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Sulphate Reduction in Partially Sterilized Soil Exposed to Air

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SUMMARY: H_2S was evolved from soil treated with CCl_4 when moistened with sucrose and $(NH_4)_2SO_4$ solution and incubated aerobically. H_2S formation took place when the soil moisture was less than field capacity, and over a range of pH values from 5 to 8. The organism responsible was isolated and identified as *Bacillus megaterium*. Several strains of this organism reduced sulphate in well-aerated sterilized soil and liquid media but not in soil or liquid incubated anaerobically.

The action of CCl_4 in fresh soil is to check or destroy certain fungi and bacteria which normally inhibit sulphate reduction by *B. megaterium*. Some of these organisms were isolated and shown to be sensitive to CCl_4 and to inhibit sulphate reduction by *B. megaterium* in sterilized soil. The isolates did not exhibit antibiotic action when grown in certain defined media.

The reduction of sulphate in soil is known to be achieved by *Desulphovibrio* species, and although there have been reports of other micro-organisms able to reduce sulphate (Nadson, 1904; Nastukoff, 1895; Sawjalow, 1913; Shturm, 1948; Tanner, 1918), the widely held view is that only *Desulphovibrio* spp. have been shown with certainty to bring about this reduction (Bunker, 1936; Butlin, Adams & Thomas, 1949; Starkey, 1950; Starkey & Wight, 1945; Young, 1936).

Desulphovibrio spp. are obligate anaerobes and utilize sulphate and other inorganic sulphur compounds as specific hydrogen acceptors during the oxidation of organic energy sources (Baars, 1930). The sulphur compounds are reduced to sulphides. Shturm (1950) claims to have obtained growth of *Desulphovibrio* spp. under aerobic conditions in meat extract broth without added sulphates, but the reduction of sulphate under aerobic conditions has not been reported in the literature as far as is known to the author. The present author observed that soil after treatment with CCl_4 released H_2S from sucrose + $(NH_4)_2SO_4$ solution under what appeared to be well-aerated conditions. When this observation is considered in the light of existing literature the problem arises of how sulphate reduction can occur under such conditions. This paper describes experiments to discover whether anaerobic conditions were developing in the apparently well-aerated soil, the nature of the micro-organisms responsible for the sulphate reduction and the effect of CCl_4 on the process.

METHODS

The soils listed in Table 1 were crushed with pestle and mortar and passed through a nest of sieves of 3, 2, 1 and 0·5 mm. mesh. The particles remaining on the 3 mm. mesh were discarded and the particles on the 2, 1 and 0·5 mm. mesh collected and kept separate. The size of the particles of soil referred to

below are defined by the diameter of the mesh which held them back; thus soil composed of particles which will pass through 3 mm. but not 2 mm. holes is referred to as 2 mm. soil.

Hydrogen sulphide evolution was detected with lead acetate paper. For pH determinations 5 or 10 g. of soil were suspended in 12·5 or 25 ml. distilled water respectively and shaken 20 times every ¼ hr. during 2 hr., after which the pH value was obtained by means of a glass electrode. Soil was sterilized by autoclaving 5 g. lots of dry sieved soil in 100 ml. conical flasks for 30 min. at 20 lb./sq.in.

Culture media

Sucrose $(NH_4)_2SO_4$ *solution.* Sucrose, 5·0 g.; $(NH_4)_2SO_4$, 1·25 g.; distilled water, 100 ml.; autoclaved 10 min. at 10 lb./sq.in. This solution was only used for the Rothamsted soil and was usually applied in the proportion of 2 ml./5 g. soil. For other soils the amounts of sucrose and $(NH_4)_2SO_4$ in 100 ml. of water depended on the water-holding capacity of the soil.

Sulphate reduction medium (Baars, 1930). K_2HPO_4, 0·05 g.; sodium lactate (70 % solution), 0·50 g.; NH_4Cl, 0·10 g.; $CaSO_4$, 0·10 g.; $MgSO_4.7H_2O$, 0·20 g.; $FeSO_4(NH_4)_2SO_4.6H_2O$, 0·05 g.; tap water, 100 ml.; pH 7·2; autoclaved 15 min. at 15 lb./sq.in.

Czapek agar, modified. $NaNO_3$, 0·2 g.; KCl, 0·05 g.; $MgSO_4.7H_2O$, 0·05 g.; $FeSO_4.7H_2O$, 0·001 g.; KH_2PO_4, 0·1 g.; distilled water, 100 ml.; sucrose, 3·0 g.; agar, 2·0 g.; pH 4·2; autoclaved 10 min. at 10 lb./sq.in.

Sucrose phosphate peptone agar (SPP). Sucrose, 0·5 g.; K_2HPO_4, 0·5 g.; Difco peptone, 0·5 g.; distilled water, 100 ml.; agar 2·0 g.; pH 7·5; autoclaved 15 min. at 15 lb./sq.in.

Medium (M) for H_2S *evolution by* Bacillus megaterium. KH_2PO_4, 0·5 g.; NaCl, 0·02 g.; $CaCl_2$, 0·01 g.; $(NH_4)_2SO_4$, 1·25 g.; sucrose, 5·0 g.; distilled water, 100 ml.; Difco yeast extract, 0·3 g.; $CaCO_3$, 0·1 g.; pH 6·5; autoclaved 15 min. at 15 lb./sq.in.

Medium for testing antagonisms. Sucrose, 1·0 g.; $(NH_4)_2SO_4$, 0·2 g.; Difco yeast extract, 0·05 g.; KH_2PO_4, 0·05 g.; $MgSO_4.7H_2O$, 0·02 g.; distilled water, 100 ml.; agar, 2·0 g.; pH 6·5; autoclaved 15 min. at 10 lb./sq.in.

RESULTS

Conditions under which H_2S *evolution occurs in* CCl_4*-treated soil*

The effect of sucrose and $(NH_4)_2SO_4$ *concentration on the evolution of* H_2S. Ten g. samples of 1 mm. mesh Rothamsted soil were spread evenly over the bottom of 100 ml. conical flasks. Duplicate flasks received the following amounts of sucrose and $(NH_4)_2SO_4$ made up in 3·6 ml. distilled water:

$(NH_4)_2SO_4$	0·05 g.	0·025 g.	0·005 g.	0·0025 g.
Sucrose	0·2 g.	0·1 g.	0·02 g.	0·01 g.

All the eight flasks then received 1 ml. CCl_4 and strips of lead acetate paper were hung in the flasks to within 1 cm. of the soil surface. After 7 days' incubation at 30° H_2S was detected in all flasks except those containing 0·01 g. sucrose and 0·0025 g. $(NH_4)_2SO_4$.

By keeping the sucrose at 0·2 g./flask and adding concentrations of $(NH_4)_2SO_4$ decreasing from 0·05 g./10 g. soil to zero, it was found that H_2S was evolved from all cultures except those to which no $(NH_4)_2SO_4$ was added. Similarly, by keeping the $(NH_4)_2SO_4$ at 0·05 g. and adding sucrose in concentrations ranging from 0·2 g./10 g. soil to zero, H_2S was evolved from all flasks except those containing no added sucrose. When the $(NH_4)_2SO_4$ was replaced by NH_4Cl, no H_2S was evolved. This result, together with the observation that no H_2S appeared when $(NH_4)_2SO_4$ was omitted, indicated that the sulphur in the H_2S came from the sulphate. An experiment described later supports this view.

Effect of soil type. To observe the effect of soil type on H_2S evolution, soils differing in texture and pH value were treated as follows: duplicate 10 g. samples of 1 mm. soil particles were spread evenly over the bottom of 100 ml. conical flasks and each was given 0·2 g. sucrose and 0·05 g. $(NH_4)_2SO_4$ in aqueous solution. The actual volume and hence the strength of the solution added varied with the soil. Each flask was then treated with 1 ml. of CCl_4. The texture, amount of solution added, the evolution of H_2S and the initial and final pH of the soils after 7 days' incubation at 30°, are given in Table 1. The Old Kennington and Rothamsted soils showed marked evolution of H_2S, Bones Close and Harpenden Common a trace, and Derby and Exton Park soils none.

Table 1. *Changes in* pH *values and presence of* H_2S *evolution from various soils moistened with sucrose + ammonium sulphate solution*

Incubation for 7 days at 30°.

Soil	Texture	Volume of solution added (ml.)	Initial pH value	Final pH value		H_2S produced (+) or not (−)	
				Control soil	Soil treated with CCl_4	Control soil	Soil treated with CCl_4
Old Kennington, Berks.	Sandy clay	2	6·3	5·7	6·4	+	+
Rothamsted, Herts.	Clay	4	6·5	6·8	6·5	−	+
Bones Close, Rothamsted, Herts.	Clay	4	7·0	7·0	7·0	−	±
Harpenden Common, Herts.	Clay	3·5	4·0	3·5	3·4	−	±
Exton Park, Rutland	Clay	4	7·2	7·1	7·2	−	−
Derby	Sandy loam	3	4·8	4·7	4·3	−	−

Effect of pH *value.* To find the pH range at which H_2S was evolved from Rothamsted soil 1 ml. distilled water and 0·25–2·0 ml. of N-HCl or N-NaOH were added to duplicate 5 g. samples of soil. The soil samples were allowed to dry at 40° for 48 hr. and then treated with sucrose + $(NH_4)_2SO_4$ solution and CCl_4. After 4 days' incubation at 30° the evolution of H_2S was noted and pH determinations made on the soils. Hydrogen sulphide was evolved between pH 5·0 and 7·7 and was greatest between pH 5·5 and 7·0. From the results of this and the previous experiment it was difficult to see why H_2S should not be

produced in Exton Park soil and to a greater degree in Bones Close soil. It was realized that factors affecting H_2S evolution could be different from those affecting sulphide formation, and that the H_2S once produced might form a non-volatile sulphide.

Effect of $CaCO_3$. It was noticed that Exton Park and Bones Close soils contained visible amounts of $CaCO_3$ and that Rothamsted and Old Kennington soils did not. To see what effect this $CaCO_3$ might have on the evolution of H_2S from Rothamsted soil, increasing quantities of $CaCO_3$ were added to duplicate 5 g. soil samples; the soils were then treated with sucrose + $(NH_4)_2SO_4$ solution and CCl_4, incubated for 4 days at 30° and then examined for H_2S evolution and their pH values determined. As little as 0·05 g. $CaCO_3$/5 g. soil decreased the evolution of H_2S and 0·2 g. prevented it; the pH values of the treated soils were between 7·0 and 7·5.

To see whether sulphide was present in the soils receiving 0·2, 0·3 and 0·5 g. $CaCO_3$ the suspensions used for pH determinations were acidified with concentrated H_2SO_4, and in all cases sufficient H_2S was released to darken lead acetate paper. Soils which did not receive sucrose + $(NH_4)_2SO_4$ solution did not give a positive test for H_2S on acidification. To see whether H_2S evolution could be obtained from Exton Park soil, 1·5 ml. 2N-HCl was added to 5 g. of the soil to decrease the amount of $CaCO_3$, the soil allowed to dry at 40° and then sucrose + $(NH_4)_2SO_4$ solution and CCl_4 applied; after 4 days at 30°, H_2S was evolved.

The effect of soil particle size, moisture content and amount of soil. The effect of particle size on H_2S evolution was studied by spreading evenly duplicate 5 g. samples of 2, 1 and 0·5 mm. mesh Rothamsted soil over the bottoms of 100 ml. conical flasks and treating them with sucrose + $(NH_4)_2SO_4$ solution and CCl_4. H_2S was evolved in all flasks after 4 days at 30°.

The effect of moisture content was studied as follows. To duplicate 10 g. samples of air-dried 1 mm. mesh with Rothamsted soil in 100 ml. conical flasks, increasing volumes from 0·5 to 8·0 ml. of sucrose + $(NH_4)_2SO_4$ solution were added. This experiment did not test the effect of moisture content alone, as the quantities of sucrose and $(NH_4)_2SO_4$ also varied. However, even in 0·5 ml. of the sucrose + $(NH_4)_2SO_4$ solution, there was 0·05 g. sucrose and 0·006 g. $(NH_4)_2SO_4$ which, from the experiment recorded above, was sufficient to allow H_2S evolution.

The soils which received from 0·5 to 2·0 ml. of solution were shaken to mix the wet and dry soil thoroughly, and then all soils received CCl_4. H_2S was evolved from all soils except those which received 0·5, 0·75 and 8·0 ml. of solution. The air-dried soil contained 2·5 % moisture, and the soil when at field capacity contained 25 % moisture. H_2S was evolved from Rothamsted soil which contained as little as 12·5 % moisture.

The effect of varying the amount of soil per flask was studied by spreading evenly 1 mm. mesh Rothamsted soil in duplicate amounts of 1–20 g. over the bottoms of 250 ml. conical flasks. The soils were moistened with sucrose + $(NH_4)_2SO_4$ solution in the proportion 0·4 ml./g. soil and then treated with 1·0 ml. of CCl_4. H_2S was detected in all flasks after 4 days at 30°.

Organisms responsible for sulphate reduction

An examination of the microflora in soil after treatment with CCl_4. It was possible that the CCl_4 may have stimulated the sulphate-reducing bacteria (*Desulphovibrio* spp.). To ascertain whether this were so, soil treated with sucrose + $(NH_4)_2SO_4$ solution and CCl_4, and soil treated with sucrose + $(NH_4)_2SO_4$ solution only, were incubated until H_2S was detected in the CCl_4-treated soil (2 days at 30°), after which time serial dilutions from 1/10 to $1/10^7$ of both soils were made in sterile water. Baars's sulphate reduction medium in test tubes and as agar plates was inoculated with 1 ml. of each of the dilutions and incubated under hydrogen in anaerobic jars for 10 days at 30°. With either soil treatment H_2S was found in liquid medium only when given 1/10 dilution of the soil.

To obtain some indication of the qualitative effect of CCl_4 on the microflora the following experiment was conducted. Two 10 g. samples of soil were each moistened with 4 ml. sucrose + $(NH_4)_2SO_4$ solution, and one soil sample received in addition 1 ml. CCl_4. They were incubated at 30° for 48 hr. and H_2S appeared in the CCl_4-treated soil after this time. To each soil sample 100 ml. sterile-distilled water was added and the samples shaken 100 times, allowed to stand for 10 min. and then shaken another 100 times. Serial dilutions up to $1/10^7$ were made from each suspension. The second shaking after 10 min. was necessary because the soil which did not receive CCl_4 was difficult to wet and remained in clumps bound by fungal growth after the first shaking. One ml. of dilutions of each soil sample were pipetted aseptically into sterile Petri dishes and mixed with melted (45°) sterile Czapek agar or SPP agar. The SPP agar plates were incubated aerobically and anaerobically at 30° and the Czapek plates aerobically at 30°. A general description of the growth developing on these plates is given below. Platings from the CCl_4-treated soil showed no fungi, few iridescent bacterial colonies and many white opaque colonies on the aerobic plates, and very little growth or gas formation on the anaerobic plates. Platings from the untreated soil showed abundant fungi, iridescent and opaque bacterial colonies on the aerobic plates. Iridescent and opaque colonies also appeared on the anaerobic plates and were accompanied by abundant gas formation.

To determine whether the organisms responsible for the H_2S evolution from the CCl_4-treated soil were developing on these two media, loopfuls of growth from various aerobic plates were suspended in sucrose + $(NH_4)_2SO_4$ solution, and 2 ml. of each of these suspensions were used to inoculate duplicate 5 g. samples of sterile Rothamsted soil. Two soil samples in duplicate were inoculated from each suspension, and one of these soil samples also received 1 ml. CCl_4. The results after aerobic incubation are given in Table 2.

H_2S was evolved from sterile soil inoculated with organisms from plates which received dilutions of CCl_4-treated soil, irrespective of whether the inoculated soil received CCl_4 or not. The addition of CCl_4 delayed the appearance of H_2S by 24 hr. Sterile soil inoculated with the organisms from plates which received dilutions of untreated soil only showed strong evolution of H_2S when

the soil received CCl_4. In the absence of CCl_4 these soils had a mat of fungal mycelia over their surfaces and gave only a slight trace of H_2S.

Thus the organisms responsible for H_2S evolution and the organisms which inhibit H_2S evolution both developed on these media; CCl_4 destroyed inhibitory organisms, which may be fungi or CCl_4-sensitive bacteria.

Table 2. *Hydrogen sulphide production from sterilized Rothamsted soil moistened with sucrose + ammonium sulphate solution, + or − CCl_4, and inoculated with organisms from various plates as shown below*

Part 1 Source of inocula for Part 2			Part 2 Sterile Rothamsted soil and nutrients + or − CCl_4			
			+		−	
			Time after inoculation with organism from plates of Part 1 aerobic, 30° (hr.)			
Soil and sucrose + $(NH_4)_2SO_4$ solution with (+) or without (−) CCl_4; 30° for 48 hr.	Suspension dilution (reciprocal)	Medium plates on 30° aerobic	24	48	24	48
			H_2S production (+ or −)			
+	10^4	Czapek	+	+	−	+
+	10^5	Czapek	+	+	−	+
+	10^5	SPP	+	+	−	+
+	10^6	SPP	+	+	−	+
−	10^4	Czapek	−F	−F	−	±
−	10^5	Czapek	−F	−F	−	±
−	10^5	SPP	−F	−F	−	+
−	10^6	SPP	−	−	−	+

F = fungal growth on soil surface.

Isolation and identification of the H_2S-producing micro-organisms. From previous experiments it was found that the organism which produced H_2S grew aerobically at 30° on SPP agar and survived CCl_4 treatment. Enrichment cultures were obtained in the following manner: 5 g. fresh Rothamsted soil were treated with 1 ml. CCl_4 and moistened with 2 ml. sucrose + $(NH_4)_2SO_4$ solution. The mixture was incubated at 30° for 3 days, suspended in 20 ml. sterile-distilled water and a loopful of the suspension plated on SPP agar. After 48 hr. at 30° a loopful of growth from the plate was suspended in 2 ml. sucrose + $(NH_4)_2SO_4$ solution and added to 5 g. sterile Rothamsted soil. The inoculated soil was treated with 1 ml. CCl_4 and incubated until H_2S was evolved. The re-inoculation procedure was repeated three times and from the plating of the suspension from the final enrichment flask, single colonies of different characteristics were picked off and were replated on SPP agar to test purity. Suspensions of these isolates in sucrose + $(NH_4)_2SO_4$ solution were used to inoculate samples of sterile soil with and without CCl_4. Of five isolates tested two produced H_2S. In soil cultures of these two isolates the evolution of H_2S appeared 24 hr. later when CCl_4 was added.

Identification of the isolates. The two organisms were similar in biochemical reactions. One was identified as *Bacillus megaterium* (Smith, Gordon & Clark, 1946).

Ten strains of *B. megaterium*, nos. 7851, 2605, 2607, 5635, 5636, 5637, 6007, 6095, 6347, 6005, 6094; two of *B. cereus*, nos. 8035, 7587, and two of *B. mycoides*, nos. 926, 7586, were obtained from the National Collection of Type Cultures. The ability of each of these strains to produce sulphide from $(NH_4)_2SO_4$ was tested on sterile soil. The soil was moistened with a suspension of each strain in sucrose + $(NH_4)_2SO_4$ solution, incubated at 30° and tested for H_2S evolution. H_2S was evolved from soil inoculated with all strains of *B. megaterium* but not from soil inoculated with the *B. cereus* or *B. mycoides* strains.

Experiments with pure cultures of Bacillus megaterium

Reduction of sulphate. An experiment was made to see whether the sulphur of the H_2S originated from the added sulphate. Radioactive sulphur as $(NH_4)_2{}^{35}SO_4$ was added to the sucrose + $(NH_4)_2SO_4$ solution so that 2 ml. of solution contained 5 μc. of ^{35}S. The solution was autoclaved, inoculated with *B. megaterium* and 2 ml. of it added to triplicate 5 g. samples of sterile Rothamsted soil contained in wide-mouthed 100 ml. conical flasks. The lead acetate paper which collected the radioactive H_2S was in the form of a 1·5 cm. diam. disk with a thin strip 6 cm. long projecting from it. The strip of paper was inserted into a piece of glass tubing so that the disk of paper protruded from one end of the tube. The other end of the tube was wrapped centrally inside a cotton-wool plug. The moist lead acetate paper when mounted in this way could be inserted and withdrawn without touching the inside of the flask and thereby avoided possible contamination of the paper with radioactive sulphate. The flasks were incubated for 24 hr. at 30°. The disks of lead acetate paper were completely black after this period.

The disks were removed, dried and then counted; the mean count rates were 993, 960 and 628 counts/min. To obtain some indication of the amount of sulphide trapped by the lead acetate paper, disks of filter-paper were moistened with 0·015, 0·02 and 0·05 ml. of the 5μc. ^{35}S solution, dried and counted. The mean count rates were 1004, 1367 and 2727 counts/min. respectively. The count rate of the lead sulphide disks approximated to that of 0·015 ml. of original solution which thus represented nearly 0·75 % of the added ^{35}S.

The effect of calcium carbonate, soil pH *value and low moisture content on hydrogen sulphide evolution.* The experiments on the effect of $CaCO_3$, pH value and moisture content on H_2S evolution, from unsterile soil treated with CCl_4 and sucrose + $(NH_4)_2SO_4$ solution, were repeated on sterile Rothamsted soil inoculated with a suspension of *B. megaterium* in sucrose + $(NH_4)_2SO_4$ solution; 0·05 g. added $CaCO_3$/5 g. soil greatly decreased the amount of H_2S evolved, and 0·2 g. $CaCO_3$/5 g. soil prevented it. When these cultures were acidified with H_2SO_4, H_2S was released in sufficient amounts to darken lead acetate paper. The pure culture of *B. megaterium* evolved H_2S from sterile soil at pH values between 5 and 8 and gave the greatest evolution between pH 5·5 and 7·0. Hydrogen sulphide was evolved from 10 g. sterilized soil moistened with as little as 1 ml. sucrose + $(NH_4)_2SO_4$ solution and inoculated with *B. megaterium*. These results are in agreement with those obtained earlier with the CCl_4-treated fresh soil.

Reduction of sulphate in soils of high moisture content. The evolution of H_2S was studied in soils of high moisture content. Duplicate 10 g. samples of sterile Rothamsted soil in 100 ml. conical flasks were moistened with 5 ml. *B. megaterium* suspension in sucrose + $(NH_4)_2SO_4$ solution, and also received 1–5 ml. sterile distilled water; another set of 10 g. samples of Rothamsted soil received 10–20 ml. of the *B. megaterium* suspension; a final set received 10 ml. suspension and 10 ml. sterile distilled water.

The soils which received 6–10 ml. of liquid were completely saturated but had soil crumbs projecting above the surface of the solution; those which received 15 and 20 ml. had depths of 2–3 and 4–5 mm., respectively, of liquid above the soil.

H_2S was evolved in sufficient amounts to blacken lead acetate paper completely from all flasks except those in which the soil was completely submerged. In the latter the edges of the lead acetate paper only turned a faint brown. There was no increase in the colour of the lead acetate paper after acidification of the cultures in these flasks.

Reduction of sulphate in liquid media. An attempt was made to obtain H_2S evolution from liquid media. The media were dispensed in duplicate 3 ml. lots in 100 ml. conical flasks, and a strip of lead acetate paper was suspended in each flask. One drop of a suspension of a 48 hr. SPP agar slope culture of *B. megaterium* was added to each flask and the contents incubated aerobically or anaerobically for 4 days at 30°. A number of different media were tested and only medium M was found to give H_2S evolution under aerobic incubation. No H_2S was evolved when Difco yeast extract was omitted or replaced by amino-acids or when $(NH_4)_2SO_4$ was replaced by NH_4Cl. Baars's medium was unsuitable for sulphate reduction by this organism.

Aeration experiments. Sterile Rothamsted soil was inoculated with a suspension of *B. megaterium* in sucrose + $(NH_4)_2SO_4$ solution and incubated: (*a*) *anaerobically* for 7 days under hydrogen in a McIntosh & Fildes jar; (*b*) *aerobically* (1) in a conical flask stoppered with a cotton-wool plug, (2) in a conical flask with a stream of water-washed air blowing on to the surface of the soil, (3) in a glass tube in which a layer of soil 1 cm. thick was supported on glass-wool half-way up the tube and water-washed air was passed through it from below. The water-washing of the air was to remove dust and to minimize evaporation from the soil. The air was delivered from an aquarium aeration pump at a rate of 100 ml./min. The soil which was incubated anaerobically for 7 days did not release H_2S but did so when subsequently incubated aerobically for another 2 days. With the three aeration treatments enough H_2S was released completely to blacken the lead acetate papers.

B. megaterium was plated on SPP agar and incubated aerobically or anaerobically. The anaerobic plates showed no growth whilst the aerobic plates showed abundant growth; the anaerobic plates developed abundant growth after a further 24 hr. aerobic incubation. To test for the presence of *Desulphovibrio* spp. as contaminants in the 'sterilized' soil and *B. megaterium* culture, sterilized soil and fresh soil were moistened with sterile Baars's medium and with Baars's medium inoculated with *B. megaterium*. The soils were incubated

aerobically and anaerobically for 10 days at 30°. H_2S was evolved only from the anaerobic fresh soil, which indicated that both the sterilized soil and the culture were free from *Desulphovibrio* spp.

The effect of carbon tetrachloride on the evolution of hydrogen sulphide from soils

Comparison with other volatile substances. Duplicate 5 g. samples of Rothamsted soil were treated with 2 ml. of one of the following substances: diethyl-ether, CCl_4, chloroform, toluene, xylene, 95 % (v/v) ethanol, benzene; or with 0·8 ml. of 2 % (v/v) phenol or 40 % formalin. H_2S was evolved from those soils treated with ether, CCl_4, chloroform or benzene. Similar experiments were repeated, but instead of using equal volumes of the volatile compounds the volumes used were the same as those lost by evaporation after 24 hr. These volumes were estimated by measuring changes in volume due to evaporation from a free surface 1 in. in diameter at hourly intervals. In this case the soils treated with ether, CCl_4, chloroform, toluene, xylene or benzene all produced an increase in H_2S evolution. A further attempt was made to get a similar effect with formalin, ethanol and phenol. Duplicate 5 g. samples of air-dried Rothamsted soil in Petri dishes were treated with 1 ml. of 1 % formalin, 2 % (v/v) phenol or 95 % ethanol, or subjected to the vapours from filter-paper moistened with 0·1 ml. 40 % formalin. The soils remained open to the air until the ethanol and formalin could not be detected by smell and until the phenol-treated soil was air dry. The soils were then moistened with sucrose + $(NH_4)_2SO_4$ solution and tested for H_2S. Only the ethanol-treated soils evolved H_2S on incubation.

To determine whether H_2S was evolved while CCl_4 was present or only after it had evaporated, three sets of flasks in duplicate containing 5 g. Rothamsted soil were treated with sucrose + $(NH_4)_2SO_4$ solution and 2 ml. CCl_4. The first set received no further treatment; the second had fresh lead acetate paper added every 24 hr.; the third had an additional 3 ml. CCl_4 added daily. H_2S was evolved in the first and second sets after 48 hr. incubation and continued to be evolved for another 48 hr. No H_2S was evolved from the third set during 7 days; after this time the daily additions of CCl_4 were stopped and 2 days later H_2S was evolved.

In another experiment, as the amount of CCl_4 was increased from 0·01 to 5 ml./5 g. soil the time for H_2S to be evolved increased from 1 to 3 days and the amount of H_2S increased too, as judged by the degree of darkening of the lead acetate paper. Thus it appeared that the H_2S was released after the CCl_4 had evaporated. H_2S was evolved from heated soil, heated at 70° for 1 hr. whether or not CCl_4 was added, but not from autoclaved soil. Thus it appeared that the effect of CCl_4 on H_2S formation was similar to a partial sterilizing effect.

Inhibition of the H_2S*-producing* Bacillus *by other micro-organisms*

Earlier experiments showed that H_2S evolution in soil could be inhibited by CCl_4-sensitive micro-organisms (see Table 2).

Isolation of inhibitory micro-organisms. In a preliminary experiment it was found that *B. megaterium* would not grow on SPP agar containing 1 part in 750,000 of crystal violet. The following experiment was planned to find whether the organisms which developed on crystal violet SPP agar were CCl_4-sensitive. Two Rothamsted soil samples received sucrose + $(NH_4)_2SO_4$ solution and were incubated for 3 days; one sample also received CCl_4. Crystal violet SPP and SPP agar plates were inoculated with 1 ml. of $1/10^4$ to $1/10^8$ dilutions of these soil samples. One lot each of crystal violet SPP and of SPP agar plates were flooded with 1 ml. CCl_4 immediately after inoculation and another lot was left untreated. The duplicate plates were incubated at 30° for 96 hr. The growths which resulted from the $1/10^8$ soil dilution is given in Table 3

Table 3. *Growth of* SPP *agar and crystal violet* SPP *agar inoculated with* $1/10^8$ *dilutions of soils after various treatments*

Initial treatment of soil in addition to sucrose + $(NH_4)_2SO_4$ solution	Medium inoculated	Treatment given to plate	Type of growth
CCl_4, 1 ml.	SPP	1 ml. CCl_4	Large white to buff, smooth and rough, shiny and dull, opaque bacterial colonies. No iridescent colonies.
CCl_4, 1 ml.	Crystal violet SPP	1 ml. CCl_4	No growth
CCl_4, 1 ml.	SPP	None	Large white buff, smooth, rough, shiny, dull, opaque bacterial colonies. Granular iridescent colonies
CCl_4, 1 ml.	Crystal violet SPP	None	No growth
None	SPP	1 ml. CCl_4	White, buff, smooth, shiny, dull, rough, opaque colonies and granular iridescent colonies
None	Crystal violet SPP	1 ml. CCl_4	No growth
None	SPP	None	Fungi, white, buff, opaque colonies, large mucoid colonies and non-granular, bluish, iridescent colonies
None	Crystal violet SPP	None	Fungi, large mucoid, some opaque bacterial colonies, also bluish, iridescent colonies

The results for a given treatment were similar at all dilutions. The micro-organisms which developed on crystal violet + SPP agar were CCl_4-sensitive and the micro-organisms which were CCl_4-resistant were crystal violet-sensitive. At the above dilutions there were no crystal violet-tolerant micro-organisms in soil treated with CCl_4, whereas in the untreated soil there were some micro-organisms which were sensitive and some which were tolerant to either crystal violet or CCl_4. CCl_4-sensitive bacteria were isolated from the crystal violet + SPP agar plate which received the $1/10^8$ dilution of untreated soil. The isolates

were replated to test their purity and checked for CCl_4 sensitivity in the following manner; SPP agar was plated with the isolate, flooded immediately with 1 ml. CCl_4 and incubated, after the CCl_4 had evaporated, for 72 hr. at 30° and then examined for growth. Fungi were isolated from the $1/10^4$ dilution of the same soil on Czapek agar and tested for CCl_4 sensitivity in the same way. In the above manner six cultures of CCl_4-sensitive bacteria and four cultures of CCl_4-sensitive fungi were obtained.

The effect of CCl_4*-sensitive micro-organisms on* H_2S *evolution by* B. megaterium. To determine whether these micro-organisms inhibited the evolution of H_2S, 5 g. samples of sterile Rothamsted soil were inoculated with a mixed suspension of *B. megaterium* and the micro-organisms to be tested; some of the soil samples also received CCl_4. A micro-organism was considered to be inhibitory when H_2S was evolved from the CCl_4-treated soil but not from the untreated soil to which it had been added. The four fungi and six CCl_4-sensitive bacteria were tested in this way against *B. megaterium*; one fungus and three bacteria inhibited the evolution of H_2S. The fungus was identified as a member of the genus *Mucor*, two of the inhibiting bacteria as species of *Pseudomonas*, and the other a species of *Bacterium*. One of the CCl_4-sensitive bacteria which did not inhibit H_2S formation by *B. megaterium* belonged to the genus *Achromobacter*, and the other two to the genus *Aerobacter*. Thus the inhibitory bacteria did not belong to the same species or even the same genus and not all the CCl_4-sensitive bacteria inhibited the formation of H_2S by *B. megaterium*.

An attempt was made to see whether the inhibition of H_2S evolution was due to an antibiotic effect of the fungus and one of the bacteria on the *B. megaterium*. Plates of sucrose + $(NH_4)_2SO_4$ medium were inoculated, either over the whole surface with a mixture of the test organism and *B. megaterium* or inoculated in straight lines with the inhibitory organism at right angles to the *B. megaterium*, and incubated for 5 days at 30°. No antagonism was observed on the plates inoculated by streaking. On the mixed inoculum plates the *B. megaterium* formed slightly smaller colonies when grown in the presence of the bacterium as compared with the colonies of the pure culture of *B. megaterium*; the fungus showed no inhibition on the mixed plates.

The effect of phenol, formalin and ethanol on B. megaterium *and on the inhibitory micro-organisms.* Phenol, formalin and ethanol are known to have a partial sterilizing effect when applied to soil, but in the experiment described earlier phenol, formalin or ethanol in the presence of water did not exhibit the same partial sterilizing effect as CCl_4. To see what effect these substances had on *B. megaterium* and the inhibitory organisms, SPP agar plates were inoculated with these micro-organisms and flooded immediately after with 1 ml. of 2 % phenol, 1 % formalin or 95 % alcohol, and examined for growth after 7 days' incubation at 30°.

The inhibitory bacteria and the fungi were killed by all three solutions, *B. megaterium* only by formalin. *B. megaterium*, however, failed to grow on SPP agar which contained 0·7 % phenol or 30 % (v/v) ethanol in water. These results explained why phenol, formalin and ethanol solutions did not behave in the same way as CCl_4. Formalin kills *B. megaterium* as well as the inhibitory

micro-organisms; phenol and ethanol, however, because of their miscibility with water, remain in the soil, and although they do not kill the spores of *B. megaterium* they prevent their germination.

DISCUSSION

Previously the biological reduction of sulphate to H_2S has been associated with anaerobic conditions and the activities of anaerobic sulphate-reducing *Desulphovibrio* spp. The results of the present work show that sulphate can be reduced under well-aerated conditions by the aerobic *Bacillus megaterium*. From the fact that sulphide was produced in only one of a number of liquid media it would appear that to get a complete understanding of the activities of soil micro-organisms it is necessary to study their behaviour in soil as well as in laboratory media. The high sugar and $(NH_4)_2SO_4$ concentrations used in the experiments makes it unlikely that this type of reduction occurs to any marked extent in normal soils, but a study of reduction in the presence of other carbon and nitrogen sources might help in assessing its importance in nature.

The part played by CCl_4 is one of partial sterilization. As a result of this, a number of different micro-organisms are killed, and amongst these are some which inhibit the evolution of H_2S by *B. megaterium*. These results indicate that in soil the type of reaction carried out by a particular micro-organism may depend, in part, on the types of accompanying micro-organisms; and that a number of processes normally not active in soil may become intensified as a result of the simplification of the soil microflora by partial sterilization. The way in which some of the CCl_4-sensitive micro-organisms inhibit sulphate reduction by *B. megaterium* is not known; no antibiotic effect could be demonstrated.

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