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Bacteriology of Manganese Nodules

IV. Induction of an MnO_2-Reductase System in a Marine Bacillus[1]

R. B. TRIMBLE[2] AND H. L. EHRLICH

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

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Bacillus 29, isolated from a ferromanganese nodule from the Atlantic Ocean, was shown to possess an MnO_2-reductase system which is induced in the presence of manganous ion. Maximal activity of the enzyme system was induced in about 5 hr in the presence of 4.35 mM $MnSO_4$ and was minimally dependent on the presence of either glucose or peptone and oxygen. Induction of optimal activity required the simultaneous presence of glucose and peptone. At least 30% of maximal activity was induced in 5 hr in the presence of 0.4 μM $MnSO_4$. Actinomycin D (5 μg/ml) or chloramphenicol (35 μg/ml), when added to the induction medium, inhibited approximately 90% of MnO_2-reductase synthesis and incorporation of uracil-2-^{14}C or leucine-1-^{14}C. Cell-free extracts having MnO_2-reductase activity were prepared by sonic disruption of cell suspensions of induced *Bacillus* 29. Such extracts used glucose metabolism as a source of electrons. They had an average specific activity of 1.15 nmoles of Mn^{II} produced per mg of protein per hr at 25 C. They had a temperature optimum of 18 C for reductase activity and retained 50% of their activity at 4 C, the approximate temperature of the natural habitat of the organism. Extracts were stable for several days at 4 C but rapidly lost over 50% of their activity on freezing and thawing. Over 90% of the activity of the extract could be destroyed by heating in a boiling-water bath for 5 min. At a concentration of 1 mM, $HgCl_2$ and atebrine dihydrochloride inhibited MnO_2-reductase activity by at least 50%, but sodium azide was ineffective. The MnO_2-reductase activity of induced cells and extracts from them was no greater in the absence of oxygen than in its presence, confirming an earlier observation that MnO_2 and O_2 do not compete as terminal electron acceptors in the respiratory activity of this organism.

A number of heterotrophic and two autotrophic organisms are known to transform large amounts of inorganic materials, such as sulfates, nitrates, and carbon dioxide, by using them as terminal hydrogen acceptors (12, 14, 17). A lesser known phenomenon of microbial interaction with inorganic materials is the reduction and solubilization of oxides of iron (1, 2, 13, 16, 19, 20) and manganese (4, 9, 11, 18, 19, 20). Although some of the earlier reports did not associate the solubilization of these metal oxides directly with enzymatic activity, recent evidence has suggested that it does occur (2, 5, 13, 18, 20; A. F. de Castro, Ph.D. Thesis, Rensselaer Polytechnic Institute, Troy, N.Y., 1969).

In previous work, it was shown that growing cultures of *Bacillus* 29, isolated from a ferromanganese nodule from the Atlantic Ocean, are able to reduce MnO_2 in the presence of glucose after a 1-day lag, and the lag can be eliminated by adding ferricyanide to the culture medium (5, 18). It was also shown that resting cells of *Bacillus* 29, previously grown in the absence of MnO_2, are unable to reduce MnO_2 in 3 hr in the absence of an electron carrier, either ferri- or ferrocyanide (5). They do possess the necessary enzyme system to convey electrons from glucose oxidation to ferricyanide and from ferrocyanide to MnO_2 (5). From these observations, it was hypothesized that growing cultures, initially unable to reduce MnO_2 without an added electron carrier, develop this ability during the 1-day lag as a result of an "adaptation" (5) by synthesizing one or more components that in unadapted cells are replaced by the ferri-ferrocyanide couple. The enzymatic nature of MnO_2-reduction by *Bacillus* 29 was previously suggested by the observation that 1 mM $HgCl_2$ rapidly stops MnO_2 reduction by growing cultures and that boiled or $HgCl_2$-treated cells are unable to reduce MnO_2 in resting-cell experiments (5, 18). The probable

[1] Part of a dissertation submitted by the senior author to the Department of Biology of Rensselaer Polytechnic Institute in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201.

chemical mechanism of MnO_2 reduction by *Bacillus* 29 was previously discussed (18). The present investigations show that *Bacillus* 29 reduces MnO_2 with the help of a partially inducible MnO_2-reductase system.

MATERIALS AND METHODS

Bacterial cultures. *Bacillus* 29, originally isolated from an Atlantic ferromanganese nodule (H. L. Ehrlich, Bacteriol. Proc., p. 42–43, 1964), was used exclusively in this work. The organism is a motile, gram-positive, sporeforming rod which produces acid from glucose but not lactose. It forms acetoin in glucose-peptone water and peptonizes litmus milk. It does not hydrolyze starch, reduce nitrate, or produce indole. It is able to grow in nutrient broth containing 10% NaCl. Stock cultures were grown and stored at 25 ± 1 C on Stock Culture Agar (Difco) prepared in distilled water.

An "adapted" *Bacillus* 29 culture was prepared by growing the parent strain in liquid induction medium (described below) and was maintained by subculture on Stock Culture Agar (SCA) containing about 5 mM $MnSO_4$ (J. T. Baker Chemical Co., Phillipsburg, N.J.). This medium was prepared by adding 1 ml of a separately sterilized 0.1 M $MnSO_4$ solution to 20 ml of SCA, after first cooling both to 43 to 46 C. The term "adapted" indicates that the culture was capable of MnO_2 reduction in the absence of the artificial electron carrier, ferricyanide.

Both adapted and unadapted cultures of *Bacillis* 29 were checked periodically for purity, as described elsewhere (18).

Inocula. Cells of *Bacillus* 29 for experimentation were grown on Roux bottle slants containing 100 ml of seawater-nutrient agar (SWNA), prepared as previously described (18). After 24 hr of incubation at 25 ± 1 C, the cells were harvested and washed three times by centrifugation at 1,500 × *g* in 10-fold diluted seawater at room temperature, aseptically if necessary.

Cells of adapted *Bacillus* 29 for experimentation were grown in the same manner as the parent strain, except that SWNA containing about 4.7 mM $MnSO_4$ was used. This medium was prepared by adding 5 ml of sterile 0.1 M $MnSO_4$ solution to 100 ml of melted SWNA in a Roux bottle. The cells were harvested like those of the unadapted parent strain, except that cells were washed repeatedly at 6,000 × *g* until 1 ml of the supernatant fluid was free from manganese by the assay described below (usually three to five washings).

MnO_2. Pure, reagent-grade manganese dioxide (J. T. Baker Chemical Co.), mineralogically equivalent to pyrolusite, was prepared as previously described (18).

Anaerobiosis. Anaerobic incubation was carried out in vacuum desiccators under nitrogen, as previously described (18).

Liquid induction medium. For induction studies, a basal liquid medium consisting of natural seawater containing 0.88% glucose (Difco) 0.044% peptone (Difco), and an appropriate concentration of $MnSO_4$ was used. For radiolabel incorporation studies, one of the following was also added to the medium: 0.025 μc (34 μc/μmole) of leucine-*1*-^{14}C per ml of medium, or either 0.21 or 0.08 μc (2.7 μc/μmole) of uracil-*2*-^{14}C per ml of medium. The leucine-*1*-^{14}C was a generous gift from C. Hurwitz and the uracil-*2*-^{14}C was from D. E. Wilson, Jr. When studying the effect of inhibitors of ribonucleic acid (RNA) and protein synthesis on MnO_2-reductase induction, the basal medium contained 35 μg of chloramphenicol (CAP; Parke, Davis & Co., Detroit, Mich.) per ml or 5 μg of actinomycin D (AMD) per ml. The AMD was a generous gift from D. E. Wilson, Jr.

Induction of MnO_2-reductase. To study MnO_2-reductase induction, unadapted cells of *Bacillus* 29 were suspended in liquid induction medium of appropriate composition, using 57.5 ml of medium for each cell crop from a Roux slant to give a final titer of about 5×10^8 organisms per ml. This suspension was incubated for a specific length of time at 25 ± 1 C. Then, if required, cells were harvested and washed repeatedly by centrifugation at 6,000 × *g* in 10-fold-diluted seawater until 1 ml of the supernatant fluid was free from manganese as indicated by the manganese assay. In experiments in which AMD treatment followed CAP treatment, the cells were harvested and washed at 37 C in 10-fold-diluted seawater supplemented with 0.88% glucose, 0.044% peptone, and 1.5 μg of AMD per ml until the supernatant fluid was free from manganese.

Test for MnO_2-reductase activity. To test for MnO_2-reductase activity in induced cells or cell-free extracts, 3-hr activity assays were done by the short-term assay of Trimble and Ehrlich (18), but the 0.1 ml of 0.01 M potassium ferricyanide was replaced by an equal volume of 10-fold-diluted seawater. Uninduced cells or cell-free extracts were tested by the unmodified assay (18). When the activity of cell extracts was measured, 1 ml of extract was used in place of cell suspension. Cell suspensions tested by the assay were adjusted turbidimetrically at 540 nm to contain a final titer of about 8×10^8 organisms per ml. All assays were incubated at room temperature (24 to 25 C) or at 18 C, as specified in the experiments.

Radioactivity measurements. For determining radioleucine incorporation, appropriately sized samples were collected on 0.45-μm filters (Gelman Instrument Co., Ann Arbor, Mich.; Millipore Filter Corp., Bedford, Mass.) by using an interchangeable syringe and a Swinney Filter Unit (Millipore Filter Corp.). Filters were washed first by twice passing 2-ml volumes of cold, 10% trichloroacetic acid and then distilled water through them. For the determination of radiouracil incorporation, cells were collected by centrifugation at 4,000 × *g* at room temperature and resuspended for 15 min in a few milliliters of ice-cold, 10% trichloroacetic acid. A portion of this suspension was collected on 0.45-μm filter membranes and washed twice with 2-ml volumes of distilled water. After the final washing in distilled water, all filters were air-dried and then cemented by their periphery to copper planchettes. In all radiolabel incorporation studies, cell titers of different suspensions were equalized immediately before sample collection. Radioactivity counting was carried out in a model PC-1 Gas Flow Proportional Counter (Nuclear Measurements Corp., Indianapolis, Ind.) for a length of time which resulted

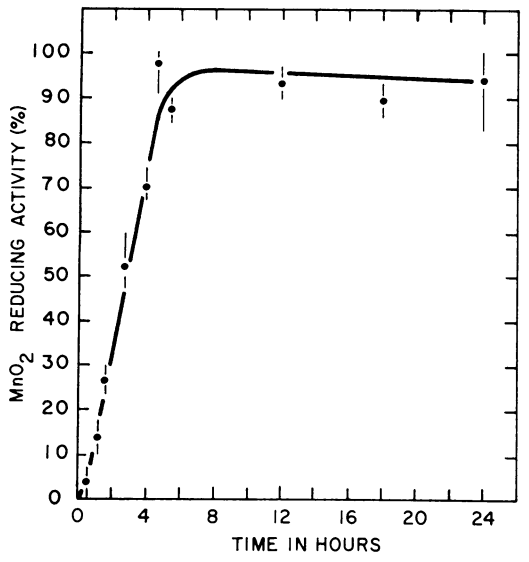

FIG. 1. *Levels of MnO_2-reducing activity of resting cells of Bacillus 29 after different periods of incubation in liquid induction medium containing 4.35 × 10^{-3} M $MnSO_4$. The per cent activities were calculated on the basis of a maximum of 24.0 nmoles of Mn^{2+} per ml released in the 3-hr activity assay. Vertical bars indicate spread of values about the mean for each point.*

in a 2-sigma counting error of less than 1%. All data were corrected for background counts and nonspecific binding of radioactive material to the filter membranes.

Cell-free extracts. Cell-free extracts of adapted and unadapted *Bacillus* 29 were prepared by sonic disruption, by using a Biosonic BP-III generator equipped with a BP-III probe assembly (Bronwill Scientific, Inc., Rochester, N.Y.). For disruption, the washed harvest from two to three Roux bottle slants of *Bacillus* 29 (adapted or unadapted) was suspended in 5 to 7 ml of 10-fold-diluted seawater and sonically treated in an ice-water bath at an intensity of 55 to 60 scale units for a total of 5 min in 1-min intervals interspaced by 0.5-min rest periods to facilitate cooling. The crude sonic-treated material was centrifuged at 10,000 × *g* for 20 min at 4 C. The supernatant fluid, which contained the enzyme activity, was separated from the precipitate by decantation and designated "crude extract."

Manganese determination. To measure the amount of MnO_2 reduced by cells or cell-free extracts in the MnO_2-reductase assay, the method of Ehrlich (5) was used, except that no 1.5-ml sample of supernatant fluid was removed before acidification with 0.05 ml of 10 N H_2SO_4.

Protein determination. Protein content of crude extracts was measured by the method of Lowry et al. (10). Developed color was read at 680 nm in a Coleman Junior spectrophotometer (Coleman Instruments, Inc., Maywood, Ill.). The assay was standardized by use of 5× recrystallized bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio).

RESULTS

Induction of MnO_2-reductase activity. Twenty-four-hour-old cells of *Bacillus* 29, unadapted for MnO_2-reduction, were incubated aerobically at 25 C for various lengths of time in liquid induction medium containing 4.4 mM $MnSO_4$ and then harvested, washed, and tested for their ability to reduce MnO_2. The results (Fig. 1) show that some MnO_2-reducing activity was formed after 0.5 hr of contact with $MnSO_4$, reaching a peak of activity after about 4.5 hr of incubation. Control experiments in which the bacteria were incubated for 24 hr in modified induction medium showed that, for $MnSO_4$ to induce MnO_2-reductase activity in *Bacillus* 29, both oxygen and glucose or peptone were required (Table 1). When peptone was the only organic substrate in the induction medium, the level of MnO_2-reductase formed was twice that found when glucose was the only organic substrate, suggesting a need for protein synthesis for induction to take place. Optimal MnO_2-reductase induction occurred in the combined presence of glucose and peptone. Cells incubated in the absence of glucose and peptone but in the presence of $MnSO_4$, although uninduced, were still able to reduce MnO_2 in the presence of the electron carrier ferricyanide, indicating that their incomplete MnO_2-reductase system was still active. In similar experiments, iron, furnished either as limonite ($Fe_2O_3 \cdot nH_2O$), a component of some manganese nodules, or as $FeSO_4$, did not replace $MnSO_4$ as an inducer of

TABLE 1. *Level of MnO_2-reductase activity in Bacillus 29 after 24 hr of incubation in the presence of $MnSO_4$ under different experimental conditions*

Condition	Avg activity (%)
Complete medium[a]	100 (4)[b]
Complete medium − air	3 (2)
Complete medium − glucose	67 (2)
Complete medium − peptone	36 (2)
Complete medium − glucose − peptone	0 (2)
Complete medium − glucose − peptone[c]	122 (2)

[a] Complete medium consisted of 4.4 mM MnSO, 0.88% glucose, and 0.044% peptone in seawater in air.

[b] Values in parentheses indicate number of experiments reported.

[c] Cells tested in the presence of $Fe(CN)_6^{3-}$.

MnO_2-reductase activity in the organism. Cultures of *Bacillus* 29 adapted in the liquid induction medium could be maintained in the induced state by cultivation on SCA or SWNA containing 4.7 mM $MnSO_4$ (*see above*).

Effect of $MnSO_4$ concentration on MnO_2-reductase induction. When unadapted bacteria were incubated for 5 hr at 25 C in liquid induction medium at various concentrations of $MnSO_4$, the levels of MnO_2-reductase activity shown in Fig. 2 were obtained. The highest level of activity was observed at an $MnSO_4$ concentration of 4.35 mM or 240 μg of Mn^{2+} per ml. A gradual increase in activity occurred between 0.435 μM and 4.35 mM. Thereafter, a further 10-fold increase in $MnSO_4$ concentration caused a strong suppression of induction. It is noteworthy that 30% of maximal activity still formed after 5 hr of contact with 0.435 μM Mn^{2+} or 0.024 μg of Mn^{2+} per ml. This is within the range of maximum concentration of 0.01 μg of manganese per ml of open ocean water and 6.6 μg of interstitial water per ml of an ocean sediment (15, 21).

Requirement for protein and RNA synthesis in MnO_2-reductase induction. To determine whether the adaptation to MnO_2-reduction was truly an induction of missing enzyme protein (or proteins)

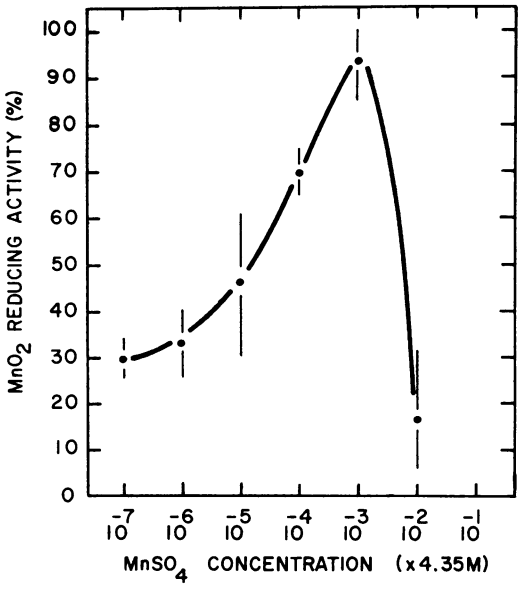

FIG. 2. *Induction of MnO_2-reducing activity in resting cells of Bacillus 29 by different concentrations of $MnSO_4$ in liquid induction medium. Incubation at each $MnSO_4$ concentration was for 5 hr. The per cent activities were calculated on the basis of a maximum of 24.0 nmoles of Mn^{2+} per ml released in the 3-hr activity assay. Vertical bars indicate spread of values about the mean for each point.*

in the electron transport chain to MnO_2, experiments were conducted in which either CAP or AMD was incorporated into the induction medium with an appropriate, radiolabeled compound. Prior control experiments showed that CAP at a concentration of 35 μg per ml of liquid induction medium inhibited about 95% of protein synthesis in *Bacillus* 29 during 5 hr of incubation as measured by leucine-*1*-^{14}C incorporation. At this concentration, CAP did not significantly affect RNA synthesis as measured by the incorporation of uracil-*2*-^{14}C into acid-insoluble material.

Additional control experiments revealed that the protein synthesis inhibition caused by CAP could be removed by washing the cells in fresh, inhibitor-free medium consisting of 10-fold-diluted seawater containing 0.88% glucose and 0.44% peptone and then incubating them in wash medium containing leucine-*1*-^{14}C. The washed cells incorporated the radiolabel rapidly and continuously into cellular material. The rate of reversal of drug inhibition measured by this technique was temperature-dependent, being at least twofold greater at 37 C than at room temperature (20 C), which is in agreement with similar studies conducted with *Escherichia coli* (8).

Since uracil may act as a precursor in the synthesis of the thymine component of deoxyribonucleic acid (DNA), a control experiment testing for radiolabel incorporation into DNA was performed. Two batches of *Bacillus* 29 (about 5 $\times$ 10^9 organisms each), grown in the presence of uracil-*2*-^{14}C, were collected by centrifugation. One batch was digested by suspension in 1 ml of 0.3 N NaOH at room temperature, whereas the other was suspended in 1 ml of distilled water and maintained in an ice bath. After 2.5 hr of incubation, 5 ml of ice-cold, 10% trichloroacetic acid was added to each batch, and an equal sample was removed from each and collected on 0.45-μm filter membranes. The membranes were washed with 10% trichloroacetic acid and then distilled water and prepared for counting. The results indicated that less than 6% of the incorporated label was found in DNA.

After ascertaining that CAP functioned specifically as an inhibitor of protein synthesis in *Bacillus* 29 under the experimental conditions employed, the effect of this drug and that of AMD was tested on the formation of MnO_2-reductase activity. Table 2 summarizes five similar experiments in which duplicate batches of unadapted cells of *Bacillus* 29 were incubated for 5 hr at 25 C in flasks with liquid induction medium containing 4.35 mM $MnSO_4$. One flask of each pair contained either 35 μg of CAP per ml or 5 μg of

TABLE 2. *Effect of chloramphenicol and actinomycin D on the incorporation of radioactive label and the formation of MnO_2-reductase activity in unadapted Bacillus 29 during 5 hr of incubation under inducing conditions*[a]

Inhibitor[b]	Radiolabel	Avg per cent inhibition	
		Radioactivity incorporation[c]	MnO_2-reductase activity[d]
Chloramphenicol	Leucine-*1*-^{14}C	92 (2)[e]	89 (3)
Actinomycin D	Uracil-*2*-^{14}C	91 (2)	95 (2)

[a] *See* footnote *a*, Table 1.
[b] Chloramphenicol, 35 μg/ml; actinomycin D, 5 μg/ml.
[c] Technique of determination of incorporation described in Materials and Methods.
[d] As determined by the 3-hr activity assay.
[e] Numbers in parentheses represent number of experiments reported.

AMD per ml as indicated, whereas the other flask was free from inhibitor. At the end of the incubation period, the differently treated batches of cells were harvested separately, washed at room temperature in 10-fold-diluted seawater, and tested for MnO_2-reductase activity. The results (Table 2) are consistent with the notion that both RNA synthesis and protein synthesis are required for the formation of MnO_2-reductase activity in unadapted *Bacillus* 29.

It was reasoned that if *Bacillus* 29 became adapted for MnO_2 reduction by a classical induction mechanism as implied by the results of the foregoing experiments, a demonstration of the appearance of MnO_2-reductase activity in unadapted cells as a result of sequential synthesis of messenger RNA followed by that of protein might be possible. The hypothesis was tested by first incubating unadapted cells in the induction medium in the presence of 35 μg of CAP per ml for 5 hr at 25 C. Then AMD was added to a final concentration of 5 μg per ml, and the cells were quickly harvested and washed repeatedly at 37 C with $MnSO_4$-free medium supplemented with AMD until the final wash fluid was free from manganese. These cells were now tested for their ability to reduce MnO_2. Results of repeated experiments indicated that the cells were, indeed, able to reduce MnO_2 without ferricyanide after these sequential treatments. The level of MnO_2-reductase activity in such cells was approximately half of the maximal level found when cells were induced in the absence of the drugs.

Effect of oxygen on MnO_2-reductase activity in whole, induced cells of Bacillus 29. The cells for this experiment were grown on Roux bottle slants of SWNA containing 4.7 mM $MnSO_4$. Activity tests for MnO_2-reductase carried out in air or in a nitrogen atmosphere showed no difference in activity (Table 3).

MnO_2-reductase activity in cell-free extracts from induced Bacillus 29. A number of crude extracts of induced cultures were tested for MnO_2-reductase by the 3-hr activity assay. Enzyme activity, expressed as nanomoles of manganese solubilized per hour at 25 C in the 3-hr assay, was determined relative to protein concentration in the extract. Although the total activity of different preparations varied, the specific activity consistently fell in the range of 0.9 to 1.3 (average 1.15) nmoles of Mn^{II} per mg of protein per hr. The MnO_2-reductase activity of a given crude extract was directly proportional to enzyme concentration over the range tested. It was the same in air and under nitrogen (Table 3). In control experiments, crude extracts from unadapted bacteria failed to reduce MnO_2 in 3 hr unless ferricyanide was present in the reaction medium.

The temperature optimum of MnO_2-reductase activity in the cell-free preparation was determined by assaying the activity of 1-ml portions of a given extract at different incubation temperatures. Figure 3 summarizes the results of several experiments. The optimal temperature was found to be 18 C.

The dependence of MnO_2-reductase on an oxidizable substrate (glucose) was determined by

TABLE 3. *MnO_2-reduction by induced cells of Bacillus 29 and extracts from them in the presence and absence of oxygen*

Experimental conditions	Manganese in supernatant fluid (nmoles/ml)[a]	
	Aerobic	Anaerobic
Medium[b] − cells + MnO_2.....	3.2	0
Medium + cells − MnO_2.....	4.8	4.8
Medium + cells + MnO_2.....	27.5	27.5
Medium − extract + MnO_2....	4.8	4.8
Medium + extract − MnO_2....	6.4	6.4
Medium + extract + MnO_2....	27.2	27.2

[a] After acidification of the reaction mixture at the end of the 3-hr assay.
[b] The medium consisted of 1 ml of 25 mM $NaHCO_3$ (*p*H 7) containing 1% glucose, 5 ml of 10-fold diluted seawater, and 0.1 g of MnO_2, where noted.

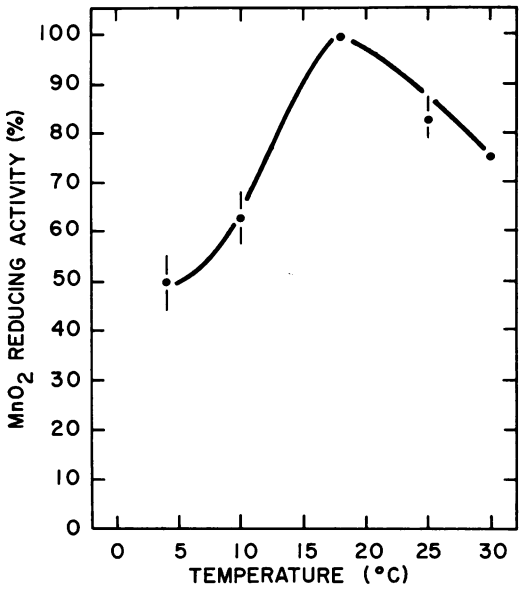

FIG. 3. *Relative MnO_2-reductase activity of cell-free extracts of induced Bacillus 29 at various temperatures. Vertical bars indicate spread of values about the mean for each point.*

testing 1-ml portions of cell-free extract by the 3-hr assay in the presence and absence of glucose. Initial experiments indicated that crude extracts of *Bacillus* 29, prepared immediately after harvesting, were only 35 to 45% dependent on glucose for the reduction of MnO_2. This low dependence was thought to be a result of utilization of reduced metabolic intermediates as well as endogenous stores liberated into the extract during sonic disruption. To test this hypothesis, cells were stored in 10-fold-diluted seawater at 25 C for 30 min after harvesting. Crude extracts from cells treated in this manner exhibited at least 60% glucose dependence in the reduction of MnO_2 in comparison to substrate-free controls.

The effect of temperature on the stability of MnO_2-reductase activity in cell-free preparations was determined by heating the enzyme in a boiling-water bath and by storing it at 4 C and at −20 C. It was found that the extract lost 58% of its activity after 2 min and 91% after 5 min of boiling. Further decrease in activity after the first 5 min of boiling was slow, probably due to the presence of nucleic acid which may protect some of the enzyme proteins from denaturation (6). The MnO_2-reductase activity appeared stable for at least 4 days at 4 C and still retained 54% of its activity after 7 days. It lost activity on freezing, probably due to salt effects, as extracts were prepared in 10-fold-diluted seawater.

Inhibitor studies on MnO_2-reductase activity in cell-free extracts showed that at a concentration of 1 mM, $HgCl_2$ caused 75 to 85% inhibition and atebrine dihydrochloride caused 50% inhibition in 3-hr activity assays. Sodium azide had no effect at this concentration.

DISCUSSION

These investigations show that manganous ion, or possibly some derivative thereof, acts as an inducer in the formation of a ferricyanide-independent MnO_2-reductase system in *Bacillus* 29. Accompanying sulfate ions can be ruled out as an inducer since seawater alone contains a higher sulfate concentration than that contained in the added $MnSO_4$. Yet, MnO_2-reductase activity is not induced in cells growing in seawater containing glucose and peptone but no $MnSO_4$.

Since Mg^{2+} and Mn^{2+} may substitute for one another in the activation of some enzymes (3), Mg^{2+} may be expected to substitute for Mn^{2+} as an inducer of MnO_2-reductase. This possibility can be ruled out because of the high concentration of Mg^{2+} in seawater, which is about 125,000 times greater by weight than that of manganese (21). Contact with it in an $MnSO_4$-free medium does not induce the enzyme.

If manganous ion as such is the inducer, it would represent an unusual example of induction because manganous ion is a product of the induced enzyme reaction rather than the substrate. This is, however, not impossible since in nature manganous manganese is usually associated with manganic oxides through adsorption, and we assume that manganous manganese from this source is the natural inducer. The actual induction may involve a derepression as a result of reaction of the repressor with Mn^{2+} and its resultant inactivation.

It may be argued that the true inducer is actually an oxidation product of Mn^{2+}, namely tri- or tetra-valent manganese formed by oxidation of Mn^{2+}, since oxygen was required in these experiments. However, Mn^{3+} ions can be ruled out since they are very unstable in aqueous solution (7), and Mn^{4+} ions can be ruled out since they are not known to occur in aqueous solution (7). One way in which oxidation of Mn^{II} may be involved in the induction phenomenon is by conversion of an Mn^{II}-repressor complex to an Mn^{III}- or Mn^{IV}-repressor complex, which may be more stable than the Mn^{II}-repressor complex. On the other hand, the oxygen requirement may be related to the energy need for the synthesis of the one or more missing enzyme components. The results of the present

study do not permit a choice among these alternative explanations.

The inducibility of MnO_2-reductase explains why growing cultures of unadapted *Bacillus* 29 are able to release Mn^{II} from MnO_2 after a 1-day lag (18); namely, they are induced by small amounts of manganous ion normally associated with MnO_2 in the first day of growth. Manganese dioxide itself is too insoluble to act as inducer.

The observations that the appearance of MnO_2-reductase in *Bacillus* 29 incubated in induction medium was prevented when RNA or protein synthesis was inhibited show that a classical induction mechanism, rather than a mutation and selection phenomenon or an allosteric rearrangement of an existing cellular component, was involved. The induction of maximal activity in a large population of unadapted cells in a brief span of 5 hr also rules out a mutation and selection phenomenon. No significant change in cell numbers took place during induction of *Bacillus* 29 in the liquid induction medium, as measured by turbidity changes. Since the initial cell concentration in the induction medium was usually around 10^8 to 10^9 cells per ml, which is the maximum cell concentration achieved by a growing culture of *Bacillus* 29 in this medium, we would not expect significant multiplication during the induction period. The finding that extracts from induced but not uninduced bacteria contain ferricyanide-independent MnO_2-reductase activity is consistent with the induction hypothesis.

The enzymatic nature of the reductase system is indicated by the susceptibility of cell-free extracts to heat inactivation and their inhibition by $HgCl_2$ and atebrine dihydrochloride, and also by the dependence of activity on extract concentration.

The ability of induced whole cells or extracts prepared from them to carry out MnO_2-reduction at the same rate whether oxygen was present or not confirms the earlier finding that oxygen and MnO_2 do not compete as terminal electron acceptors in the respiratory activity of *Bacillus* 29 (18). This represents an interesting case with respect to other organisms which use inorganic terminal electron acceptors. Whereas the sulfate reducers and methane formers are obligately anaerobic, *Bacillus* 29 is unaffected by the presence or absence of oxygen in its ability to reduce MnO_2 once the cells are induced. Dissimilatory nitrate reducers represent an intermediate case in that they are competitively inhibited in their ability to reduce nitrate by the presence of oxygen.

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