,, ...	4·31	4·31	4·31	2·08	100·60	100·60	100·60	92·73	44·63	2·4	0·96	890
16 ,, ...	6·12	5·30	5·71	3·48	143·38	145·03	144·21	136·34	39·13	1·8	1·30	1,080
23 ,, ...	7·80	5·52	6·66	4·43	212·04	212·04	212·04	204·17	46·07	1·6	1·65	1,520
30 ,, ...	7·44	6·72	7·08	4·85	236·77	234·58	235·68	227·81	46·95	1·6	1·79	1,600
37 ,, ...	8·55	8·76	8·66	6·43	270·30	270·30	270·30	262·43	40·83	1·4	2·04	1,760
45 ,, ...	10·08	10·38	10·23	8·00	370·26	370·26	370·26	362·39	45·29	1·4	2·63	2,310
								Average	40·77			

TABLE XI.

The Sulphur-Carbon Ratio with Involution forms.

Days	Mgms. of Carbon in 100 c.c. of medium			Mgms. of carbon assimilated	Mgms. of sulphur as SO_4 in 100 c.c. medium			Mgms. of sulphur oxidised	Ratio of Sulphur to Carbon	P_H	Titer N/10 NaOH per c.c. of medium	Total solids, mgms. in 100 c.c.
	1	2	Average		1	2	Average					
0 Control	1·65	1·96	1·80	...	8·79	8·36	8·57	...	...	5·0	0·22	360
20 ,, ...	2·50	2·18	2·34	...	8·79	9·18	8 98	...	...	5·0	0·23	366
Average ..	...	...	2·07	...	...	...	8·77	...	...	...	...	...
5 Inoculated	2·28	2·46	2·37	0·30	22·41	21·32	21·86	13·11	43·92	3·4	0·31	400
9 ,, ...	2·75	2·71	2·73	0·66	37·14	37·36	37·25	28·49	43·35	3·0	0 40	440
16 ,, ...	3·34	3·34	3·34	1·27	52·74	52·52	52·63	43·88	34·68	2·5	0·50	526
30 ,, ...	3·37	3·65	3·51	1·43	58·23	59·88	59·06	50·30	35 12	2 3	0·55	560
40 ,, ...	4·52	4·99	4·76	2·69	82·40	83·50	82·95	74·20	27·63	2 0	0·72	672

volume-weight of the organisms both of normal and involution forms is considered from the point of view of the total surface, it will be evident that the involution forms would naturally possess much less surface than the normal ones. As the rate of the reaction is proportional to the reacting surface, the energy requirements of the involution forms will be proportionately much less than those of the normal ones. Hence it is but natural to expect less intake of carbon by the involution forms with a corresponding decrease in the amount of sulphur oxidised. The velocity of oxidation in the latter instance has been reduced by 33·8 per cent. during the period of observation.

SUMMARY.

The cultural and physiological characteristics of a sulphur-oxidising organism isolated from activated sludge are described.

The organism develops on washed agar, silica jelly and gypsum blocks presenting both normal and involution forms; the characteristics and cultural behaviour are different from those observed by Joffe and co-workers. It withstands P_H 6·6 and can utilise carbohydrates to some extent, oxidising more sulphur in their presence. Certain inorganic salts also have a favourable effect, the influence of sodium silicate being very marked.

Additional air resulting in disturbance of the culture retards the progress of sulphur-oxidation.

The influence of vegetable and animal charcoals has been studied and is found to accelerate oxidation.

The sulphur-carbon ratio of the organism is 40·75; that of Joffe's 31·8. Thus the organism is much more economical in the utilisation of energy.

Peculiarities in the morphological and physiological behaviour, the higher sulphur-carbon ratio and other similar considerations afford data sufficient to classify the organism as a new species, capable of oxidising elemental sulphur to sulphuric acid and not accumulating sulphur within or without the cells.

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