OXIDATION OF MANGANESE BY SOIL MICROORGANISMS

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Summary

Oxidation of manganous sulphate by an association of two bacteria and by a newly-isolated strain of a *Corynebacterium* sp. was studied in an artificial medium containing 0·005 per cent. yeast extract, 0·005 per cent. manganous sulphate, and various other inorganic salts.

The organisms produced an insoluble manganese oxide which was intimately mixed with bacterial cells. Oxidation of manganese occurred in growing cultures of the new isolate just after the organism reached the stationary phase in its growth-cycle and it appeared that the organism did not grow autotrophically during the period of oxidation. This period coincided with the maximum oxidizing activity of cells grown in manganese-deficient medium. Cells harvested from this medium were readily able to oxidize a 0·005 per cent. manganous sulphate solution; this reaction was inhibited by boiling and by various poisons, especially catalase inhibitors and hydrogen peroxide, and was not accelerated by any of the substrates tested. The mechanism of oxidation is discussed.

I. Introduction

Manganous sulphate is readily oxidized when it is added to neutral soils (Gerretsen 1937; Leeper and Swaby 1940; Mann and Quastel 1946). This oxidation is the result of the activity of soil microorganisms (Gerretsen 1937; Bromfield and Skerman 1950; Timonin 1950*b*).

Manganese-oxidizing organisms have been isolated from soil using various media. Söhngen (1914) used a medium containing calcium malate, and later, Gerretsen (1937), McLachlan (1941), and Timonin (1946) used a medium containing calcium citrate. Söhngen concluded that malate and other hydroxyacids, including citrate, catalyse the oxidation of manganese and that the reaction is favoured by an increase in alkalinity. He suggested that the microbial oxidation of manganese in the presence of citrate is due to the production of an alkaline pH, which accelerates this catalytic oxidation.

Bromfield and Skerman (1950) showed that many organisms which oxidize manganese in the presence of citrate cannot oxidize manganese in soil, and they concluded that a medium containing citrate was undesirable for isolating the manganese-oxidizing organisms active in soil. They used a soil-water-extract agar in preference to the citrate medium and isolated a pair of bacteria which could oxidize manganese added to soil. Others have also observed oxidation in media which do not contain citrate. Beijerinck (1914) used a manganous carbonate agar, Thiele (1925) employed peat extract and low concentrations of peptone, and Timonin (1950*a*, 1950*b*) observed oxidation in the presence of agar alone and with various nitrogen sources. Numerous fungi and some bacteria have been isolated on these non-citrate media, but these isolates have not been studied in detail, and the mechanism of oxidation is still obscure. Beijerinck (1914) suggested that the oxidation resulted from autotrophic

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growth. Waksman (1940) and Starkey (1955), however, concluded that this question was still unanswered. Beijerinck (1914) and Thiele (1925) observed that the oxide was deposited outside the mycelia of oxidizing fungi, and postulated that an extracellular enzyme was involved. Beijerinck (1914) claimed that the oxidation product was manganese dioxide.

This paper describes the preparation of a medium suitable for studying manganese oxidation, factors affecting oxidation by pure cultures of bacteria, and a study of the mechanism of oxidation.

II. Media and Methods

Mixed cultures of species of *Corynebacterium* and *Chromobacterium*, previously studied by Bromfield and Skerman (1950), were grown in a manganese medium of the following composition:

Distilled water	100 ml	$Ca_3(PO_4)_2$	0·010 g
KH_2PO_4	0·005 g	"Difco" yeast extract	0·005 g
$MgSO_4.7H_2O$	0·002 g	$MnSO_4.4H_2O$	0·005 g
$(NH_4)_2\,SO_4$	0·010 g		

The pH of the medium was adjusted to 6·0 and was autoclaved at 10 lb/sq. in. for 15 min. The medium was also used in a solid form after the inclusion of 2 per cent. "Difco" agar.

This manganese medium was preferred to the soil-extract manganese medium of Bromfield and Skerman (1950) because it was more defined and easier to prepare. The manganese remained unoxidized in the sterile medium and in this regard it is to be preferred to a citrate medium since oxidized manganese can be detected in the latter.

The liquid medium was dispensed in either $6 \times \frac{3}{4}$ in. test tubes or 1-l. beakers and inoculated with one loopful or 1 ml, respectively, of a suspension of a 24-hr culture grown on nutrient agar (0·5 per cent. sucrose, 0·3 per cent. "Difco" yeast extract, and inorganic salts as above). The inoculated medium was incubated at 27°C for periods up to 21 days during which time a dark brown deposit was formed which gave a deep blue colour with 1 per cent. (w/v) benzidine hydrochloride solution in 2N acetic acid, indicating that the manganese had been oxidized.

Determinations of pH were made with a glass electrode. Manganese determinations were made colorimetrically by oxidizing manganous ion to permanganate with periodate. Manganese in the deposit was reduced to manganous ion with sodium sulphite and sulphuric acid before the addition of periodate. The permanganate colour was measured in a "Unicam" spectrophotometer at 530 mμ.

Viable cell counts were determined by mixing serial 10-fold dilutions of the culture with nutrient agar and counting the number of colonies which developed after 48 hr.

Total cell counts were made on samples of 5 ml of culture stained with 1 drop of 1 per cent. aq. Victoria blue 4R. In oxidizing cultures the oxide cementing material was dissolved with acidified sodium sulphite solution before staining. The cells were then counted on a haemocytometer slide.

Cell suspensions which oxidize manganese solutions were prepared using the cells harvested by centrifugation from cultures grown in a manganese free medium. The cells were suspended in distilled water to give a suspension 50-fold more concentrated than the original culture. A cell suspension prepared in this way oxidized manganese within 5 min at 35°C. Attempts to obtain large amounts of active cell material from the growth which occurred on ordinary nutrient media failed. Activity of cell suspensions was determined by mixing 1 ml of the suspension with 1 ml of 0·01 per cent. (w/v) manganous sulphate solution in a $6 \times \frac{3}{4}$ in. test tube, incubating in a water-bath at 40°C, and noting the time for the appearance of manganese oxide, or alternatively, noting the relative amount of oxidation after a given time.

III. Experimental Results

(*a*) *Experiments with Manganese Medium*

The results of the experiments about to be described formed the basis for the composition of the manganese medium used in this investigation.

Table 1

EFFECT OF VARIOUS CONCENTRATIONS OF MANGANESE AND YEAST EXTRACT ON THE TIME FOR THE APPEARANCE OF, AND DEGREE OF, MANGANESE OXIDATION

N = No oxidation after 21 days; P = poor oxidation; G = good oxidation

Concentration of Yeast Extract (g %)	Time in Days for, and Degree of, Oxidation							
	Concentration of $MnSO_4.4H_2O$ (g %)							
	0·00	0·001	0·005	0·01	0·02	0·03	0·05	0·1
0·00	N	N	N	N	N	N	N	N
0·001	N	6, P	6, P	6, P	6, P	6, P	N	N
0·005	N	1, P	1, G	1, G	2, G	6, G	16, P	N
0·01	N	1, P	1, G	1, G	2, G	6, G	16, P	N
0·5	N	N	N	N	N	N	N	N

(i) *Effect of Varying the Composition of the Manganese Medium on the Oxidation of Manganese.*—The concentrations of manganous sulphate and "Difco" yeast extract were varied over a range of 0–0·1 per cent. (w/v) and 0–0·5 per cent. (w/v) respectively. The effect this variation had on oxidation is given in Table 1.

Most rapid and complete oxidation of manganese occurred in the media which contained 0·005–0·01 per cent. manganous sulphate and 0·005–0·01 per cent. yeast extract. With higher or lower concentrations of either of these substances oxidation was retarded or did not occur at all. Oxidation was absent in media without yeast extract and in media containing 0·5 per cent. yeast extract or 0·1 per cent. manganous sulphate. The inhibition of oxidation in media containing 0·1 per cent. manganous sulphate was due to inhibition of growth. In the media containing non-

inhibitory amounts of manganous sulphate and 0·5 per cent. yeast extract, growth was very good but oxidation of manganese could not be detected. A concentration of 0·005 per cent. (w/v) for both manganous sulphate and "Difco" yeast extract was used in the manganese medium in further experiments.

Calcium phosphate was replaced by other sources of calcium in equivalent amounts and it was found that oxidation occurred equally well with calcium carbonate, decreased with calcium chloride (pH of medium adjusted to 6·0 with sodium hydroxide), and did not occur at all with calcium chloride (pH 5·3). When ammonium sulphate was replaced by ammonium nitrate or sodium nitrate the oxidation of manganese decreased. The omission of one or other of the inorganic salts from manganese medium either prevented oxidation or delayed its onset 7–8 days With the complete inorganic salt complement oxidation commenced within 24 hr.

To determine at what pH values oxidation occurred in this medium, the pH was adjusted with 0·1N hydrochloric acid, and 0·1N sodium hydroxide to give a pH range of 3·7–8·4. The media were inoculated and incubated for 1 week, after which the cultures were examined for oxidation and growth and final pH values were determined. Oxidation occurred in media with an initial pH value of between 4·2 and 7·0, but not at 3·7 or above 7·6. During growth the pH increased by 0·6 of a unit in the more acid media, and decreased by 0·7 of a unit in the alkaline media. In media with an initial pH of 5·4 there was no change in pH during oxidation. Growth was comparable at all pH values except below 4 and above 8, in which cases it was poorer. It appears that a pH value between 5 and 6 is most suitable for oxidation in this medium.

"Difco" yeast extract was replaced in liquid manganese medium by the organic substances listed in Table 2. The final concentration of organic substance was 0·005 per cent. and the pH of the medium adjusted to 6·0. The relative amount of oxidation after 1, 3, and 21 days is also given in the table.

Oxidation was greatest in the "Difco" yeast extract, glucose, and mixed amino acid media but oxidation occurred more rapidly in the first. Slight to moderate oxidation occurred when various amino acids, α-ketoglutaric acid, and pyruvic acid were present, but other amino acids and organic acids were ineffective. The mineral-salts control also gave no oxidation. It was found subsequently that the addition of various growth factors and vitamins with the above substrates had no stimulating effect on the oxidation of manganese by the bacteria. It is of interest to note at this stage that oxidation occurred after 14 days on a mineral-salts control medium solidified with agar. This means that agar itself provided sufficient substrate to enable the organisms to oxidize manganese.

(ii) *Behaviour of Manganese-oxidizing Bacteria Isolated on Gerretsen's Medium.*— To determine whether these bacteria could also oxidize manganese in the "new" manganese medium, previously-isolated manganese-oxidizing bacteria (Bromfield and Skerman 1950) were plated on it. Of the six bacteria tested only one was able to oxidize on the new medium. This indicates that it is more selective than Gerretsen's and that oxidation on it may be a specific property of the bacterium and not due merely to the production of an alkaline pH.

(*b*) *Studies on the Relationship between* Corynebacterium *sp. and* Chromobacterium *sp. in the Oxidation of Manganese*

It has been shown (Bromfield and Skerman 1950) that oxidation of manganous sulphate occurs in a mixed but not in a single pure culture of the above two organisms. Experiments were made to find whether this was due to the production of a readily diffusible substance by one organism, which enabled the other to oxidize manganese.

TABLE 2

RELATIVE AMOUNTS OF MANGANESE OXIDE PRODUCED IN LIQUID MANGANESE MEDIUM CONTAINING VARIOUS SUBSTRATES

− = No oxidation; + = faint deposit, light brown; ++ = moderate deposit, brown; +++ = heavy deposit, dark brown

Organic Substance (0·005%)	Relative Amounts of Oxide after:		
	1 Day	3 Days	21 Days
"Difco" yeast extract	++	+++	+++
Davis gelatin	+	+	++
Glucose	−	++	+++
DL-Alanine	−	+	+
DL-Serine	−	−	∓
DL-Aminobutyric acid	−	−	−
DL-Threonine	−	−	−
DL-Valine	−	−	−
L-Leucine	+	+	±
L-Asparagine	+	+	+
L-Glutamic acid	+	+	++
L-Lysine HCl	−	+	+
L-Histidine HCl	−	+	+
L-Cystine	−	−	−
DL-Methionine	−	−	−
DL-β-Phenylalanine	−	+	++
L-Tyrosine	+	+	++
L-Tryptophan	−	−	−
Mixed amino acids	++	++	+++
α-Ketoglutaric acid	−	+	++
Pyruvic acid	−	−	+
Citric acid	−	−	−
Succinic acid	−	−	−
Fumaric acid	−	−	−
Malic acid	−	−	−
No substrate	−	−	−

Cultures of the two organisms were grown separately in 10 ml of manganese medium for 3 days at 27°C; they were then either heated at 70°C for 3 min, filtered through a Seitz sterilizing filter, and treated with 1 ml ether, or left untreated and then re-inoculated with the other bacterium. After 10 days at 27°C only the untreated, doubly-inoculated cultures gave oxidation. Sterility checks showed that in all the treated tubes only the organism used for the second inoculation was present.

Following this experiment, the two bacteria were grown in the same medium but in such a way that pure cultures were separated from one another by a collodion membrane. In this case oxidation did not occur unless both organisms were present together on one side of the membrane. It was shown that the constituents of the medium could diffuse through the membrane. This was done by separating the sterile medium from doubly-inoculated distilled water. In this case oxidation occurred in the side containing the distilled water.

In order to determine whether active growth by both organisms was necessary for oxidation, the two species were grown successively on agar plates. The first organism was allowed to grow for 3 days and then treated with 1 ml ether. After the ether had evaporated the plates were re-inoculated with the second organism and incubated for a further 3 days. The ether treatment did not kill all the organisms and, as a result, oxidation occurred on the doubly-inoculated plates but only where the surviving organisms developed. A microscopic examination of these sites of oxidation revealed that the oxide was dense and confined within the surviving *Corynebacterium* colonies, but was only scattered thinly above the *Chromobacterium* colonies. The deposition of oxide in *Corynebacterium* colonies could be demonstrated in another way by streaking the two organisms in parallel lines on the same medium. The *Chromobacterium* sp. due to its spreading habit of growth on manganese medium, eventually overgrew the *Corynebacterium* colonies and where this happened oxidation occurred (Plate 1, Fig. 1). This figure also illustrates how intimate is the association of the two organisms leading to oxidation, a colony of *Corynebacterium* sp. which is three-quarters covered by the spreading *Chromobacterium* sp. shows oxidation only in that portion.

To determine whether mixtures of cell suspensions of *Corynebacterium* sp. and *Chromobacterium* sp. could oxidize a manganous sulphate solution, these organisms were grown in pure and mixed culture in manganese-deficient medium. The cells were harvested by centrifugation when a manganese medium control started to show oxidation. Four cell suspensions were prepared from the harvested cell material: (1) a suspension of *Corynebacterium*, (2) a suspension of *Chromobacterium*, (3) a mixed suspension prepared from the above two, and (4) a suspension of the growth from the mixed culture. These suspensions were corrected for dilution and incubated at 35°C with 0·005 per cent. (w/v) manganous sulphate. After 1 hr incubation, only suspension (4) produced oxidation. This result also indicates that the two organisms have to be growing together to produce the oxidizing system.

(c) *Isolation of a Single Pure Culture of a Bacterium Capable of Oxidizing Manganese in Manganese Medium*

It was observed on plates of manganese medium inoculated from single colonies of the *Corynebacterium* sp. (subsequently referred to as strain A) that a few single colonies showed segments which contained manganese oxide (Plate 1, Fig. 2). On plating these segments, completely brown and completely white colonies developed (Plate 1, Fig. 2). The brown colonies, which will be called strain B, usually gave colonies on re-plating which were completely brown, although sometimes white segments or wholly white colonies developed, especially in old cultures (14 days) (Plate 1,

Fig. 4). These white colonies were shown to be similar to strain A. Strain A and strain B were subjected to the standard biochemical tests used for identification and were found to differ in these tests only in their ability to oxidize manganese. Strain B when inoculated into sterile soil alone and treated according to Gerretsen's technique (Gerretsen 1937) produced a brown-ring deposit of manganese oxide.

(*d*) *Oxidation of Manganous Sulphate by Growing Cultures of* Corynebacterium *Strain B*

Experiments were next undertaken to determine the extent of oxidation which occurred in growing cultures of strain B, and to find at what stage during its growth oxidation took place.

(i) *Extent of Oxidation.*—To determine the amount of oxide which had formed at various times during the growth of strain B, the amounts of manganese in solution and in the deposit were measured at 2-hourly intervals over a period of 48 hr. The inoculum used was 1 ml of suspension per 500 ml of medium. The results of these determinations are presented in Figure 1.

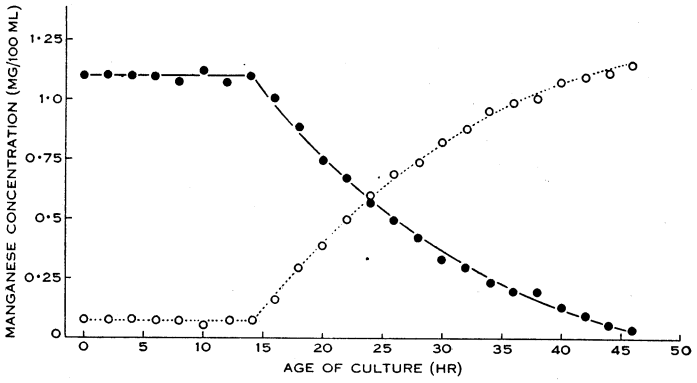

Fig. 1.—Rate of oxidation of manganous sulphate in manganese medium by *Corynebacterium* strain B. ● Mn^{++} in solution. ○ Mn^{++} in deposit after reduction. Mn determined as permangate.

Oxide commenced to form after an initial incubation period of 15 hr. The rate at which it formed declined progressively during the course of the experiment and after about 48 hr oxidation was virtually complete. Qualitative tests for higher valency manganese were made on the supernatant of centrifuged samples during the experiment, to see if oxidized manganese was in solution. However, this could not be demonstrated with benzidine hydrochloride.

(ii) *Nature of Growth Curve during Oxidation.*—To ascertain at what stage during the growth of the culture oxidation commenced, viable cell counts were made on the supernatant of cultures similar to the above. The results of these counts are presented in Figure 2 together with a brief description of the appearance of the culture at arbitrary stages indicated in the figure by the letters A to H.

The pertinent observation is that the appearance of oxide in the culture occurred just after the onset of the stationary phase in the growth curve, i.e. after 18- to 20-hr

incubation. This apparently coincides with the onset of the active oxidative period determined in the previous experiment.

(iii) *Examination of the Apparent Fall and Rise in the Number of Viable Cells during Oxidation.*—The dip during the interval D to F (Fig. 2) was confirmed in other experiments although its magnitude was not constant. It was of special interest since the rise might have been due to autotrophic growth. If cell multiplication had taken place during this period there should be approximately twice as many cells

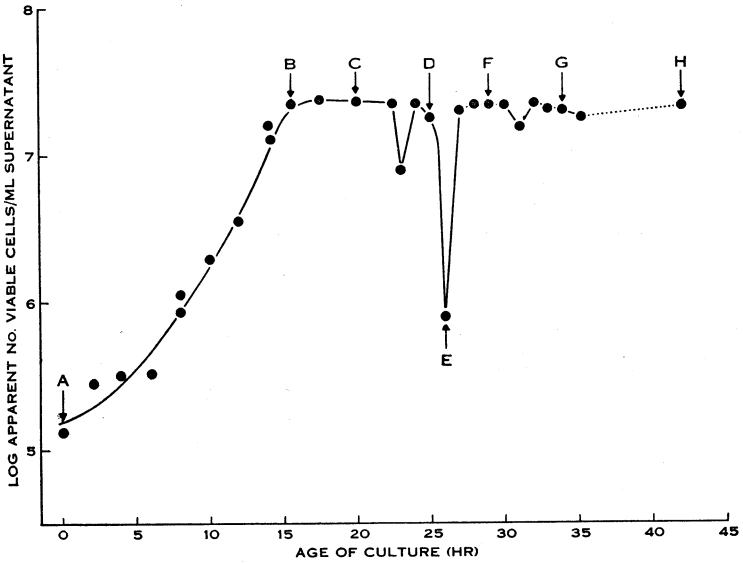

Fig. 2.—Number of viable cells in supernatant of a culture of *Corynebacterium* strain B growing in manganese medium. Appearance of the culture: *A-B*, increasing milky turbidity; *B-C*, culture becomes very pale yellow; *C-D*, culture light brown with small brown particles in suspension, slight deposit; at *E*, light brown, fairly large brown particles in suspension which are visibly settling out, moderate deposit; *E-F*, increasing deposit, supernatant lighter in colour but still containing suspended brown particles; *F-G*, supernatant turbid, milky with very few brown particles, heavy dark brown deposit; at *H*, supernatant turbid, milky, no obvious brown particles in suspension, heavy dark brown deposit.

in the culture at F as at D. Total cell counts at C and at G were next undertaken to determine whether multiplication had, in fact, taken place. The experimental procedure was modified to overcome the interference of calcium phosphate particles in the direct counting. The cultures were grown for 17 hr in the complete medium at which time the supernatant was transferred to sterile 1-l. beakers and given further incubation. Direct counts were made of the number of oxide clumps and free cells in the suspended culture and also of the total number of cells after removal of oxide. Counts of viable cells were also made at C and G. For comparison, manganese-deficient medium was also examined in the same way. The various determinations are presented in Table 3.

The counts show that the total numbers of cells in the manganese medium did not increase greatly during the course of the oxidation and were virtually the same as the total number of cells in the manganese-free medium. The organism, therefore,

apparently does not derive energy for growth during the oxidation of manganese: this energy must come wholly from the yeast extract. The fact that there was no gross change in the total number of cells indicates that the dip in viable cells during

TABLE 3

NUMBERS OF OXIDE CLUMPS, CELLS IN SUSPENSION, TOTAL CELLS, AND VIABLE CELLS OF CORYNEBACTERIUM STRAIN B GROWN IN MANGANESE-DEFICIENT AND MANGANESE MEDIA

Age of Culture (hr)	Stage (see Fig. 2)	Numbers/Ml ($\times 10^{-5}$)					
		Manganese Medium				Manganese-deficient Medium	
		Oxide Clumps	Cells in Suspension	Total Cells	Viable Cells	Total Cells	Viable Cells
17	*C*	4	287	323	201	334	195
41	*G-H*	8	228	375	64	370	104

the interval D to F was an apparent one, due possibly to the adsorption and subsequent release of large numbers of viable cells by the surface of the oxide clumps. The observed fall in the number of viable cells (Table 3) is attributed to the increased aeration the cultures received during transferring and suspending procedures.

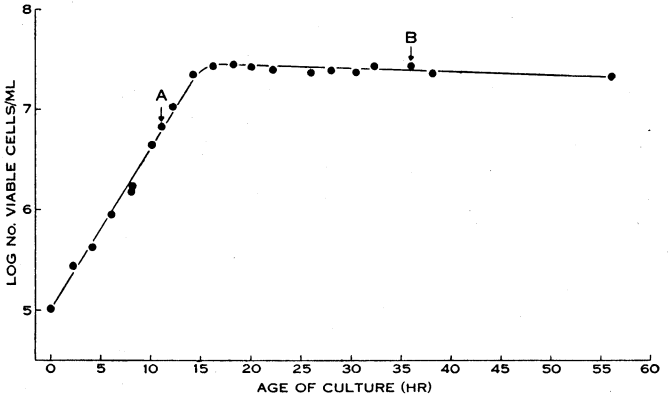

Fig. 3.—Number of viable cells in supernatant of a culture of *Corynebacterium* strain B grown in manganese-deficient medium. See also Table 4 for description of oxidizing activity of cells harvested during period *A-B*.

(iv) *Nature of the Oxide Deposit.*—A microscopic examination of the brown material which formed in the above cultures revealed that individual cells ($1 \times 2\ \mu$) first became coated with a thin layer of brown material, these cells then formed aggregates (about $10\ \mu$ in diameter) which in their turn came together and formed a loose sponge-like deposit (Plate 2, Figs. 1, 2, and 3). The precipitate is thus an intimate mixture of bacterial cells and manganese oxide.

(e) *Manganese Oxidation with Cell Suspensions*

In an attempt to elucidate the nature of the oxidation, experiments were undertaken with cell suspensions.

(i) *Age of Culture.*—To determine at what stage of growth strain B exhibited maximum oxidizing activity, its cells were harvested and tested at various stages during its growth. Activity was measured by noting whether oxidation had taken

TABLE 4

OXIDIZING ACTIVITY OF CELLS HARVESTED FROM MANGANESE-DEFICIENT MEDIUM AT VARIOUS STAGES OF GROWTH OF CORYNEBACTERIUM STRAIN B

− = No oxidation; ± = query oxidation; + = oxidation

Stage (see Fig. 3)	Age of Culture (hr)	Fresh Cells			Cells Rested 30 Min		
		Oxidation after Incubation with Manganese for:			Oxidation after Incubation with Manganese for:		
		5 Min	15 Min	30 Min	5 Min	15 Min	30 Min
A	11	−	−	−	−	−	−
	14	−	−	−	−	−	−
	16	−	−	−	−	−	−
	18	−	−	+	−	+	+
	20	−	−	+	+	+	+
	22	−	+	+	+	+	+
	24	−	+	+	+	+	+
	26	+	+	+	+	+	+
	28	+	+	+	+	+	+
	30	−	+	+	−	±	+
	32	−	+	+	−	+	+
	34	−	−	+	−	−	+
B	36	−	−	−	−	−	−

place after 5, 15, and 30 min. Viable cell counts were made at the time of sampling and the cells from the centrifuged sample were tested both as fresh cells and after allowing them to stand for 30 min. The results of this experiment are illustrated in Table 4 for the period of growth between A and B in Figure 3. The manganese-oxidizing activity of the cells coincided with the onset of the stationary phase and first appeared 18 hr after inoculation with a viable cell count of $2{\cdot}8 \times 10^7$ cells per ml. The activity of the harvested cells increased to a peak during the next 8 hr and then lost activity until, at 36 hr, activity was almost nil. The activity of the cells at 18, 20, 22, and 24 hr was increased by allowing them to stand for 30 min at room temperature before testing. After 24 hr the activity did not increase on standing. These results indicate that to obtain cell suspensions with good oxidizing activity the cells should be harvested 2–8 hr after the onset of the stationary phase and allowed to stand for 30 min.

(ii) *Effect of Temperature.*—To determine whether cell suspensions of cultures grown at 27°C could oxidize manganese at temperatures which inhibited oxidation by growing cultures, cell suspensions and inoculated medium were incubated at 27, 35, 45, and 100°C. Oxidation occurred in cell suspensions at all temperatures except 100°C, whereas the inoculated medium only gave oxidation at 27°C; at 35°C the cultures grew sparsely, and at 45 and 100°C the inoculum was killed. The fact that cells from cultures grown at 27°C can oxidize manganese at 45°C but not at 100°C suggests that a heat labile enzyme system is involved.

(iii) *Addition of Organic Substances.*—It was thought that manganese oxidation might occur concurrently during the oxidation of an organic substance, or that the manganous ion by itself, could act as an electron donor. To invesitgate these possibilities, oxidation by cell suspension was studied in the presence of the following oxidizable organic substances:

L-Lactate	DL-Histidine	DL-Phenylalanine
Citrate	L-Histidine	L-Tyrosine
Succinate	L-Phenylalanine	L-Glutamate
Malate	L-Arginine	Yeast extract
Pyruvate	L-Leucine	Glucose
Fumarate	L-Methionine	*p*-Cresol

All the substances were used as neutral 0·005 per cent. (w/v) solutions; glutamate and yeast extract were also used at a concentration of 0·05 per cent. The test system contained 1 ml of substrate solution, 1 ml cell suspension, and 1 ml 0·01 per cent. manganous sulphate. Oxidation occurred in 10 min with all substances except 0·05 per cent. yeast extract, L-arginine, and L-histidine. The water control gave as good, or better, oxidation than did any of the substances tested. It was thought that the inhibition of oxidation by L-arginine and L-histidine might have been due to their preventing, by competition, electron transfer from manganous ion to an electron acceptor, i.e. the amino acids themselves might have given up an electron more readily than the manganous ion. To test this, the amino acid oxidase activity of the cell suspension in the presence of 0·005 per cent. L-arginine, L-histidine, L-methionine, L-phenylalanine, and L-leucine was determined by Thunberg's methylene blue reduction technique. The amino acid oxidase activity of the cell suspension was found to be slight; the times for complete reduction of 1/6000 methylene blue at 37°C being 2 hr or more in all cases: L-methionine, 120 min; L-leucine, 116 min; L-phenylalanine, partial reduction after 120 min; L-histidine, partial reduction after 120 min; L-arginine and water control, no reduction after 120 min. That somewhat greater amino acid oxidase activity occurred in the presence of L-methionine and L-leucine than with L-arginine and L-histidine suggests that the observed inhibition of manganese oxidation is due to some cause other than electron competition. It is concluded from these results that the complete oxidizing system is present in the cell suspension, and that it is unlikely that L-amino oxidase or the intermediate acid dehydrogenases tested are involved in the electron transfer when manganous ion is oxidized.

(iv) *Effect of Enzyme Poisons.*—In an attempt to elucidate the nature of the system responsible for the oxidation of manganese the effects of various poisons on the

oxidizing activity of cell suspensions were studied. The poisons used and their effect on oxidation are listed in Table 5. They were added directly to a mixture of 1 ml of cell suspension and 1 ml of 0·01 per cent. manganous sulphate and incubated for various intervals. Mercuric chloride, copper chloride, potassium cyanide, and sodium azide completely inhibited oxidation at certain concentrations as also did benzene treatment and boiling. The other substances, with the exception of *n*-octyl alcohol and carbon monoxide, partially inhibited oxidation.

TABLE 5

EFFECT OF VARIOUS POISONS AND TREATMENTS ON THE OXIDATION OF MANGANESE BY CELL SUSPENSIONS OF CORYNEBACTERIUM STRAIN B

Treatment	Final Concentrations	Incubation Time (min)	Oxidation*
Control	—	10	++
Boiled cells	—	24 Hr	−
$HgCl_2$	M/2700	24 Hr	−
$CuCl_2$	M/300	24 Hr	−
$CuCl_2$	M/2000	1 Hr	+
$MnSO_4$	M/4500	10	++
$MnSO_4$	M/450	10	++
$MnSO_4$	M/225	10	+
$MnSO_4$	M/45	10	±
CO in dark	—	10	++
KCN	M/13000	10	+
KCN	M/1300	10	+
KCN	M/650	10	±
KCN	M/65	10	−
NaN_3	M/13000	10	+
NaN_3	M/1300	10	−
H_2O_2	M/400	10	++
$KCN+H_2O_2$	M/13000+M/400	10	−
$NaN_3+H_2O_2$	M/13000+M/400	10	−
Ethanol	33% v/v	20	+
Methanol	33% v/v	20	±
Benzene	Sat. soln.	20	−
Toluene	Sat. soln.	20	+
CCl_4	Sat. soln.	20	++
Chloroform	Sat. soln.	20	++
n-Octyl alcohol	Sat. soln.	20	++

* Colour relative to control produced on addition of 1 drop benzidine reagent to cell system.

These results again indicate that dehydrogenases and L-amino oxidase are not involved in the oxidation since it occurred in the presence of chloroform, toluene, and *n*-octyl alcohol. The inhibition by copper and mercury salts confirms the enzymatic nature of the oxidation. The inhibition by azide and cyanide suggest that a heavy metal-containing enzyme is involved. The absence of inhibition by carbon monoxide, in the dark, indicates that cytochrome oxidase is not part of the oxidation system.

It is of interest to note also that low concentrations of cyanide and of azide, which by themselves result in slight inhibition, can, in the presence of 0·0025M hydrogen peroxide, itself a slight inhibitor, completely inhibit oxidation. It was possible that this inhibition was due to catalase inactivation and that the hydrogen peroxide persisted and adversely affected the oxidizing system.

(v) *Effect of* 2, 4-*Dichlorophenol.*—To see whether catalase was involved in the oxidizing system, 2, 4-dichlorophenol, a specific inhibitor for catalase (Goldacre and Galston 1953), was added to cell suspensions to give final concentrations of 1×10^{-3}, 1×10^{-4}, and 1×10^{-5}M. Oxidation was adversely affected at all concentrations and was completely inhibited at the highest. This result suggested that catalase was involved in the oxidizing system.

(vi) *Tests for Catalase and Peroxidase.*—Since oxidation was affected by poisons which inhibit catalase and possibly other heavy metal-containing enzymes, tests were made for these two enzymes. Catalase was readily demonstrated in suspensions of cells. Peroxidase however could not be detected in either whole cells, ground cells, or cells incubated with toluene. Saturated aqueous guaiacol solution was used as the peroxidase substrate and the final concentration of peroxide was 0·025M.

(vii) *Effect of Hydrogen Peroxide on the Oxidation Product.*—It is known that hydrogen peroxide can reduce manganese oxides. To see if the inhibitory effect of hydrogen peroxide could be due to it reducing the oxidation product, 4-ml samples of a light suspension of oxide, collected from oxidizing cultures of strain B, were mixed with 1 ml of 0·05, 0·005, or 0·0005M hydrogen peroxide solution and allowed to stand for 20 min at 30°C. It was found that hydrogen peroxide at final concentrations of 0·01 and 0·001M completely reduced and dissolved the oxide suspension and at 0·0001M caused partial reduction. It was found also that this reduction could be prevented, or decreased, by adding a cell suspension of strain B showing good catalase activity, or by adding a purified catalase preparation to the oxide suspension.

It appears that the inhibition of oxidation by hydrogen peroxide could be due to peroxide preventing the accumulation of oxidized manganese, by reducing it as fast as it is formed.

IV. Discussion

The inhibition of manganese oxidation by moderately high concentrations of yeast extract (Table 1) confirms a similar observation by Beijerinck (1914). The reason for this inhibition is not known but it appears that oxidation only occurs when the organism is grown under starvation conditions. It is possible that oxidation of manganese in soil could be inhibited by the addition of organic materials which enhance the growth of the organisms.

Mann and Quastel (1946) suggested that the oxidation of manganese in unsterile soil was due to actively proliferating microorganisms since the oxidation curve was similar to a growth curve. The results of the present work indicate, however, that oxidation only occurs when the organism is in the stationary phase (Figs. 1, 2, and 3; Table 4).

Beijerinck (1914) proposed that the oxidation of manganese provided energy for the autotrophic growth of the oxidizing organism. The bacterium studied in the present investigation does not appear to grow autotrophically since it does not grow in the absence of an organic substrate (Table 2), and since the total number of cells which grow in the manganese medium and in the manganese-deficient medium are virtually the same and do not increase significantly during oxidation (Table 3).

The mechanism of oxidation by mixtures of *Corynebacterium* strain A and *Chromobacterium* sp. was not unravelled, but it was shown that they have to be growing together to cause oxidation, and that strain A was the main member of the pair since it gave rise to a strain which could oxidize manganese in pure culture (Plate 1, Figs. 2–4). It may be that the *Chromobacterium* sp. induces the formation of, or supplements, an enzyme system in strain A.

The studies on the mechanism of manganese oxidation by *Corynebacterium* strain B revealed that an intracellular enzyme system was involved. This system is different from the manganese-oxidizing peroxidase system described by Kenten and Mann (1950) since peroxidase activity could not be detected in cell suspensions and since hydrogen peroxide, in the absence of catalase, inhibited oxidation. The observed inhibition by peroxide and the need for catalase in the oxidizing system coincides with a similar observation by Andreae (1955). He studied the photo-induced oxidation of manganese in a system containing manganese, pyrophosphate, riboflavin, a hydrogen donor such as *p*-cresol, catalase, and air, and found that the catalase was necessary to decompose the hydrogen peroxide which formed when reduced riboflavin was reoxidized. He postulated that the manganese was oxidized by the oxidation product of the hydrogen donor. It is tempting to suggest a system similar to that of Andreae to account for the need for catalase in *Corynebacterium* strain B. However, the failure of various organic hydrogen donors, including *p*-cresol, to enhance oxidation by cell suspensions, together with the observation that the activity of cells often improved on standing, suggests that oxidation proceeds without an organic hydrogen donor. If this is so then it is likely that manganese itself is the specific electron donor in the oxidizing system. To decide this issue it will be necessary to use cell free preparations.

Since oxidized manganese was not detected in the supernatant of centrifuged oxidizing cultures it is probable that the manganese is oxidized inside the cell and that it deposits as the oxide on the surface of the cells as it diffuses out. The manganous ion may first form an organic complex, perhaps with the enzyme itself, which is more easily oxidized than the free manganous ion (Martell and Calvin 1952, p. 58). It is proposed to investigate the oxidation product and determine its availability to plants.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—Photomicrograph showing deposition of manganese oxide in colonies of *Corynebacterium* strain A which have been overgrown by the spreading colony of *Chromobacterium* sp. × 10.

Fig. 2.—Photomicrograph showing sector of *Corynebacterium* strain B. × 10.

Fig. 3.—Photomicrograph of *Corynebacterium* strain A and *Corynebacterium* strain B isolated from sector shown in Figure 2. × 10.

Fig. 4.—Photomicrograph showing reversion of *Corynebacterium* strain B to *Corynebacterium* strain A. × 20.

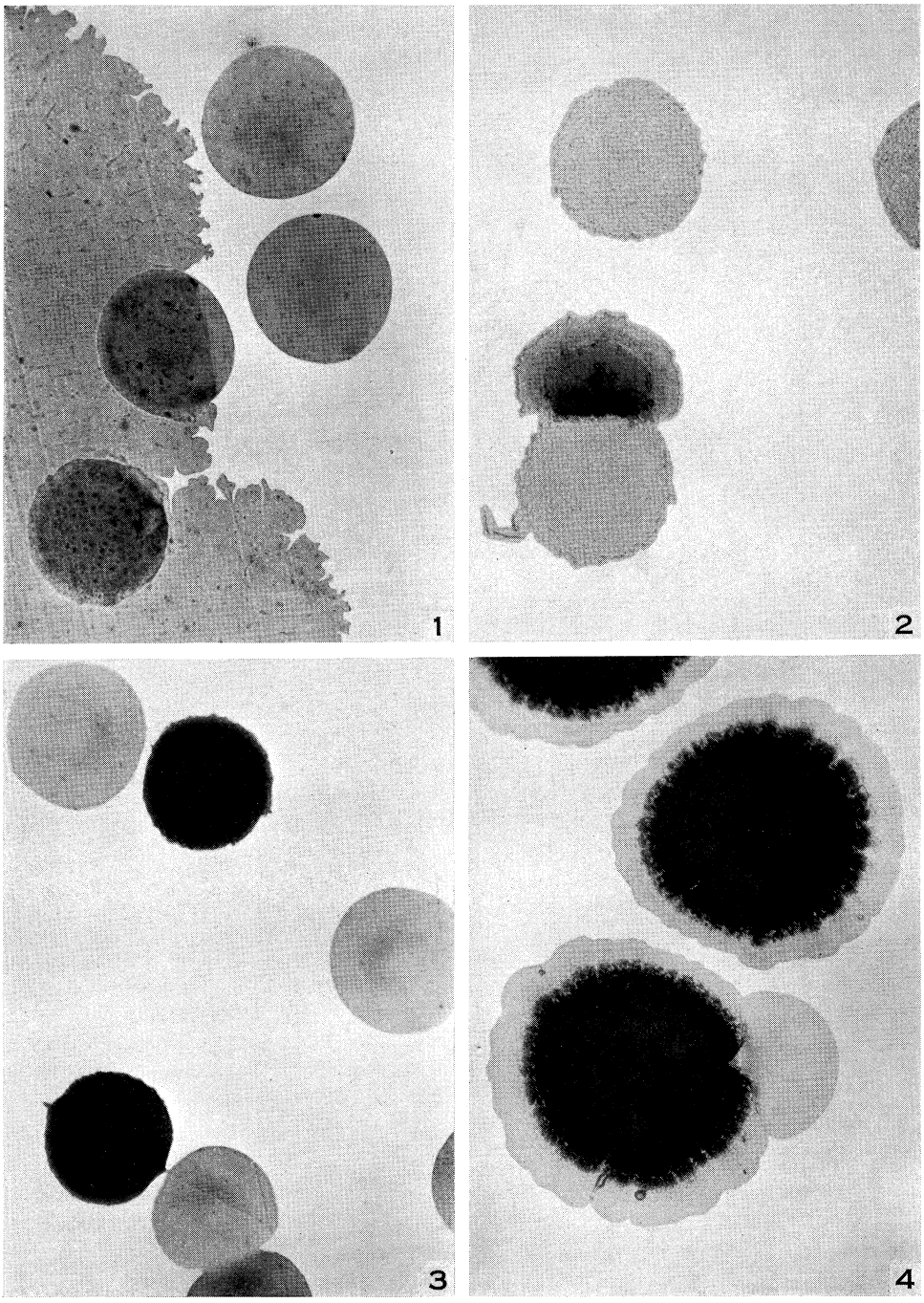
PLATE 2

Fig. 1.—Photomicrograph of the aggregates in the oxidation product showing the intimate mixture of oxide and bacterial cells. × 2125.

Fig. 2.—Photomicrograph showing the aggregates which clump together to form the sponge-like oxidation product. × 1125.

Fig. 3.—Photomicrograph of the product of biological oxidation of manganese showing its sponge-like nature. × 30.

OXIDATION OF MANGANESE BY SOIL MICROORGANISMS



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