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# Manganese Oxidation by Spores and Spore Coats of a Marine *Bacillus* Species

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***Bacillus* sp. strain SG-1 is a marine bacterial species isolated from a near-shore manganese sediment sample. Its mature dormant spores promote the oxidation of $Mn^{2+}$ to $MnO_2$. By quantifying the amounts of immobilized and oxidized manganese, it was established that bound manganese was almost instantaneously oxidized. When the final oxidation of manganese by the spores was partly inhibited by $NaN_3$ or anaerobiosis, an equivalent decrease in manganese immobilization was observed. After formation of a certain amount of $MnO_2$ by the spores, the oxidation rate decreased. A maximal encrustment was observed after which no further oxidation occurred. The oxidizing activity could be recovered by reduction of the $MnO_2$ with hydroxylamine. Once the spores were encrusted, they could bind significant amounts of manganese, even when no oxidation occurred. Purified spore coat preparations oxidized manganese at the same rate as intact spores. During the oxidation of manganese in spore coat preparations, molecular oxygen was consumed and protons were liberated. The data indicate that a spore coat component promoted the oxidation of $Mn^{2+}$ in a biologically catalyzed process, after adsorption of the ion to incipiently formed $MnO_2$. Eventually, when large amounts of $MnO_2$ were allowed to accumulate, the active sites were masked and further oxidation was prevented.**

A wide variety of different bacteria have the ability to catalyze the reduction or oxidation of manganese (7, 12, 14). The question arises whether these manganese transformations may be involved in the primary metabolism of bacterial cells. Chemolithotrophic or mixotrophic growth sustained by $Mn^{2+}$ ions has been suggested for several manganese-oxidizing bacterial strains (1, 6). However, so far no unambiguous evidence to support this idea has been presented (14, 20). Some species of manganese-reducing organisms may use manganese oxides as alternative electron acceptors in the absence of oxygen (3, 8). Few species of microorganisms have been reported which have the ability to catalyze both the oxidation of $Mn^{2+}$ and the reduction of $MnO_2$. Bromfield and David (2) described a soil *Arthrobacter* species which oxidized $Mn^{2+}$ in the presence of oxygen, but reduced the formed oxide under anaerobic conditions. A marine sporeforming *Bacillus* species, strain SG-1, promotes the oxidation of $Mn^{2+}$ or the reduction of $MnO_2$, depending on its life stage. Rosson and Nealson (17) demonstrated that mature dormant spores of this species promote the oxidation of $Mn^{2+}$ to $MnO_2$. The $MnO_2$ was precipitated on the spore surface. The oxidation was inhibited by high temperatures, $NaN_3$, $HgCl_2$, and KCN. The authors suggested that $Mn^{2+}$ was complexed by a spore component; once bound, the manganese was rapidly oxidized. In a previous report (4) we demonstrated that vegetative cells of the same *Bacillus* strain reduced $MnO_2$ under low-oxygen conditions. Under oxic conditions the $MnO_2$ reduction could be induced by addition of $NaN_3$. Endogenous reduced cytochromes could be oxidized in situ by addition of $MnO_2$ to the cells. These data suggested that the vegetative cells of *Bacillus* sp. strain SG-1 may use $MnO_2$ as an electron acceptor. Therefore this species may benefit from the oxidizing activity of its spores upon germination in a low-oxygen environment.

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In the present study we investigate the mechanism of the oxidation reaction and the localization of the activity in more detail.

## MATERIALS AND METHODS

**Organism and its cultivation.** *Bacillus* sp. strain SG-1, isolated by Nealson and Ford (15), was grown at room temperature on a modified K medium (see reference 17): 2 g of peptone (Difco Laboratories), 0.5 g of yeast extract (Difco), 10 μg of ferri-EDTA, and 100 μg of $MnCl_2 \cdot 4H_2O$ (filter sterilized) per liter of 50 mM Tris in 80% natural seawater, pH 7.0. For the isolation of spores, aerated batch cultures of 8 liters were used. After approximately 10 days 95% sporulation was achieved.

**Preparation of spore suspensions.** Spores were isolated and purified according to the method of Goldman and Tipper (9) as modified by Rosson and Nealson (17). Purified spores were stored in deionized water at 4°C. For storage periods of longer than 1 week, spores were frozen in 10% dimethyl sulfoxide at −80°C. Spores could be stored this way for at least 2 months without significant loss of oxidizing activity.

**Preparation of spore coats.** Spore coats were prepared essentially as described by Goldman and Tipper (9). Suspensions of purified spores in 10 mM Tris buffer, pH 7.0, were supplemented with an equal volume of glass beads (10 to 50 μm in diameter) and sonicated for 15 min at 30-s intervals at 0°C, using an ultrasonic cell disruptor at maximum amplitude. The glass beads were allowed to settle down and the supernatant containing the spore coats was removed. The glass beads were washed twice with an equal volume of 10 mM Tris, pH 7.0. The original supernatant and the supernatants of the washes were combined and subsequentally centrifuged at 15,000 × *g* for 15 min. The sediment was suspended in 10 mM Tris buffer, pH 7.5, and treated with lysozyme (100 μg/ml) for 30 min at 37°C to lyse the cortex peptidoglycan. After centrifugation the spore coats were

washed in the following sequence: once with 1 M NaCl, once with 0.14 M NaCl, once with 1% sodium dodecyl sulfate, and finally five times with deionized water. All treatments were carried out at room temperature, and except for the sodium dodecyl sulfate and deionized water washes, all solutions were supplemented with EDTA (10 mM; pH 7.5) and phenylmethylsulfonyl fluoride (0.3% wt/vol) to inhibit protease activities.

**Stock suspensions of spores and of spore coats.** Stock spore suspensions contained 0.1 g (wet weight) of spores per ml. They contained $1.5 \times 10^{10} \pm 0.2 \times 10^{10}$ spores per ml as determined with a Burker counting chamber and 3.2 ± 0.6 mg of dipicolinic acid, a specific spore component, as determined with the dipicolinic acid assay according to Janssen et al. (10). One milliliter of a stock spore coat suspension contained the coats isolated from $1.5 \times 10^{10}$ spores.

**Determination of manganese binding and oxidation.** Manganese binding to spores or spore coats was measured essentially as described by Rosson and Nealson (17). Spores or spore coats were suspended in 80% natural seawater, containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5 ($3 \times 10^9$ spores or spore coats isolated from that amount of spores per ml, unless stated otherwise), supplemented with 100 μM $MnCl_2$ and 10 μCi of $^{54}Mn^{2+}$ (New England Nuclear Corp.). At specified times duplicate samples of 0.2 ml were filtered through 0.2-μm nitrocellulose filters (Millipore Corp.). The filters were rinsed twice with 5 ml of seawater, pH 7.5. The filters and 0.2 ml of the filtrates were transferred to counting vials. Filters were treated with 0.2 ml of 1% (wt/vol) $NH_2OH$ solution to reduce the manganese oxide formed. Significant absorbance of $^{54}Mn^{2+}$ radiation occurred when this treatment was omitted. Subsequently 9 ml of scintillation cocktail (Rialuma; 3 M Co.) was added and the radioactivity of filters and filtrate samples was measured in a liquid scintillation counter (Isocap). Counting efficiency was approximately 30%. The recovery of radioactivity thus determined amounted to at least 95%.

The amount of manganese oxide formed by spores or spore coats was measured with a colorimetric assay, using Leukoberbelin blue (LBB), as described before (4, 11). Samples to determine manganese binding and manganese oxidation were taken simultaneously. The exact nature of the manganese oxide produced has not been investigated so far. For convenience it is referred to as $MnO_2$ in this paper.

**Determination of proton release during $Mn^{2+}$ oxidation by spore coats.** In these experiments suspensions of spore coats in unbuffered deionized water (pH adjusted to 7.5 with diluted HCl or NaOH) were used. These suspensions were supplemented with $MnCl_2$ (final concentration, 100 μM), and the pH decrease during $MnO_2$ formation was recorded (approximately 0.3 pH unit per 100 μM $MnO_2$ formed). The relation between pH decrease and $H^+$ production under these conditions was quantified by measuring the pH decreases after stepwise additions of 10-μl amounts of a 10 mM HCl solution to 5 ml of identical unbuffered suspensions of nonencrusted spore coats or, alternatively, spore coats encrusted with 100 μM $MnO_2$. Since the latter had a slightly higher buffer capacity than the nonencrusted coat suspensions, corrections had to be made for the degree of encrustment during the oxidation reaction.

**Determination of oxygen consumption during $Mn^{2+}$ oxidation by spore coats.** Oxygen consumption during $Mn^{2+}$ oxidation in spore coat suspensions was measured at 25°C, using a Clark oxygen electrode in a closed cell with a volume of 1 ml. The oxygen concentration at 100% saturation was taken to be 260 μM.

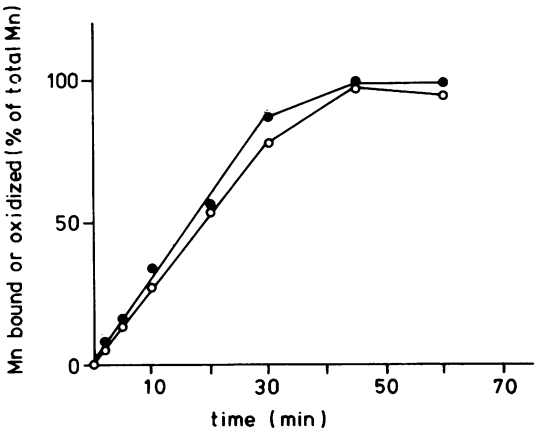


FIG. 1. Manganese binding and oxidation by spores of *Bacillus* sp. strain SG-1. Spore suspensions of $3 \times 10^9$ spores per ml in 50 mM HEPES-buffered seawater, pH 7.5, were supplemented with $MnCl_2$ (final concentration, 100 μM) and 10 μCi of $^{54}Mn$ per ml. At specified times the amounts of manganese immobilized (●) and oxidized (○) were measured. The amounts are expressed as a percentage of the total manganese added. For further experimental details see Materials and Methods.

## RESULTS

**Maganese binding and oxidation by spores of *Bacillus* sp. strain SG-1.** Spores of *Bacillus* sp. strain SG-1 efficiently remove $Mn^{2+}$ ions from their medium (see reference 17). At a concentration of $3 \times 10^9$ spores per ml, 100 μM $Mn^{2+}$ was completely bound within 1 h (Fig. 1, closed circles). The amount of oxidized $Mn^{2+}$ was quantified by reaction with the redox dye LBB (Fig. 1, open circles). The rate of $MnO_2$ formation closely followed that of manganese binding. It should be kept in mind that the amount of manganese bound represents the total amount of manganese immobilized at the spore surface: it thus includes oxidized $Mn^{2+}$, $Mn^{2+}$ bound to the spores, and $Mn^{2+}$ adsorbed to the oxide already formed (see below).

**Effect of $MnO_2$ encrustment on $Mn^{2+}$ binding and oxidizing activity.** It is well known that the adsorption of $Mn^{2+}$ ions on manganese oxides may promote the autocatalytic oxidation of $Mn^{2+}$ (13). Therefore, experiments were performed to test whether the oxide formed by spores catalyzed a rapid abiological oxidation of $Mn^{2+}$. Spore suspensions were incubated with various amounts of $Mn^{2+}$ (concentrations varying from 0 to 7 mM). After 48 h of incubation the amount of $MnO_2$ formed was determined (Fig. 2). The maximum concentration of $Mn^{2+}$ that the spores could oxidize was 1.3 mM (Fig. 2). At concentrations higher than 1.3 mM $Mn^{2+}$ no further oxidation could be measured.

In another experiment spore suspensions in 5 ml of HEPES-buffered seawater, pH 7.5 ($3 \times 10^9$ spores per ml), were incubated with 0, 0.1, or 1 mM $MnCl_2$ for 48 h. Subsequently, from each sample two 2-ml portions were taken. The portions were made up to 5 ml, one portion with HEPES-buffered seawater and the other with HEPES-buffered seawater containing $NaN_3$ (final concentration, 50 mM). Azide inhibits $Mn^{2+}$ oxidation by spores (17), but does not interfere with $Mn^{2+}$ adsorption on manganese oxides (18). The suspensions were supplemented with $MnCl_2$ (final concentration, 100 μM) and 10 μCi of $^{54}Mn^{2+}$ per ml, and

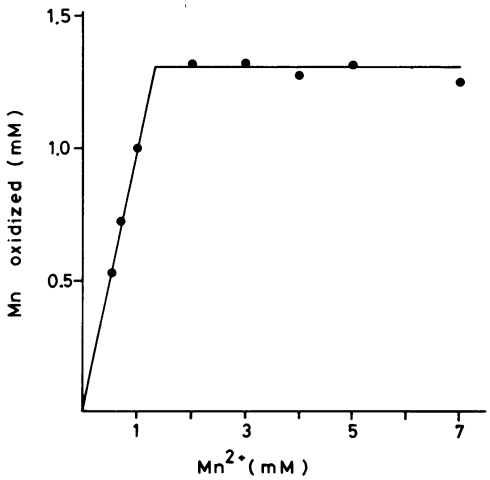


FIG. 2. $Mn^{2+}$ oxidation by spores of *Bacillus* sp. strain SG-1 at different initial $Mn^{2+}$ concentrations. Spore suspensions of $3 \times 10^9$ spores per ml in 80% natural seawater containing 50 mM HEPES, pH 7.5, were supplemented with various concentrations of $MnCl_2$. After 48 h of incubation the $MnO_2$ formed was measured with the LBB assay. The concentration of $MnO_2$ is plotted as a function of the initial $Mn^{2+}$ concentration.

manganese binding and oxidation were measured (Fig. 3). When manganese oxidation by nonencrusted spores was inhibited by $NaN_3$, the amount of manganese bound decreased accordingly, (Fig. 3A). The oxidation rates of spores encrusted with 100 μM $MnO_2$ did not differ significantly from those of nonencrusted spores (Fig. 3B). In the presence of $NaN_3$, the encrusted spores showed slightly enhanced manganese binding, probably due to $Mn^{2+}$ adsorption to the preformed oxide. This adsorption was less pronounced in the absence of $NaN_3$. Manganese oxidation was clearly inhibited when spores were encrusted with 1 mM $MnO_2$, in spite of increased binding of $Mn^{2+}$ (Fig. 3C). In the presence of $NaN_3$ no oxidation could be measured at all, whereas the binding of $Mn^{2+}$ was significant (Fig. 3C). Apparently, $Mn^{2+}$

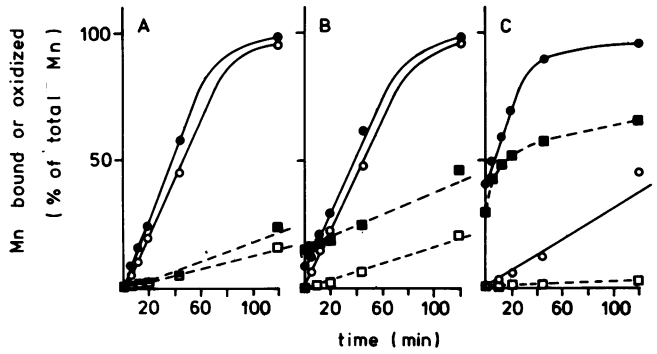


FIG. 3. Effect of $MnO_2$ encrustment on $Mn^{2+}$ binding and oxidation by spores. Spores which had previously oxidized certain amounts of $Mn^{2+}$ were suspended in 80% natural seawater containing 50 mM HEPES, pH 7.5, in either the absence or presence of 50 mM $NaN_3$ (circles and squares, respectively). These spore suspensions were supplemented with 100 μM $MnCl_2$ and 10 μCi of $^{54}Mn^{2+}$ per ml and the $Mn^{2+}$ binding (closed symbols) and oxidation (open symbols) were measured. $Mn^{2+}$ binding and oxidation are expressed as a percentage of the total amount of $Mn^{2+}$ added at time zero. Preformed $MnO_2$: 0 (A), 0.1 (B), and 1.0 (C) mM per $3 \times 10^9$ spores. Spore concentration: $1.2 \times 10^9$ spores per ml.

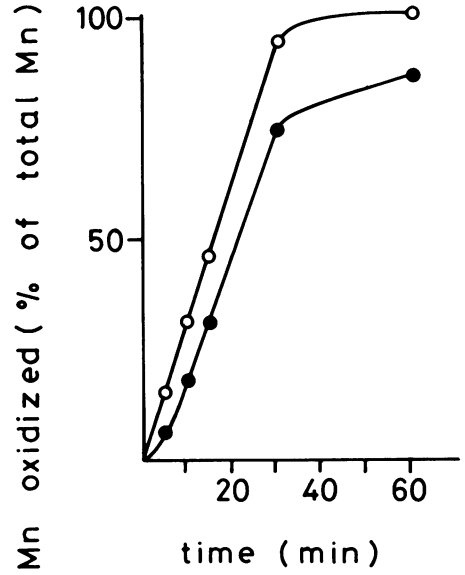


FIG. 4. Recovery of $Mn^{2+}$-oxidizing activity after reduction of the $MnO_2$ of encrusted spores. Spores maximally encrusted with $MnO_2$ (cf. Fig. 2) were treated with 1% (wt/vol) $NH_2OH$ in deionized water to reduce the $MnO_2$. After extensive washing, the $Mn^{2+}$-oxidizing activity of these spores was measured with the LBB assay (●) and compared with that of control spores (○). Spore concentration: $3 \times 10^9$ spores per ml.

was adsorbed by $MnO_2$ formed by the spores. This process, however, did not induce rapid abiological $Mn^{2+}$ oxidation.

The apparent maximum amount of $Mn^{2+}$ that could be oxidized by the spores and the decrease in $Mn^{2+}$ oxidation rate with increasing amounts of $MnO_2$ on the spore surface indicate masking of active sites or an irreversible conversion of the oxidizing factor in a stoichiometric reaction with $Mn^{2+}$. To discriminate between these possibilities, the following experiment was performed. Maximally encrusted spores were treated with $NH_2OH$ solution to reduce the $MnO_2$. After washing the spores extensively with deionized water, the kinetics of $Mn^{2+}$ oxidation were compared with that of untreated spores (Fig. 4). Apart from a short initial lag phase, the rate of oxidation of treated spores did not differ markedly from that of untreated spores.

**Manganese binding and oxidation by spore coats of *Bacillus* sp. strain SG-1.** To investigate whether the manganese-oxidizing activity is associated with the spore coats, suspensions of spore coats were prepared and the binding and oxidation of manganese were measured. With phase-contrast and electron microscopy, no intact spores could be observed in the spore coat preparations. The dipicolinic acid content of the spore coats was <1% of that of the original spores. The rates of manganese binding (Fig. 5A, closed circles) and manganese oxidation (Fig. 5A, open circles) of these spore coat preparations did not differ significantly from those of intact spores (cf. Fig. 1). In many cases spore coats showed an initial lag phase in $Mn^{2+}$ oxidation, the length of which varied with batch activity. The manganese-oxidizing activity of spore coats was inhibited by KCN, $NaN_3$, $HgCl_2$, and pre-encrustment with $MnO_2$ at concentrations needed to inhibit the oxidation of complete spores (results not shown). Apparently the manganese-oxidizing activity is located on the spore coats or on the exosporium, if present.

**Mechanism of the manganese oxidation reaction.** When a spore coat suspension was continuously sparged with nitro-

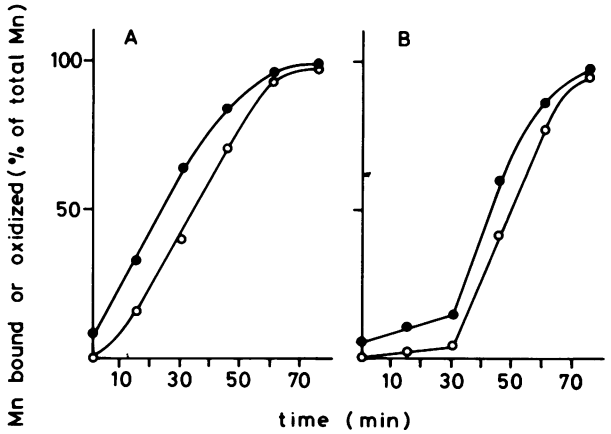


FIG. 5. Manganese binding and oxidation by spore coats. Manganese immobilization (●) and oxidation (○) by spore coats were measured as in the legend to Fig. 1. The coat suspensions contained, per milliliter, coats isolated from $3 \times 10^9$ spores. (A) Aerobic conditions and (B) anaerobic conditions by sparging the suspension with nitrogen. After 30 min the suspension was vigorously shaken to reestablish aerobic conditions.

gen, both manganese binding and oxidation were strongly inhibited (Fig. 5B). When aerobic conditions were reestablished by shaking the suspension vigorously for several minutes, the manganese binding and oxidation regained its normal rates. Apparently the manganese oxidation reaction promoted by the spore coats needed molecular oxygen. Measurement of the oxygen concentration in spore coat suspensions during the oxidation of manganese revealed that molecular oxygen was consumed. The rates of $O_2$ consumption and $MnO_2$ formation were 2.1 and 4.3 μM/min, respectively.

In Fig. 6 the concentrations of $MnO_2$ (open circles) and $H^+$ (closed circles) formed by spore coats in unbuffered deionized water supplemented with 100 μM $Mn^{2+}$ are depicted. Clearly $H^+$ is released during the manganese oxidation reaction. The ratio between the amount of $H^+$ and of $MnO_2$ formed during the process was approximately 1.5.

## DISCUSSION

A marine *Bacillus* sp., strain SG-1, isolated from a manganese sediment, is able to catalyze the reduction or the oxidation of manganese under suitable conditions. In a previous report (4) we demonstrated the reduction of $MnO_2$ by vegetative cells. Rosson and Nealson (17) demonstrated the oxidation of $Mn^{2+}$ by mature dormant spores. The two processes seem to be functionally related. The experiments on the $MnO_2$ reduction suggest that the oxide accumulated in the dormant life stage can be used as a terminal electron acceptor in the primary metabolism of the vegetative cells. This implicates that the oxidation by spores has a selective advantage. An organism carrying an alternative electron acceptor might successfully compete with species depending on oxygen only. In this study this oxidation by spores of *Bacillus* sp. strain SG-1 is investigated in more detail.

The data in this paper support the suggestion of Rosson and Nealson that the oxidizing factor is located on the spore coat, or the exosporium, if such a structure is present in *Bacillus* sp. strain SG-1 spores. Isolated coats oxidized $Mn^{2+}$ at a similar rate as intact spores. Moreover, KCN, $NaN_3$, and $HgCl_2$ which are known to have inhibitory effects on manganese oxidation by spores, had similar effects on the manganese oxidation by spore coats. Apparently these inhibitors do not act as metabolic poisons, as also proposed by Rosson and Nealson. Whereas KCN probably inhibits $Mn^{2+}$ oxidation through its $Mn^{2+}$-complexing ability (18), $NaN_3$ and $HgCl_2$ may affect manganese oxidation through their interaction with one or more coat proteins, which possibly contain sulfur or metal components.

By using higher spore concentrations than reported by Rosson and Nealson (or spore coats isolated from these spore suspensions), the period in which 100 μM $Mn^{2+}$ was completely oxidized was reduced from several days to approximately 1 h. By measuring the amount of manganese immobilized by spores with $^{54}Mn$ as a tracer and quantification of the $MnO_2$ produced with the redox dye LBB, the kinetics of $Mn^{2+}$ binding and $Mn^{2+}$ oxidation could be compared. Initially the increase in bound as well as oxidized $Mn^{2+}$ was linear with time. The amount of $Mn^{2+}$ oxidized as a percentage of the total immobilized manganese varied between 90 and 100% over the total experimental period. Rosson and Nealson suggested that $Mn^{2+}$ is complexed by a spore coat component prior to oxidation. Our data indicate that the process is more complicated. Under conditions where the oxidation step was strongly inhibited, only minor binding of $Mn^{2+}$ to the spores was observed. Consequently, if an $Mn^{2+}$-binding substance is present on the spore surface, it has only a low binding capacity. On the other hand, spores encrusted with $MnO_2$ bound significant amounts of $Mn^{2+}$ also when oxidation was inhibited. Probably $Mn^{2+}$ is adsorbed preferentially to the $MnO_2$ formed by the spores rather than to the spore surface itself. Under the experimental conditions, autocatalytic manganese oxidation was negligible. Apparently a spore coat component is needed for the oxidation per se. This component probably serves as a catalyst for the oxidation reaction. It is unlikely that it is converted in a stoichiometric reaction with manganese. Two arguments support this assumption. First, the $MnO_2$ of maximally encrusted spores could be dissolved with a reducing agent, with almost complete recovery of the manganese-oxidizing activity. Second, molecular oxygen is consumed during the oxidation reaction in a ratio of approximately 0.5

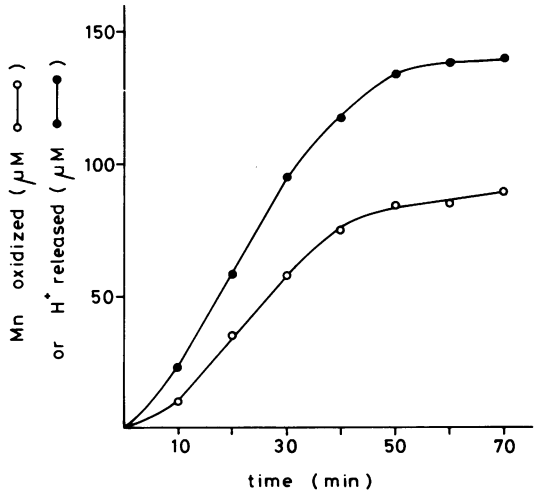


FIG. 6. Acid release during $Mn^{2+}$ oxidation by spore coats. Spore coat suspensions in unbuffered deionized water, pH 7.5, were supplemented with $MnCl_2$ (final concentration, 100 μM). At 10-min intervals the $MnO_2$ formation was measured. The $H^+$ formation was determined with the use of a pH electrode. For further experimental details see Materials and Methods.

mol of $O_2$ per mol of $MnO_2$ formed. The decrease in oxidizing activity with increasing amounts of $MnO_2$ formed may be explained by a decreased accessibility of the active sites for the substrate, because the oxide adheres to the spore surface (17).

The consumption of $O_2$ and the production of protons during manganese oxidation roughly agree with the following overall equation: $Mn^{2+} + 1/2\ O_2 + H_2O \rightarrow MnO_2 + 2H^+$. Since $Mn^{2+}$ is probably oxidized after adsorption to $MnO_2$, the overall reaction may involve at least two processes, as also suggested by Ehrlich (5). First, $Mn^{2+}$ is adsorbed by hydrated $MnO_2$ with concomitant proton release. This is followed by a rapid oxidation of the adsorbed $Mn^{2+}$. This sequence suggests that the oxidation will proceed optimally after a small amount of $MnO_2$ has been formed. The lag phase in oxidation, often observed in spore coat suspensions, may represent the time needed for this initial $MnO_2$ formation. Like spores, spore coats encrusted with $MnO_2$ oxidized $Mn^{2+}$ at a lower rate than nonencrusted spores and a lag phase was never observed. Whether the incipient $MnO_2$ production is catalyzed by the spore coats or is caused by chemical oxidation of $Mn^{2+}$ remains to be established. That the lag phase was not observed during $Mn^{2+}$ oxidation by intact spores may be due to the presence of traces of $MnO_2$ on the spore surface. Spores contain manganese which is released upon germination (21). As reported before, some spores of *Bacillus* sp. strain SG-1 may germinate even in the absence of nutrients (4). The released manganese will be oxidized by nongerminated spores. Note that intact spores treated with the reductant $NH_2OH$ appeared to oxidize $Mn^{2+}$ after a short lag phase (Fig. 4). A comparable reaction sequence as described above has been described in more detail for manganese oxidation by the freshwater species *Leptothrix discophora* (F. C. Boogerd, E. W. de Vrind-de Jong, J. P. M. de Vrind, and P. Westbroek, submitted for publication).

The recovery of the $Mn^{2+}$-oxidizing activity from spore coat preparations is a first step towards the purification and characterization of the oxidizing factor. However, the solubilization of the latter in an active form may prove to be difficult. Spore coats are highly resistant structures, containing a high percentage of cross-linked proteins (9). Release of spore coat proteins is usually achieved under strongly denaturing conditions, using buffers with high concentrations of dithioerythritol or mercaptoethanol and sodium dodecyl sulfate (9, 16). Preliminary experiments indicated that about 5% of the coat proteins of *Bacillus* sp. strain SG-1 were solubilized under these conditions. But neither the extracted coats nor the extracts showed any oxidizing activity after removal of the extraction buffers. At present we are attempting to find alternative conditions for the solubilization of spore coat components.

## ACKNOWLEDGMENTS

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