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Bacteriology of Manganese Nodules

III. Reduction of MnO_2 by Two Strains of Nodule Bacteria[1]

R. B. TRIMBLE AND H. L. EHRLICH

Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181

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MnO_2 reduction by aerobic growing cultures of *Bacillus* 29 and coccus 32, isolated from ferromanganese nodules, was assessed for 7 days. A 1-day lag was observed before the onset of MnO_2 reduction by either culture. Addition of $HgCl_2$ to a final concentration of about 10^{-3} M caused a rapid cessation of MnO_2 reduction by the growing cultures. Neither culture reduced MnO_2 when grown under continued anaerobiosis from the start of an experiment. However, if conditions were made anaerobic after MnO_2 reduction was initiated, reduction continued at a rate only slightly lower than that under aerobic conditions. Resting-cell cultures reduced MnO_2 equally well aerobically and anaerobically, provided that ferricyanide was present to serve as electron carrier. These findings showed that oxygen is needed for culture adaptation to MnO_2 reduction, and that oxygen does not interfere with microbial MnO_2 reduction itself. Both cultures caused sharp drops in the *p*H of the medium during MnO_2 reduction: with coccus 32, during the entire incubation time; with *Bacillus* 29, for the first 3 days. The E_h of the medium fluctuated with either culture and never fell below 469 mv with *Bacillus* 29 and below 394 mv with coccus 32. The rates of glucose consumption and Mn^{2+} release by *Bacillus* 29 and coccus 32 were fairly constant, but the rates of lactate and pyruvate production were not. Although acid production undoubtedly helped in the reduction of pyrolusite (MnO_2) by the bacteria, it did not appear to be important in the reduction of manganese oxide in ferromanganese nodules, as shown by the results with a nodule enrichment.

With suitable electron donors, several different soil microorganisms and bakers' yeast reduce manganese oxides to Mn^{II} (9, 10, 12, 14). To observe MnO_2 reduction by such microorganisms, Mann and Quastel (10) and Hochster and Quastel (9) employed an electron carrier and an oxygen-free atmosphere of N_2 and CO_2 in their reaction system. On the other hand, Perkins and Novielli (12) and Vavra and Frederick (14) did not use an electron carrier nor did they exclude O_2 from their system. According to generally accepted views (1), microbial MnO_2 reduction should be favored by an absence of O_2, but neither Perkins and Novielli nor Vavra and Frederick commented on this in the discussion of their work.

Reduction of manganese oxides in bacterial enrichments of marine ferromanganese nodules was first reported by Ehrlich (3). In later investigations, he demonstrated MnO_2 reduction by pure cultures of bacteria isolated from ferromanganese nodules (5; H. L. Ehrlich, Bacteriol. Proc., p. 42-43, 1964). Ehrlich's work showed that growing cultures of *Bacillus* 29 and coccus 32, as well as other microorganisms, reduced MnO_2 to Mn^{II} aerobically with glucose as electron donor. Under resting-cell conditions, he found that cultures not previously grown in the presence of MnO_2 required the addition of an electron carrier (ferricyanide) in order to reduce MnO_2 with glucose as substrate during an initial 3-hr period of incubation. Ehrlich's experiments indicated that both organisms possessed an enzyme system which reduced ferri- to ferrocyanide with electrons derived from the substrate, and another system which reduced MnO_2 to Mn^{II} with electrons derived from ferrocyanide. However, the organisms lacked one or more enzyme components necessary to convey electrons from the ferricyanide reducing system to the ferrocyanide oxidizing system. His experiments also indicated that growing cultures developed this missing component after a period of contact with MnO_2. Ehrlich called such cultures "adapted cultures." Whether this adaptation involved a mutation and

[1] Part of a dissertation submitted by R. B. Trimble to the faculty of Rensselaer Polytechnic Institute in partial fulfillment of the requirements for the M.S. degree in the Department of Biology.

selection phenomenon, an allosteric rearrangement of an existing component, or an induction was not determined.

Further details of bacterial MnO_2 reduction by growing and resting-cell preparations of *Bacillus* 29 and coccus 32 are given in this paper.

MATERIALS AND METHODS

Media. Stock cultures were carried on Difco Stock Culture Agar made up in distilled water. Inocula of desired cultures were prepared on slants in Roux bottles, each containing 100 ml of seawater-nutrient agar consisting of (in g per liter of natural seawater): peptone, 5; beef extract, 3; agar, 15.

Bacterial cultures. Two strains of bacteria, a spore-forming *Bacillus*, designated strain 29, and an unidentified coccus, called coccus 32, were used in pure culture work. The *Bacillus* and coccus had been previously isolated from ferromanganese nodules obtained from the Atlantic Ocean (3). Stock cultures were maintained at 25 ± 1 C. Frequent checks for purity were made by microscopic observation and plating.

Preparation of inoculum. Bacillus 29 or coccus 32 was grown up for 24 hr on Roux bottle slants of seawater-nutrient agar. Cells were harvested and washed three times by centrifugation in undiluted or 10-fold diluted seawater. The washing procedure was carried out aseptically, when the cells were to be used as the inoculum in growth experiments. The final cell titer of each suspension was adjusted to between 10^8 and 10^9 cells per ml. A 1-ml amount of final suspension was used for inoculation of reaction vessels.

Manganese dioxide. Reagent-grade MnO_2 (J. T. Baker Chemical Co., Phillipsburg, N.J.) was used. The MnO_2 was washed 10 times in 200-ml volumes of distilled water in order to remove fine, colloidal particles of the oxide from larger ones. It was then dried under a heat lamp and was used in experiments immediately thereafter.

Anaerobic incubation. Anaerobic conditiions were maintained in a large vacuum desiccator containing 50 ml of distilled water on the bottom for humidity control. The desiccator was evacuated to a resdual pressure of less than 60 mm of Hg with an aspirator pump. Dry, purified nitrogen gas (Union Carbide Corp., Linde Div., New York, N.Y.) was then introduced to a pressure of 760 mm of Hg. This procedure was repeated two more times, leaving a residual pressure of less than 0.15 mm Hg of O_2 (13).

Experimental procedure. Growing-cell experiments (long-term) were performed by autoclaving 1-g portions of MnO_2 under 10 ml of distilled water in an appropriate number of 125-ml Erlenmeyer flasks at 15 psi for 30 min. After sterilization, the water was aseptically removed by pipette and was replaced with 18 ml of sterile, natural seawater. To this were added 1 ml of a sterile, distilled water solution of 1.0% peptone and 1 ml of another sterile, distilled water solution of either 2, 10, or 20% glucose. A 1-ml amount of aseptically washed cell suspension was added to experimental flasks, while an additional 1 ml of sterile seawater was added to control flasks. In some experiments, additional controls of inoculated flasks with the above nutrient solution, but without MnO_2, were included. Each experimental condition, including controls, was set up in duplicate and sampled in duplicate at appropriate times. Incubation was at 25 ± 1 C.

Chemical and physical changes in the medium of growing cultures were determined in separate pairs of experimental and control flasks, at desired times, by assaying for dissolved manganese, residual glucose, and lactic and pyruvic acids. Cell populations in the supernatant fluid were estimated turbidimetrically in a Spectronic-20 colorimeter (Bausch & Lomb, Rochester, N.Y.). Determinations of *p*H and E_h were made with a model 7 or model 10 *p*H meter (Corning Glass, Corning, N.Y.). For E_h determinations, a platinum electrode against a calomel reference electrode was used. Readings of observed potentials were converted to standard potentials (E_h) by adding a value of +244 mv, the E_h of the calomel electrode at 25 C.

Resting-cell experiments (short-term) were carried out for 3 hr in 50-ml Erlenmeyer flasks. Each flask received 0.1 g of washed MnO_2, which was overlaid with 4.9 ml of 10-fold diluted seawater, 1 ml of 0.25 M $NaHCO_3$ buffer (*p*H 7.0) containing 1% glucose, 0.1 ml of 0.01 M potassium ferricyanide, and 1 ml of washed cells suspended in 10-fold diluted seawater. In control flasks, 1 ml of 10-fold diluted seawater replaced the cell suspension. Flasks to which ferricyanide reagent was not added received 0.1 ml of 10% seawater. Each experimental condition and control was set up in duplicate and incubated at room temperature for 3 hr. Immediately thereafter, a 1.5-ml sample was removed from each flask, centrifuged at 3,500 × *g*, and assayed for dissolved manganese. The residual solution in each flask was then acidified with 0.05 ml of 10 N H_2SO_4 and incubated at room temperature for 30 min. A 3-ml sample of solution was then removed from each flask, centrifuged at 3,500 × *g*, and assayed for manganese.

To determine if pyruvate would cause a dismutation between MnO_2 and Mn^{2+}, 18 ml of sterile seawater was added to six 125-ml Erlenmeyer flasks containing sterilized MnO_2. Two experimental flasks received 1 ml of sterile 2×10^{-2} M $MnSO_4 \cdot H_2O$ and 1 ml of 1.0 M sodium pyruvate; two control flasks received 1 ml of sterile 2×10^{-2} M $MnSO_4 \cdot H_2O$ and an additional 1 ml of sterile seawater; and two other control flasks received an additional 2 ml of sterile seawater each. All flasks were incubated at room temperature for a specified length of time. Samples of supernatant fluid were then assayed for manganese concentration.

Analytical methods. Manganese in solution was determined by a persulfate method described by Ehrlich (3, 5). Since this method does not discriminate among the various valence states of manganese, periodic tests with benzidine-acetate reagent were used to detect Mn^{III} and Mn^{IV} (6), for which this reagent was specific under the conditions of these experiments. Relative sensitivities of the benzidine test and the persulfate assay are of the same order of magnitude. However, the benzidine test does not lend itself to precise quantitative measurement.

Residual glucose was measured by mixing 2-ml portions of 50-fold diluted samples of culture fluid with 4 ml of anthrone reagent in test tubes (15 × 150 mm) and measuring color development at 540 mμ after 15 min (11). The anthrone reagent consisted of 0.2 g of anthrone in 100 ml of 95% H_2SO_4.

Lactic acid was determined on 1.0-, 0.2-, or 0.1-ml samples of culture fluid, depending on its concentration, according to the colorimetric method of Barker and Summerson (2). Since pyruvic acid interferes with this assay, all measurements of lactic acid were corrected for pyruvic acid present. Color development was measured at 560 mμ.

Pyruvic acid was measured on 1.0-, 0.2-, or 0.1-ml samples of culture fluid, depending on its concentration, by the colorimetric method of Friedemann and Haugen (7). Color development was measured at 520 mμ.

All color determinations were made with a Spectronic-20 colorimeter.

In the rate studies of manganese release shown in Fig. 1, changes in manganese concentration in the supernatant fluid were measured by repeated sampling of the same duplicate flasks. This required correction of the measurements for volume changes and for manganese removal resulting from repeated sampling. Corrections were made according to the following relationship, which normalized all data on the basis of an initial 21-ml volume of supernatant fluid:

$$\frac{A_n[21 - 0.4(n - 1] + 0.4(A_{n-1})}{21}$$

= corrected manganese concentration per ml

where A_n = manganese concentration of the nth sample of supernatant fluid; A_{n-1} = manganese concentration of the $(n - 1)$th sample of supernatant fluid; and n = the number of 0.4-ml samples of supernatant fluid removed from the reaction vessel. The corrections are necessary only when $A_n > A_{n-1}$. When $A_n = A_{n-1}$, the nth sample contains the same manganese concentration on a normalized basis as the $(n - 1)$th sample because MnO_2 was not reduced during this time.

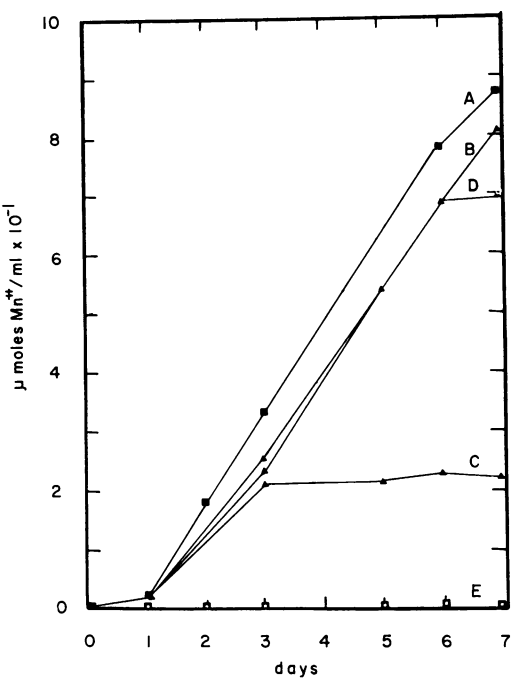

FIG. 1. *MnO_2 reduction by growing cultures of Bacillus 29 and coccus 32, and the effect of $HgCl_2$ upon the process. Curves A and B represent the activities of Bacillus 29 and coccus 32, respectively, in the absence of $HgCl_2$. Curves C and D represent the effect of adding $HgCl_2$ to a final concentration of about 10^{-3} M to a growing MnO_2-reducing culture of coccus 32 at 3 and 6 days of incubation, respectively. Curve E represents the activity in an uninoculated control.*

RESULTS

MnO_2 reduction by growing cultures. The action of *Bacillus* 29 and coccus 32 on MnO_2 in a medium containing an initial concentration of 0.95% glucose and 0.048% peptone is shown in Fig. 1, curves A and B. A fairly constant rise in the concentration of soluble manganese was noted after the first day of incubation at 25 C. No manganese was detected in the supernatant fluid of uninoculated flasks (Fig. 1, curve E). Inoculated flasks contained dissolved manganese which reacted with persulfate, but not with benzidine-acetate reagent, indicating that the valence state of the dissolved manganese was Mn^{II}.

Further manganese release stopped very soon after the addition of 0.2 ml of 10^{-1} M $HgCl_2$ (final concentration about 10^{-3} M) to growing, MnO_2-reducing cultures of either *Bacillus* 29 or coccus 32. Curves C and D (Fig. 1) show the results obtained with coccus 32 when $HgCl_2$ was added after 3 and 6 days, respectively. When the cells of coccus 32 were poisoned with $HgCl_2$ at the time of inoculation, no soluble manganese appeared throughout the 7-day period of incubation. Similar results were obtained with *Bacillus* 29.

Effect of anaerobiosis on growing and resting-cell cultures. When growing cultures of *Bacillus* 29 or coccus 32 were made anaerobic immediately after inoculation and were then kept anaerobic for 5 to 7 days, no manganese was found in the supernatant fluid. However, if growing cultures of either strain of bacteria were made anaerobic after the cells had begun to reduce MnO_2, as evidenced by finding Mn^{II} in the supernatant fluid of the reaction vessels, the reduction continued anaerobically at a rate only slightly lower than the aerobic rate during a 5-day period. At the

TABLE 1. *Aerobic and anaerobic MnO_2 reduction by resting cells of Bacillus 29 and coccus 32*

Culture	Condition	Mn (mμmoles) per ml of supernatant fluid[a]	
		Aerobic	Anaerobic
29	System[b] − cells + (a)[c]	12.8	12.0
	System + cells + (a)	12.8	12.0
	System − cells + (b)[c]	12.8	12.0
	System + cells + (b)	89.6	91.6
32	System − cells + (a)[c]	1.6	1.6
	System + cells + (a)	1.6	1.6
	System − cells + (b)[c]	1.6	1.6
	System + cells + (b)	12.9	16.0

[a] After acidification at the end of 3 hr. Acidification accomplished by addition of 0.05 ml of 10 N H_2SO_4 to each reaction vessel.

[b] System consisted of 0.1 g of MnO_2; 4.9 ml of 10% seawater; 1.0 ml of 0.25 M $NaHCO_3$ (*p*H 7.0) containing 1% glucose; and 1.0 ml of inoculum or 10% seawater.

[c] (a) 0.1 ml of 10% seawater; (b) 0.1 ml of 0.01 M $K_3Fe(CN)_6$.

end of these experiments, the *p*H was slightly higher in the flasks in which MnO_2 was being reduced anaerobically than in similar flasks incubated aerobically.

Ehrlich (5) has mentioned that preliminary work with resting cells of *Bacillus* 29, which had not been adapted to MnO_2 reduction, showed Mn^{II} release from MnO_2 anaerobically in evacuated Thunberg tubes, provided that an electron carrier, potassium ferricyanide, was present in the reaction mixture. The preliminary findings were confirmed in experiments in which 50-ml Erlenmeyer flask cultures were incubated in a vacuum desiccator under a nitrogen atmosphere. Additional experiments with coccus 32, not previously tested under anaerobic conditions, yielded similar results. Typical findings for each organism are summarized in Table 1. After acidification, some manganese appeared even in the supernatant fluid of uninoculated flasks. This manganese was not removed from the supernatant fluid by centrifugation for 15 min at 4,000 × *g* and was only detectable by the persulfate method, indicating that it was Mn^{II}. Whether this manganese was initially present in the MnO_2 substrate as an impurity or whether it resulted from chemical reduction during the experiment is not known. Before acidification, no manganese was recovered in the supernatant fluids from the experiment with coccus 32, nor, with one exception, in the supernatant fluids from the experiment with *Bacillus* 29. The unacidified supernatant fluids in flasks with the complete reaction mixture and *Bacillus* 29 contained 13.6 mμmoles of Mn per ml after aerobic incubation and 7.2 mμmoles of Mn per ml after anaerobic incubation. As will be discussed later, the recovery of less Mn^{II} from the anaerobic than from the aerobic reaction of *Bacillus* 29 prior to acidification was probably due to the formation of less acid by the cells in the supernatant fluid under anaerobic conditions.

Chemical and physical changes in the culture medium during bacterial MnO_2 reduction. Studies of chemical, physical, and population changes in the presence and absence of MnO_2 reduction by *Bacillus* 29 at an initial glucose concentration of 0.48% and by coccus 32 at an initial glucose concentration of 0.60% are summarized in Table 2. *Bacillus* 29 and coccus 32 consumed more glucose with MnO_2 than without MnO_2. The rates of glucose consumption and Mn^{II} release by *Bacillus* 29 and coccus 32 were relatively constant for most of the observation period. Lactate production by *Bacillus* 29 reached a peak on the fourth day of incubation and thereafter leveled off. The total lactate produced by *Bacillus* 29 with MnO_2 was greater than without MnO_2. Pyruvate production by *Bacillus* 29 increased continually with MnO_2 but leveled off after the fourth day without MnO_2. Lactate production by coccus 32 was negligible with or without MnO_2. Pyruvate production by coccus 32 increased progressively with or without MnO_2, but the total yield was greater with MnO_2 than without MnO_2. It is also seen in Table 2 that *Bacillus* 29 in the presence of MnO_2 caused a sharp *p*H drop to 5.4 in 3 days followed by a slow rise. This *p*H pattern was even more apparent in an experiment with an initial glucose concentration of 0.95%. With coccus 32, the *p*H tended to drop continually during the entire experimental period. With both *Bacillus* 29 and coccus 32, *p*H dropped more in the absence of MnO_2 than in its presence. Observed changes in E_h, fluctuating between 469 and 617 mv with MnO_2 and 474 and 594 mv without MnO_2 in the case of *Bacillus* 29, and between 394 and 519 mv with MnO_2 and 419 and 549 mv without MnO_2 in the case of coccus 32, provided further evidence that MnO_2 reduction by bacteria is not dependent on strong reducing conditions.

Qualitatively similar results were obtained with *Bacillus* 29 at initial glucose concentrations of 0.95 and 0.095%, and with coccus 32 at 0.48%. At an initial concentration of 0.095%, *Bacillus* 29 exhausted the glucose in 3 days, requiring the addition of fresh glucose to a final concentration of 0.095% for continued activity for the next two 3-day periods.

TABLE 2. *Chemical and physical changes during growth of Bacillus 29 and coccus 32 in the presence and absence of MnO_2*[a]

Culture	Time (days)	Glucose consumed (mμmoles/ml)		Manganese released (mμmoles/ml)	Pyruvate produced (mμmoles/ml)		Lactate produced (mμmoles/ml)		E_h (mv)		pH		Cells/ml (× 10^7)	
		+MnO_2	−MnO_2	+MnO_2	+MnO_2	−MnO_2	+MnO_2	−MnO_2	+MnO_2	−MnO_2	+MnO_2	−MnO_2	+MnO_2	−MnO_2
29	0	0	0	0	0	0	0	0	469	474	7.3	7.2	1.6	1.6
	1	1,720	655	25	43	32	461	449	528	482	6.6	6.7	3.8	4.0
	3	3,060	1,239	240	190	153	1,635	1,612	502	512	5.4	5.6	4.5	4.8
	4	3,686	2,342	420	438	330	2,430	1,955	573	514	5.7	5.5	4.5	5.0
	7	6,120	3,635	1,060	705	243	2,080	1,387	615	594	5.7	5.5	4.6	5.2
	8	8,750	5,286	1,260	879	330	2,040	1,705	617	589	5.5	4.9	4.8	5.0
	11	11,893	7,281	1,830	936	225	2,430	1,761	602	589	5.8	5.1	5.0	5.0
32	0	0	0	0	0	0	0	0	426	426	7.7	7.7	2.5	2.5
	1	480	0	0	50	52	0	0	394	419	7.0	6.9	4.6	5.8
	2	1,440	1,440	0	84	75	9	5	519	490	6.8	6.5	6.2	7.0
	3			130	138	79			491	496	6.6	6.4	6.2	7.0
	7	3,000	2,650	490	1,025	575	174	68	484	495	5.7	4.7	6.0	4.8
	9	4,900	3,125	780	1,650	980	250	124	514	549	5.4	4.5	5.8	4.2
	11	5,500	3,600	1,020	2,625	1,120	250	123	475	509	5.6	4.3	5.4	4.8

[a] Composition of culture medium is described in text.

Test for possible reaction between MnO_2 and metabolic products. An experiment was performed in order to determine if pyruvate might complex Mn^{III} and thus promote the reaction, $MnO_2 + Mn^{2+} + 4H^+ = 2Mn^{3+} + 4H_2O$, which would result in solution of MnO_2. As described in Materials and Methods, the setup consisted of two experimental flasks containing MnO_2 and a seawater solution of $MnSO_4$ and sodium pyruvate, two control flasks containing MnO_2 and a seawater solution of $MnSO_4$ but without sodium pyruvate, and two more control flasks containing MnO_2 and seawater without either $MnSO_4$ or sodium pyruvate. No change in soluble manganese concentration was measured with persulfate over an 8-day period in any of the six flasks. All supernatant fluids gave a negative test with benzidine acetate reagent. The *p*H of the reaction mixture was 6.8 during this time. To determine if the dismutation between MnO_2 and Mn^{2+} would have occurred at a lower *p*H, 0.4 ml of 0.1 N H_2SO_4 was added and the *p*H in the flasks was lowered to about 4. Still, no change in soluble manganese concentration was measured with persulfate during the next 4 days. The supernatant fluids also remained unreactive with benzidine-acetate reagent. It is concluded that pyruvate cannot promote a dismutation between MnO_2 and Mn^{2+} by complexing Mn^{III}.

Chemical and physical changes in a nodule enrichment undergoing bacterial oxide reduction. A 17.6-g portion of an Atlantic ferromanganese nodule, supplied in 1961 by the Woods Hole Oceanographic Institution, Woods Hole, Mass., was aseptically crushed, placed in a sterile 3-liter Fernbach flask, and overlaid with 1 liter of sterile seawater containing 0.5% glucose and 0.05% peptone. The glucose and peptone were sterilized separately. A 5-ml sample was aseptically removed from the enrichment on successive days, examined for the presence of microorganisms growing from the nodular material, and assayed for *p*H, E_h, dissolved manganese, and residual glucose. The results of the assays are shown in Fig. 2. Unlike the experiments with pure cultures that reduced MnO_2, the initial *p*H in this enrichment did not drop when the manganese in the nodule substance began to be reduced by the outgrowing bacteria. Glucose was progressively consumed as manganese was solubilized over the 16 days of the experiment. Wet mounts of crushed nodular material revealed four distinct morphological forms of organisms: motile rods, nonmotile cocci, curved rods, and large spherical cells. Of a total of 2,640 mg of manganese (as Mn^0) in the nodule substance initially present in the flask, 248 mg were solubilized in 16 days. This represents a solubilization of about 10% of the manganese in the nodule.

DISCUSSION

The rapid cessation of Mn^{II} release after $HgCl_2$ poisoning of the bacteria suggested an enzymatic role of the cells in MnO_2 reduction (Fig. 1). A much more gradual slowing down in Mn^{II}

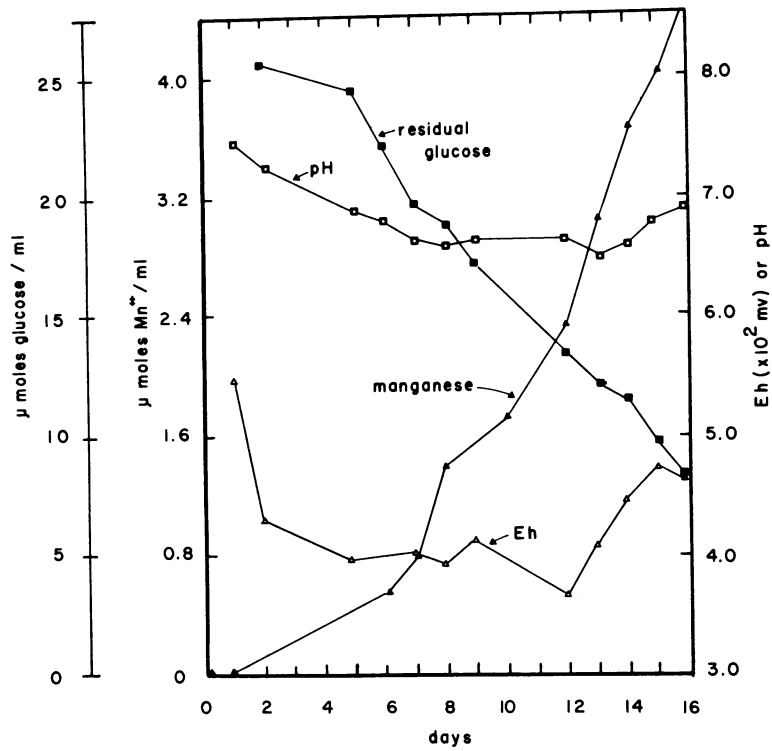

FIG. 2. *Chemical and physical changes in an enrichment culture made with a portion of an Atlantic ferromanganese nodule in the presence of glucose and peptone. Composition of the enrichment is described in text.*

release would have been expected if metabolic end products, whose reactivity was unaffected by $HgCl_2$, had been responsible for MnO_2 reduction instead of enzymatic interaction by the bacteria.

Since the cells used in these experiments were not in contact with MnO_2 while they were being cultured in the preparation of the inoculum, and since it has been previously shown that such cells cannot reduce MnO_2 in the first 3 hr of contact without added ferricyanide (5), the 1-day lag in Fig. 1 is partly attributable to a missing component in the electron transport system for MnO_2 reduction at the time of inoculation, which the culture synthesized during the lag period. As will be explained later, the lag may also be partly attributable to an initially insufficient quantity of acid for dissolving $Mn(OH)_2$, the probable direct product of MnO_2 reduction.

The observation that displacement of oxygen by nitrogen at the beginning of a growth experiment with unadapted *Bacillus* 29 or coccus 32 prevented Mn^{II} release from MnO_2 indicated that O_2 is required for adaptation of the culture to MnO_2 utilization. This conclusion was further supported by noting that displacement of air by nitrogen, after MnO_2 reduction was initiated by the culture, did not significantly reduce the rate of MnO_2 reduction. The observation that anaerobiosis did not stimulate MnO_2 reduction by these now adapted cells paralleled the observation that MnO_2 reduction by unadapted cells, utilizing ferricyanide as electron carrier, was not stimulated or inhibited by anaerobiosis (Table 1). With added ferricyanide, the unadapted organisms must have possessed a complete, oxygen-insensitive system for electron transport from electron donor to MnO_2. The observed lack of sensitivity of bacterial MnO_2 reduction to O_2 and, indeed, the absence of any significant increase in the rate of MnO_2 reduction under anaerobic conditions are contrary to the generally held notion that such reduction should proceed preferentially under anaerobic conditions (1). The results indicated that adapted cultures use MnO_2 in preference to O_2 as the terminal electron acceptor, and that in such cells MnO_2 and O_2 do not compete with each other. Trimble (M.S. Thesis, Rensselaer Polytechnic Institute, Troy, N.Y., 1967) has noted that unadapted, nongrowing cells of *Bacillus* 29, with ferricyanide as electron carrier, use significantly less O_2 in the presence of MnO_2 than in its absence.

The immediate product of MnO_2 reduction at near neutral *p*H appears to be a relatively insoluble form of Mn^{II}. It may be $Mn(OH)_2$, which would be brought into solution by acidification. The lactic and pyruvic acids produced by *Bacillus* 29 and the pyruvic acid produced by coccus 32 undoubtedly accounted for ready solubilization of Mn^{II} in growing cultures but not in resting cultures. Unlike growing cultures, resting cultures, acting on MnO_2 in a medium buffered with $NaHCO_3$, could not have produced enough free acid to release much or any Mn^{II} formed. Consequently, acidification with H_2SO_4 was required to bring the Mn^{II}, which the resting cultures had formed, into solution.

The quantitative observations on chemical and physical changes in the culture medium during MnO_2 reduction by *Bacillus* 29 and coccus 32 in air can be summarized as follows. Out of the total large population in the inoculum introduced into a reaction vessel, only a portion of the cells, whose exact number depends on available surface on the MnO_2, came into direct contact with MnO_2 and reduced it. A period of adaptation was necessary before the cells on the MnO_2 reduced it, providing that the inoculum had not been grown on MnO_2 and that ferricyanide was not added to the reaction mixture. During reduction of the MnO_2 with glucose, the *Bacillus* 29 cells on the MnO_2 produced lactic and pyruvic acid, and the coccus 32 cells mainly pyruvic acid. The cells remaining free in the liquid medium also produced the respective acids, but by reducing O_2 with glucose. The total acid thus formed caused a marked drop in *p*H. When the *p*H of the supernatant fluid had fallen below 6.0, the cells in the supernatant fluid slowed down or stopped growing and produced little acid. However, the cells on the MnO_2 continued to grow and reduce MnO_2 according to the reaction, $MnO_2 + 2(H) = Mn(OH)_2$, but they did not produce acid rapidly enough for bringing all $Mn(OH)_2$ formed into solution. Acid previously formed by the cells in the supernatant fluid was consumed in dissolving $Mn(OH)_2$ and, as a consequence, the *p*H in the supernatant fluid rose until it reached a level at which cells not in contact with MnO_2 metabolized again. The cell population in the supernatant fluid increased slowly as cells on MnO_2 multiplied and pushed at least half of the progeny of each cell generation into the medium because of the limited surface available on the MnO_2. Depending on the prevailing *p*H, the cells that had been pushed into the liquid medium may or may not have been active in oxidizing glucose to lactic and pyruvic acids. Under anaerobic conditions, cells in the supernatant fluid were inactive, but cells on MnO_2 metabolized, if they were adapted to MnO_2 reduction or if ferricyanide was present.

The interactions during MnO_2 reduction described above assign a proper function to metabolically formed acids. In general, acids can affect dissolution of MnO_2 in only two ways: (i) by reducing MnO_2 chemically to Mn^{II}, as concentrated HCl does, or (ii) by promoting the reaction $MnO_2 + Mn^{2+} + 4H^+ = 2Mn^{3+} + 2H_2O$ through complexing of Mn^{3+}, which prevents its reoxidation to tetravalent manganese. The Mn^{3+} complex would be soluble. In this study, we found that pyruvic acid, an α-keto acid, is unable to complex Mn^{3+} at either neutral or acid *p*H. It has previously been shown that pyrophosphoric acid causes an accumulation of Mn^{III} by complex formation under similar experimental conditions (4).

Although in pure culture experiments with MnO_2 acid was important for Mn^{II} release into the supernatant fluid, it appeared less important in the enrichment culture with manganese nodule substance (Fig. 2). Mn^{II} was readily released into the medium despite the fact that the *p*H during the course of manganese oxide reduction never dropped markedly below the initial *p*H. Whether the greater ease of Mn^{II} release at neutral *p*H was attributable to the different types of oxides involved, or whether a difference in microorganisms accounted for it, remains to be determined. In the open sea, a sharp drop in *p*H to below 5.0, even in a microenvironment, seems unlikely because an open system is involved. In such a system, any acid formed metabolically on a nodule surface during manganese oxide reduction may be rapidly neutralized and would diffuse or be swept away by circulating waters. Release of Mn^{2+} from the reduction process may here be promoted by the complexing of Mn^{2+} with sulfate, bicarbonate, or organic compounds, such as amino acids. Sulfate and bicarbonate complexes have been described by Hem (8).

The chemistry of MnO_2 reduction in the experiments described in this paper may be summarized by the following reaction:

$$\text{glucose} \xrightarrow{\text{bacteria}} ne^- + n\text{H}^+ + \text{end products} \quad (1)$$

$$n/2\text{MnO}_2 + ne^- + n\text{H}^+ \xrightarrow[\text{or unadapted cells} + \text{Fe(CN)}_6^{3-}]{\text{adapted cells}} n/2\text{Mn(OH)}_2 \quad (2)$$

$$n/2\text{Mn(OH)}_2 + n\text{H}^+ \rightarrow n/2\text{Mn}^{2+} + n\text{H}_2\text{O} \quad (3)$$

$$n/2MnO_2 + ne^- +$$

$$2nH^+ \xrightarrow[\text{or unadapted cells} + Fe(CN)_6^{3-}]{\text{adapted cells}} (2) + (3)$$

$$n/2Mn^{2+} + nH_2O$$

Reaction 2 may actually proceed in two steps, involving an initial reduction of MnO_2 to MnOOH, followed by a reduction of MnOOH to $Mn(OH)_2$; firm evidence for such a sequence of reactions has not been found. The protons in reaction 3 are derived from acid end products by bacteria on MnO_2 and by those not on MnO_2. When insufficient amounts of such acids are available, some or all solubilization of $Mn(OH)_2$ would have to depend on complexing by sulfate, bicarbonate, or other agents of Mn^{2+} formed by dissociation of $Mn(OH)_2$.

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LITERATURE CITED

1. Alexander, M. 1961. Introduction to soil microbiology. John Wiley and Sons, Inc., New York.
2. Barker, R. S., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. **138**:535–554.
3. Ehrlich, H. L. 1963. Bacteriology of manganese nodules. I. Bacterial action on manganese in nodule enrichments. Appl. Microbiol. **11**:15–19.
4. Ehrlich, H. L. 1964. Microbial transformations of minerals, p. 43–60. *In* H. Heukelekian and N. C. Dondero [ed.], Principles and applications in aquatic microbiology. John Wiley and Sons, Inc., New York.
5. Ehrlich, H. L. 1966. Reactions with manganese by bacteria from marine ferromanganese nodules. Develop. Ind. Microbiol. **7**:43–60.
6. Feigl, F. 1946. Qualitative analysis by spot tests. Inorganic and organic applications, 3rd ed., p. 133–135. Elsevier Publishing Co., Inc., New York.
7. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**: 415–442.
8. Hem, J. D. 1963. Chemical equilibria and rates of manganese oxidation. Geological Survey Water-Supply Paper 1667-A. U.S. Govt. Printing Office, Washington, D.C.
9. Hochster, R. M., and J. H. Quastel. 1952. Manganese dioxide as terminal hydrogen acceptor in the study of respiratory systems. Arch. Biochem. Biophys. **36**:132–146.
10. Mann, P. J. G., and J. H. Quastel. 1946. Manganese metabolism in soils. Nature **158**:154–156.
11. Neish, A. C. 1952. Analytical methods for bacterial fermentations. Report no. 46-8-3 (sec. rev.), Natl. Res. Council, Canada.
12. Perkins, E. C., and F. Novielli. 1962. Bacterial leaching of manganese ores. U.S. Bureau of Mines, Report of Investigations 6102. U.S. Govt. Printing Office, Washington, D.C.
13. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric techniques. Burgess Publishing Co., Minneapolis.
14. Vavra, J. P., and L. R. Frederick. 1952. The effect of sulfur oxidation on the availability of manganese. Soil Sci. Soc. Am. Proc. **16**:141–144.