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Manganese Oxidation by *Sphaerotilus discophorus*

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ABSTRACT

JOHNSON, A. H. (Washington State University, Pullman), AND J. L. STOKES. Manganese oxidation by *Sphaerotilus discophorus*. J. Bacteriol. **91**:1543–1547. 1966.—Cell suspensions of *Sphaerotilus discophorus* in 0.02 M potassium phosphate buffer (*p*H 7.2) readily oxidized $MnSO_4$ to dark-brown manganic oxide. The latter appeared to be MnO_2, since its color was the same as MnO_2 and, like the latter, it was readily soluble in HCl with the evolution of chlorine. The amount of O_2 consumed in the oxidation of Mn^{++} was 63% or more of theoretical. The ability of the cells to oxidize Mn^{++} was destroyed by heating the cells at 93 C for 5 min. Only cell suspensions prepared from cultures of *S. discophorus* grown with $MnSO_4$ were able to oxidize Mn^{++}. It was concluded that the oxidation of manganous ions by *S. discophorus* is catalyzed by an inducible enzyme(s).

Sphaerotilus natans oxidizes ferrous ions and *S. discophorus* oxidizes both ferrous and manganous ions. The oxides of the metals accumulate on the organic sheaths of these filamentous bacteria as characteristic, morphologically distinct, and frequently thick deposits. Sheaths with ferric oxide are yellow to golden brown and those with manganic oxide or manganic and ferric oxides are dark brown. There has been a long controversy as to whether the energy liberated in the oxidation of ferrous and manganous ions can be used by *Sphaerotilus* for growth, i.e., whether these organisms are chemoautotrophs. Winogradsky (11, 12), who used impure cultures of *S. discophorus* [*Leptothrix ochracea*; see Pringsheim (6)] concluded that the organism was autotrophic. Molisch (4) was able to grow a pure culture of the organism on organic media, and therefore questioned its ability to derive energy from the oxidation of inorganic compounds. Lieske (3) noted that *S. discophorus* (*L. ochracea*) was facultative and grew heterotrophically or autotrophically, depending on the culture conditions. He provided evidence for autotrophic growth with manganous bicarbonate. However, Lieske's results were erratic in that he frequently failed to obtain growth in inorganic media. The data of these and other investigators were carefully and critically reviewed by Cholodny (1), who concluded that the available experimental data supported Winogradsky's contention of autotrophy but in a modified form. Winogradsky (12) claimed that *S. discophorus* was a strict autotroph, whereas Cholodny, like Lieske, concluded that it could grow both autotrophically and heterotrophically. In contrast, Cataldi (Ph.D. Thesis, Univ. of Buenos Aires, Argentina, 1939) obtained growth of *S. discophorus* [*L. ochracea*, *L. crassa*, and related forms; see Pringsheim (6)] in organic media but not in inorganic media with ferrous and manganous salts. Pringsheim (6) pointed out that Cataldi's negative results with inorganic media could have been due to the absence of calcium in her media, or to the use of media that were too acid or that did not contain iron and manganese, both of which might be essential for growth. Recently Mulder (5) suggested that manganese oxidation may be carried out by the living cells and the liberated energy may possibly be used by them, or that manganese oxidation is is brought about chemically (auto-oxidation) by metabolic compounds such as hydroxycarboxylic acids secreted by the cells in which case the energy is not utilized by the cells. His experiments tended to favor the latter possibility, although autotrophy could not be entirely ruled out.

We decided, therefore, to investigate further the oxidation of manganese by *S. discophorus*. The oxidation of this metal was chosen in preference to that of iron because of the much lesser tendency of manganous ions to undergo autooxidation in air at *p*H levels near neutrality, i.e., in the *p*H range suitable for the growth of *S. discophorus*.

MATERIALS AND METHODS

Strains 35, 36, and 42 of *S. discophorus* were used. The isolation and properties of these strains and

maintenance of stock cultures have been described previously (2, 8, 9, 10).

The cultures were grown in 1-liter Erlenmeyer flasks containing 200 ml of the basal medium for 20 to 24 hr at 30 C on a rotary shaker. The basal medium consisted of 0.5% peptone, 0.05% ferric ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2$, 0.001% $FeCl_3 \cdot 6H_2O$, 0.2 μg of thiamine per ml, and 0.02 μg of biotin per ml in distilled water. The *p*H was adjusted to 7.2. $MnSO_4 \cdot H_2O$, 0.005%, was added as desired.

Inocula were prepared by suspending in 1 ml of sterile basal medium the growth from a 1- or 2-day-old agar slant culture grown at 30 C. Each flask received 0.5 ml of this suspension. The composition of the slant medium was the same as the basal medium, except that biotin and thiamine were omitted, tap water was used, and 1.3% agar was added.

After incubation, the cells were recovered by centrifugation and were washed twice in a 0.02 M KH_2PO_4-K_2HPO_4 buffer (*p*H 7.2). The washed cells were suspended in a small amount of buffer and blended for 10 sec in a Waring Blendor. The suspension then was adjusted to 425 Klett units (red filter). Dry-weight determinations indicated that the final suspension contained 1.1 mg/ml of cell material when the growth medium contained $MnSO_4 \cdot H_2O$. In media devoid of this salt, the suspension contained 4.0 mg/ml of cell material.

Heated cell suspensions were prepared by placing a 500-ml Erlenmeyer flask containing 50 ml of the standard cell suspension into a boiling-water bath. The water bath was deep enough so that the surface of the cell suspension was well below the water level. Approximately 4 min were required for the cell suspension to reach a constant temperature (93 C), and heating was continued for an additional 5 min. The suspension was constantly agitated by whirling during the heating process to prevent clumping of cell material. If more than 50 ml of heated cell suspension was required, a number of 50-ml portions were prepared in the manner described.

The oxidation of Mn^{++} by the cell suspensions of *S. discophorus* was investigated by manometric methods. Cells grown with and without $MnSO_4$ and also heated cells grown with $MnSO_4$ were used. For these experiments, the standard cell suspensions were centrifuged and resuspended in one-half the original volume of buffer. Each Warburg vessel received 2 ml of cell suspension, 0.2 ml of 10% KOH in the center well to absorb CO_2, and 0.1 ml of a $MnSO_4 \cdot H_2O$ solution (60 μmoles/ml) or 6 μmoles. The $MnSO_4$ solution was prepared by dissolving 101 mg of $MnSO_4 \cdot H_2O$ in 10 ml of distilled water. In some instances, mercuric chloride also was used, in which case 0.1 ml of a 2.2×10^{-3} M solution was added to the main chamber of the vessel to give a final concentration of 10^{-4} M $HgCl_2$. The gas phase was air, and the bath temperature was 30 C.

The conditions in the Warburg vessels were simulated in large-scale experiments. For these, the manganese solution was prepared by adding 405 mg of $MnSO_4 \cdot H_2O$ to 150 ml of 0.02 M K_2HPO_4 and adjusting to *p*H 7.2 with 0.02 M K_2HPO_4. The final volume was adjusted to 300 ml with *p*H 7.2 buffer solution, and the final concentration of $MnSO_4 \cdot H_2O$ was 8 μmoles/ml. Samples of 100 ml of this solution were placed in 1-liter Erlenmeyer flasks. The cell material from 100 ml of the desired standard cell suspension was recovered by centrifugation and resuspended in a small amount of *p*H 7.2 buffer and added to each flask of $MnSO_4$ solution. The flasks were incubated on a rotary shaker at 30 C and were observed for manganese oxidation as indicated by an increase in dark-brown pigmentation of the suspension.

RESULTS

When *S. discophorus* is grown in media containing Mn^{++} the cultures become dark brown due to the formation and deposition of manganic oxide on the sheaths of the filaments. Suspensions of such manganese-grown cells also can oxidize Mn^{++} manometrically, as evidenced by an increase in O_2 consumption above the endogenous level when exposed to Mn^{++}. Representative data are plotted in Fig. 1. Oxygen was consumed at a more rapid rate with Mn^{++} than without it. After 3 hr, the cells with Mn^{++} had consumed 238 μliters of O_2, compared with 196 μliters of O_2 in the absence of Mn^{++}. The difference of 42 μliters of O_2 appears to be due directly to the oxidation of the manganous ions rather than indirectly to Mn^{++} stimulation of oxidation of organic endogenous reserves in the cells, since there was a marked increase in the formation of the characteristic dark-brown manganic oxide in the Warburg vessels.

On the basis of the reaction $MnO + \frac{1}{2} O_2 \rightarrow MnO_2$, 3 μmoles of O_2 or 67 μliters of O_2 would

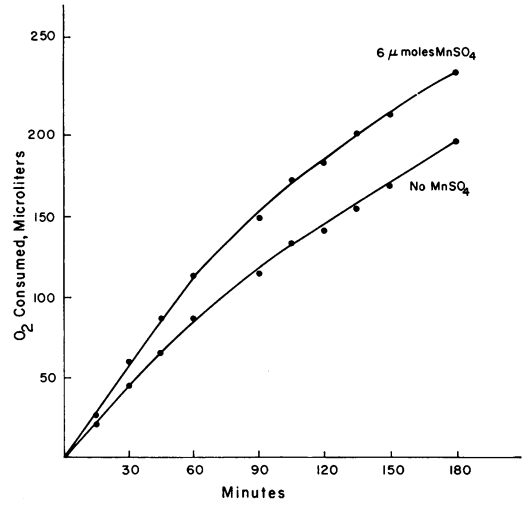

FIG. 1. *Manganese oxidation by Sphaerotilus discophorus strain 35.*

be required for the oxidation of 6 μmoles of Mn^{++}. The observed value was 42 μliters of O_2 or 63% of theoretical. In other similar experiments, O_2 values of 53 and 73 μliters or 80 and 109%, respectively, were obtained. It may be that the manganic oxide formed, although mainly MnO_2, may also include Mn_2O_3, which could account for the lower O_2 values. In general, O_2 consumption is not rapid, perhaps because of the heavy incrustation of the cells with oxides of manganese and iron which may limit the rate of reaction between Mn^{++} and the cells and also the diffusion of O_2.

A number of experiments were made to determine whether the oxidation of Mn^{++} by *S. discophorus* is an enzymatic process. Manganese-grown cell suspensions were heated for 5 min at 93 C in the manner previously described. The heated cell suspension was compared manometrically with an unheated suspension for ability to oxidize Mn^{++}. The results are plotted in Fig. 2. The unheated cell suspensions oxidized Mn^{++} in the usual fashion, as indicated by the more rapid and extensive O_2 consumption compared with the endogenous control and by the formation of dark-brown manganic oxide. In contrast, the heated cell suspension failed to oxidize Mn^{++}. Oxygen uptake was slight with and without Mn^{++} owing to the destruction by heat of the enzymes involved in both Mn^{++} oxidation and the oxidation of endogenous organic matter.

An attempt to repress Mn^{++} oxidation with 10^{-4} M $HgCl_2$ in place of heat resulted in a large decrease in O_2 consumption with and without Mn^{++}, although more O_2 was consumed with Mn^{++}. It appeared that $HgCl_2$ at the concentration employed inhibited endogenous but not Mn^{++} oxidation.

If the *S. discophorus* cells are grown in the basal medium without $MnSO_4$, the resulting cell suspensions are unable to oxidize Mn^{++} (Fig. 3). The rate and extent of O_2 consumption was the same with and without Mn^{++}. Also, there was no evidence of manganic oxide formation in the presence of Mn^{++}, i.e., the cell suspension did not darken. It appears, therefore, that Mn^{++} oxidation is mediated by an inducible enzyme (or enzymes) which is formed by the cells only when they are grown in the presence of Mn^{++}.

Manganese oxidation by *S. discophorus* and the effect of heat and manganese induction on the process is clearly shown in Fig. 4. All of the cell suspensions were in 0.02 M phosphate buffer (*p*H 7.2), and the photograph was taken after 18 hr of incubation at 30 C. The contents and treatment of the tubes were as follows: tube 1, suspension of manganese-grown cells; tube 2, same as tube 1 plus 8 μmoles of $MnSO_4$ per ml; tube 3, same as tube 2 except that the cell suspension was heated for 5 min at 93 C; tube 4, suspension of cells grown without $MnSO_4$; tube 5, same as tube 4 plus 8 μmoles of $MnSO_4$ per ml; tube 6, 0.02 M phosphate buffer (*p*H 7.2) plus 8 μmoles of $MnSO_4$ per ml (no cells).

Comparison of tubes 1 and 2 shows the marked darkening in tube 2 due to oxidation of Mn^{++} to manganic oxide. Heating of the cell suspension destroyed its ability to oxidize Mn^{++} (tube 3). Cells grown without manganese did not oxidize Mn^{++}, as indicated by the lack of darkening in tube 5 which contained Mn^{++} compared with the control tube 4 without Mn^{++}. Also, the lack of darkening of tube 6 indicated that Mn^{++} is not

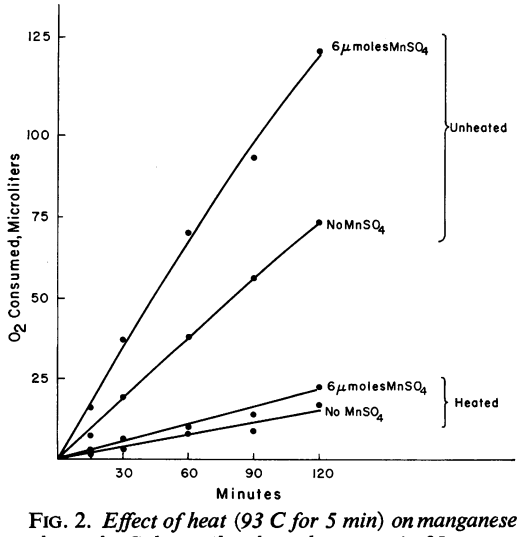

FIG. 2. *Effect of heat (93 C for 5 min) on manganese oxidation by Sphaerotilus discophorus strain 35.*

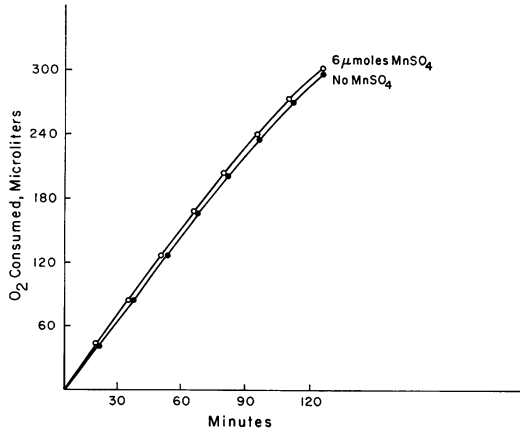

FIG. 3. *Lack of manganese oxidation by Sphaerotilus discophorus strain 35 when grown in the absence of $MnSO_4$.*

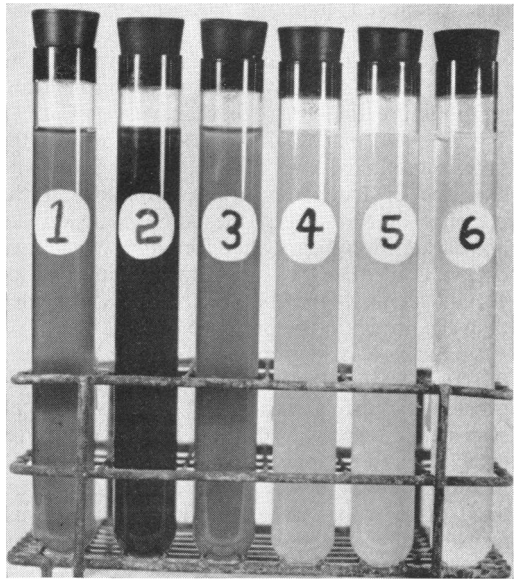

FIG. 4. *Oxidation of Mn^{++} by Sphaerotilus discophorus strain 35, and the effect of heat and growth with and without $MnSO_4$ (see text for content and treatment of the tubes).*

auto-oxidizable under the conditions of the experiment.

Reduction of the $MnSO_4$ concentration from 8 to 3 μmoles/ml resulted in a corresponding decrease in dark-brown pigmentation.

Additional evidence indicated that the dark-brown manganic oxide formed under the experimental conditions in tube 2 is MnO_2. The color was the same as that produced by a suspension of known MnO_2. Moreover, both the experimentally produced dark-brown material, and known MnO_2 readily dissolved in HCl with the evolution of chlorine.

Similar data were obtained with all three strains of *S. discophorus*.

DISCUSSION

Our data indicate that oxidation of manganous ions by *S. discophorus* is an enzymatic process. Washed cell suspensions readily oxidized Mn^{++}. Moreover, 63% or more of the theoretically required amount of O_2 was consumed in the process, and manganic oxide was formed. Mn^{++} oxidation was sensitive to heat and was destroyed by exposure of the cells to 93 C for 5 min. We also found that the cells had to be grown with manganese to be able to oxidize Mn^{++} in washed cell suspensions, which indicates that an inducible enzyme is involved.

In contrast, Mulder (5) favors the hypothesis that oxidation of manganese is an auto-oxidative process catalyzed by metabolic products which surround the sheaths of *Leptothrix* (similar or identical to *S. discophorus*). This is based on the finding that washed cell suspensions oxidized Mn^{++} to manganic oxide, even though the cells were killed by prior exposure to toluene and the deposition of MnO_2 on sheaths devoid of cells. Also, colonies of *Leptothrix* on agar media containing $MnSO_4$ produced a wide halo of dark-brown MnO_2.

It can be argued that Mulder's results do not necessarily exclude direct enzymatic oxidation of Mn^{++}. Such enzymes could act even in toluene-treated cell suspensions, and they could also be attached to empty sheaths. Although it has been assumed generally that cell walls of bacteria are metabolically inert structures, recent evidence indicates that enzymes may be present on or in cell walls (13). Conceivably, this could be true also for the sheaths of *Sphaerotilus*. The composition of the *S. natans* sheath is similar to that of the cell wall (7). Moreover, the MnO_2 halo around colonies could be due to excreted manganese-oxidizing enzyme(s).

It is not impossible that all of our results can be explained in terms of an indirect effect on Mn^{++} oxidation, in that the enzymatic process we have studied involves the formation of metabolic products which catalyze auto-oxidation of manganous ions. Our difficulty in repressing manganese oxidation with $HgCl_2$ might be considered as evidence in support of the indirect effect. It seems, however, that the preponderance of evidence supports the idea of a direct enzymatic oxidation of Mn^{++} by *S. discophorus*. Furthermore, it does not appear that auto-oxidation of Mn^{++} could have occurred in our experiments, since the cell suspensions were in phosphate buffer at *p*H 7.2. At the termination of the reactions, both in the Warburg vessels and the large-scale flasks, the *p*H had decreased 0.2 to 0.4 unit. Auto-oxidation of Mn^{++} takes place only at *p*H 10 or above, although it can be catalyzed at *p*H 8 by malic, citric, and butyric acids (5).

S. natans does not oxidize manganous salts in growing cultures. Also, suspensions of several strains of *S. natans* failed to oxidize Mn^{++} under the same conditions which led to manganese oxidation by *S. discophorus* in the present experiments.

It remains to be determined whether our strains of *S. discophorus* can utilize the energy liberated in the oxidation of Mn^{++} for growth. This aspect is now under investigation.

ACKNOWLEDGMENT

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