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Electron Transport Components of the MnO_2 Reductase System and the Location of the Terminal Reductase in a Marine *Bacillus*

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The response of MnO_2 reduction by uninduced and induced whole cells and cell extracts of *Bacillus* 29 to several electron transport inhibitors was compared. MnO_2 reduction with glucose by uninduced whole cells and cell extracts was strongly inhibited by 0.1 mM dicumarol, 100 mM azide, and 8 mM cyanide but not by atebrine or carbon monoxide, suggesting the involvement of a vitamin K-type quinone and a metalloenzyme in the electron transport chain. MnO_2 reduction with ferrocyanide by uninduced cell extracts was inhibited by 5 mM cyanide and 100 mM azide but not by atebrine, dicumarol, or carbon monoxide, suggesting that the metalloenzyme was associated with the terminal oxidase activity. MnO_2 reduction with glucose by induced whole cells and cell extracts was inhibited by 1 mM atebrine, 0.1 mM dicumarol, and 10 mM cyanide but not by antimycin A, 2n-nonyl-4-hydroxyguinoline-N-oxide) (NOQNO), 4,4,4-trifluoro-1-(2-thienyl),1,3-butanedione, or carbon monoxide. Induced cell extract was also inhibited by 100 mM azide, but stimulated by 1 mM and 10 mM azide. Induced whole cells were stimulated by 10 mM and 100 mM azide. These results suggested that electron transport from glucose to MnO_2 in induced cells involved such components as flavoprotein, a vitamin K-type quinone, and a metalloenzyme. The stimulatory effect of azide on induced cells was explained on the basis of a branching in the terminal part of the electron transport chain, one branch involving a metalloenzyme for the reduction of MnO_2 and the other involving a metalloenzyme for the reduction of oxygen. The latter was assumed to be the more azide sensitive. Spectral studies showed the presence of *a*-, *b*-, and *c*-type cytochromes in membrane but not in soluble fractions. Of these cytochromes, only the *c* type may be involved in electron transport of MnO_2, owing to the lack of inhibition by antimycin A or 2n-nonyl-4-hydroxyquinoline-N-oxide. The terminal MnO_2 reductase appears to be loosely attached to the cell membrane of *Bacillus* 29 because on cell fractionation it is found associated with both particulate and soluble fractions. Electron photomicrographs of bacilli attached to synthetic Fe-Mn oxide revealed an intimate contact of the cell walls with the oxide particles.

The reduction of MnO_2 by some bacteria isolated from marine ferromanganese nodules and deep-sea sediments has been the subject of extensive investigations in this laboratory (2-8, 16, 17). This work has established, among other things, that MnO_2 reduction by these bacteria is enzymatic. The locus of the MnO_2-reducing enzyme system in the bacterial cells is unknown. However, since MnO_2-reducing bacteria have been shown to attack solid nodular material and synthetic manganese oxides (2, 4), and since MnO_2-reducing activity from crude extracts was recovered in several different large molecular fractions during filtration through G-150 Sephadex (5), it has been thought that a terminal part of their MnO_2-reductase system resides at the cell surface, probably in the cell membrane. The type of components of the MnO_2-reductase system is also unknown. They have been assumed to include electron transport enzymes (4, 5, 16). A portion of the electron transport system in *Bacillus* 29, for which a ferri/ferrocyanide redox couple can substitute, is inducible in cells grown in the presence of manganous manganese. The induced system is inhibited by atebrine (17). MnO_2-reducing enzyme systems can operate at hydrostatic pressures of the deep sea

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(3, 6). In *Bacillus* 29, at least, MnO_2-reducing activity is influenced by variations in the concentrations of seawater cations and by temperature (8).

The present report partially identifies electron transport components of the MnO_2-reductase system in *Bacillus* 29 based on response of its electron transport system to respiratory inhibitors and on spectral properties. A model of electron transport involved in MnO_2 reduction by *Bacillus* 29 is proposed. The report also examines the location of the terminal MnO_2-reductase in cell fractions of *Bacillus* 29 and provides electron microscope evidence of cell attachment to mineral particles. Part of this work was presented in a preliminary report (W. C. Ghiorse and H. L. Ehrlich, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K80, p. 160).

MATERIALS AND METHODS

Culture preparation and activity tests. Bacterial cultures, induced and uninduced cell suspensions, and cell-free extracts were prepared as described previously by Ghiorse and Ehrlich (8). Determination of dry cell weight, protein content, specific glucose-linked, MnO_2-reductase activity of uninduced (ferricyanide-dependent) or induced (ferricyanide-independent) preparations, and specific ferrocyanide-linked activity of uninduced cell extracts were also made according to the methods of Ghiorse and Ehrlich (8), except that in some experiments nearly twice as much ferrocyanide-linked activity was attained by shaking the 50-ml reaction flasks at 200 rpm in a reciprocating water bath shaker. Specific MnO_2-reductase activities (nanomoles of Mn^{2+} released per hour per milligram of protein) were estimated by colorimetric determinations of Mn^{2+} released from MnO_2 incubated for 3 h in the presence of cells or extract as described by Ghiorse and Ehrlich (8). Unless otherwise stated, fivefold diluted seawater was used to wash cells and prepare extracts and as the basic suspension medium in reaction flasks. Incubation temperatures for all experiments were those previously determined to be optimal for the type of cell preparation and activity being tested, i.e., induced and uninduced whole cells (glucose-linked activity, 40 C), uninduced cell extracts (glucose-linked activity, 30 C; ferrocyanide-linked activity, 37 to 40 C), induced cell extracts (glucose-linked activity, 25 C) (8). Since 10 mM azide was found to stimulate MnO_2-reductase activity of induced cell extracts, it was added to the reaction mixture in experimental and control flasks in some experiments to increase the level of activity.

Inhibitor tests on MnO_2-reductase activity. The water-soluble inhibitors (NaCN, NaN_3, and atebrine dihydrochloride) were dissolved in the suspension medium before addition to the reaction flasks. Those inhibitors soluble in nonaqueous solvents [antimycin A; 2n-nonyl-4-hydroxyquinoline-N-oxide (NOQNO); 4,4,4-trifluoro-1-(2-thienyl)1,3-butanedione (TTFA)] were dissolved in 95% ethanol, and 0.1 ml of the ethanol solution was then added to the suspension medium. Dicumarol [3,3'-methylene-bis-(4-hydroxy-1,2-benzopyrone)] and *p*-chloromercuribenzoate (*p*-CMB) were dissolved in fivefold diluted seawater by adding NaOH. The pH of the solution was adjusted to 8.5, and 0.1 ml was added to the suspension medium in the reaction flasks. The pH of the final reaction mixture was unaffected by the addition of the alkaline dicumarol or *p*-CMB solutions.

Carbon monoxide was applied to the enzyme system by bubbling the gas through cell suspensions or extracts for 1 to 3 min before their introduction into reaction flasks, avoiding exposure to bright light. To test for light-reversible CO inhibition, a set of flasks containing CO-treated samples was incubated in the light and dark. Their activities were then compared with that of untreated cells or extract. In some experiments, reaction vessels were exposed to CO in CO-filled desiccators during the entire incubation period. All CO experiments were incubated on different days at a monitored temperature between 19 and 28 C.

Preparation of cell fractions. Cell fractions were made from cells washed, suspended, and sonicated in either 0.1 M phosphate buffer (pH 7.0) (spectral studies only) or fivefold diluted seawater, as described previously (8). The crude sonic extract was centrifuged at 10,000 × *g* for 10 min at 4 C in a Sorvall RC2 or RC2-B centrifuge. The resulting supernatant was centrifuged at 144,880 × *g* for 3 h at 4 C in a Beckman L2-50 ultracentrifuge using a type 40 rotor. The supernatant of this first high-speed centrifugation was designated as the soluble enzyme fraction. The pellet resulting from the first high-speed centrifugation was washed four times by centrifugation in buffer or diluted seawater at 144,880 × *g* for 1 h at 4 C. The final pellet was suspended in buffer or diluted seawater and designated as the washed particulate fraction.

Difference spectra. Difference spectra of cell fractions were obtained with a Beckman DBG dual-beam spectrophotometer by first recording a base line over the region between 350 and 650 nm with untreated samples in both cuvettes (oxidized-minus-oxidized base line). Next, a few grains of sodium dithionite were added to one of the cuvettes, and the reduced spectrum was recorded over the same region (dithionite-reduced-minus-oxidized spectrum). Cell fractions prepared in both 0.1 M phosphate buffer and diluted seawater yielded identical spectra; however, since the enzyme activity of the fractions could not be tested in phosphate buffer, diluted seawater was used for the majority of spectral analyses.

Attachment of *Bacillus* 29 to synthetic Mn-Fe oxide. The attachment of *Bacillus* 29 cells to synthetic Mn-Fe oxide was studied by mixing the cells of a 24-h culture equivalent to approximately 15 mg (dry weight) in 7.0 ml of seawater with 0.2 g of Mn-Fe oxide (80 mesh), synthesized according to the method of Ehrlich (4). After 30 min of incubation, during which extensive clumping of the mineral particles occurred, a drop of 50% (wt/vol) glutaraldehyde solution was added to prefix the cells attached to the mineral. After 2 h at room temperature, the contents of each flask were decanted into test tubes

(13 by 100 mm) and centrifuged at 4,000 × g for 10 min. The glutaraldehyde-fixed pellet, containing cells and mineral, was then subjected to osmium fixation using a modified version of the Ryter-Kellenberger procedure (14). Samples were dehydrated in ethanol and embedded in Epon 812, according to Luft (11). Thin sections, cut on a Porter-Blum Mt-1 ultramicrotome equipped with a diamond knife, were double stained with lead citrate and uranyl acetate (18). Grids carrying the sections were examined in a Siemens EM I electron microscope operated at 60 kV, using final magnifications of ×10,000 and ×20,000. Representative fields were photographed on Kodak electron image plates.

RESULTS

Effects of enzyme inhibitors. Glucose-linked, ferricyanide-dependent, MnO_2-reductase activity of uninduced whole cells and cell extracts was inhibited by cyanide and azide but not by carbon monoxide (Table 1). MnO_2-reducing activity in uninduced extracts was also inhibited by dicumarol but not by atebrine (Table 1). The ferrocyanide-linked activity (terminal MnO_2 reductase) of uninduced cell extracts was inhibited by cyanide, azide, and *p*-CMB (Tables 1 and 4), but this activity was unaffected by all other inhibitors at the concentrations tested (Table 1).

TABLE 1. *Effect of inhibitors on MnO_2-reductase activity of uninduced Bacillus 29*

Inhibitor[a]	Concn[b]	% Inhibition[c]		
		Whole cells	Extracts	
		Glucose[d]	Glucose[e]	$Fe(CN)_6^{-4}$[f]
Cyanide	0.5 mM			0
Cyanide	5 mM		78	93
Cyanide	8 mM	96	96	100
Azide	10 mM	11	0	0
Azide	100 mM	69	49	40
CO	CO[g]	0	0	0
Dicumarol	0.1 mM		45	0
Atebrine	1 mM		0	0

[a] See text for details concerning inhibitors.
[b] Final concentration in reaction flask.
[c] (100 − [Specific activity derived from experimental flasks with inhibitor/specific activity derived from experimental flasks without inhibitor]) × 100. Value is mean of at least two experiments.
[d] Glucose-linked, ferricyanide-dependent activity at 40 C. Mean specific activity without inhibitor = 758 ± 72 nmol of Mn^{2+} per h per mg of protein.
[e] Glucose-linked, ferricyanide-dependent activity at 30 C. Mean specific activity without inhibitor = 11.7 ± 1.5 nmol of Mn^{2+} per h per mg of protein.
[f] Ferrocyanide-linked activity at 40 C. Mean specific activity without inhibitor = 16.9 ± 1.1 nmol of Mn^{2+} per h per mg of protein.
[g] Flasks incubated in a CO atmosphere or inoculum treated with CO gas for 1 to 3 min.

Glucose-linked activity of induced cell preparations was inhibited by cyanide but unaffected by CO. It was inhibited by atebrine and stimulated by azide (Table 2). In extracts of induced cell preparations, glucose-linked activity was also stimulated by azide but at approximately 10-fold lower concentrations than in whole cells and was inhibited by an approximately 100 mM azide concentration (Table 2). Moreover, this activity of extracts was inhibited by dicumarol and atebrine but not by antimycin A, NOQNO, or TTFA (Table 2).

The azide stimulation of glucose-linked activity of induced cell preparations merited further investigation to determine the effect of various azide concentrations on extract activity (Fig. 1). Concentrations between 0.5 and 50 mM azide were stimulatory. Below this range activity was unaffected, and above it activity was inhibited. Stimulation by azide was optimal at a 10 mM concentration.

Since cyanide and azide are known to inhibit many of the same metalloenzymes, it was possi-

TABLE 2. *Effect of inhibitors on glucose-linked, MnO_2-reductase activity of induced Bacillus 29*

Inhibitor[a]	Concn[b]	Relative activity[c]	
		Whole cells[d]	Extracts[e]
Cyanide	0.5 mM	1.00	1.00
Cyanide	10 mM	0	0.01[f]
Azide	1 mM		1.70
Azide	10 mM	1.90	2.70
Azide	100 mM	3.70	0.40
CO	CO[g]	1.00	1.00[f]
Antimycin A	7.0 μg/mg of protein		1.00[f]
Antimycin A	11.5 μg/mg of protein		1.00[f]
NOQNO	11.5 μg/mg of protein		1.00[f]
Dicumarol	0.1 mM		0.37[f]
Atebrine	1 mM	0.27	0.27[f]
TTFA	0.1 mM		1.00[f]

[a] See text for details concerning inhibitors.
[b] Final concentration in reaction flask.
[c] (Specific activity from flasks with inhibitor/specific activity from flasks without inhibitor); value is mean of at least two experiments.
[d] Activity measured at 40 C. Mean specific activity without inhibitor = 18.3 ± 2.2 nmol of Mn^{2+} per h per mg of protein.
[e] Activity measured at 25 C. Mean specific activity without azide or other inhibitors = 1.9 ± 0.2.
[f] Ten millimolar azide (2.3 to 4.6 mg of NaN_3 per mg of protein) added to flasks to increase activity. Mean specific activity with 10 mM azide alone = 5.1 ± 0.9 nmol of Mn^{2+} per h per mg of protein.
[g] Three-minute pretreatment of bubbling CO gas through inoculum.

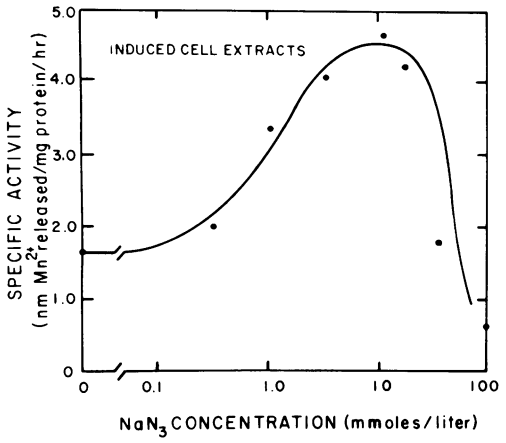

FIG. 1. *Effect of various azide concentrations on glucose-linked MnO_2-reductase activity in extracts of induced Bacillus 29. Each point is the mean of several experiments.*

ble that noninhibitory concentrations of cyanide might also stimulate glucose-linked activity of induced preparations. As the appropriate data in Table 2 show, this was not the case. Cyanide neither stimulated nor inhibited the induced system at 0.5 mM, whereas it completely inhibited it at 10 mM.

After the stimulatory effect of azide on the induced system was discovered, subsequent inhibitor experiments on extracts of induced cells were performed by adding 10 mM azide to all flasks. This permitted observations at higher levels of MnO_2-reducing activity than was otherwise possible.

Distribution of the terminal MnO_2 reductase in cell fractions of uninduced *Bacillus* 29. To locate the terminal MnO_2 reductase, uninduced rather than induced cells of *Bacillus* 29 were utilized because only in uninduced cells is it possible to study terminal MnO_2 reductase apart from the remainder of the electron transport components, especially the inducible component. Information about the location of the terminal component is important in understanding how *Bacillus* 29 can reduce an insoluble electron acceptor such as MnO_2.

Both the soluble enzyme and washed particulate fractions possessed terminal MnO_2-reductase activity (ferrocyanide-linked activity) (Table 3). In four different experiments, including those shown in Table 3, 11 to 28% of the total detected activity resided in the washed particulate fraction, whereas the remaining 72 to 89% was associated with the soluble enzyme. Similar results were obtained when cells were fractionated using lysozyme treatment followed by protoplast lysis (W. C. Ghiorse, Ph.D. thesis, Rensselaer Polytechnic Institute, Troy, N.Y., 1972). In these experiments, the activity was also associated with both the washed cell membranes and a soluble enzyme fraction. Mesosome fractions isolated from protoplasts by the method of Reavely and Rogers (12) were not active. Washed membrane fractions prepared by protoplasting did not reduce MnO_2, using glucose as electron donor, whereas the soluble enzyme fraction did, demonstrating that some or all of the enzymes for glucose oxidation linked to MnO_2 reduction are soluble.

The possibility that two different enzyme activities were being observed in the two cell

TABLE 3. *Distribution of ferrocyanide-linked activity in cell fractions of uninduced Bacillus 29*

Fraction	Expt	Total protein[a]	Sp act[b]	Total act[c, d]
Unfractionated	1	130	30.7 ± 1.7	3993 (100)
	2	220	31.3 ± 1.3	6879 (100)
	3	258	35.4 ± 0.7	9121 (100)
Particulate	1[e]	22	19.0 ± 0.9	418 (11)
	2	72	12.9 ± 0.7	929 (14)
	3	96	27.0 ± 0.4	2592 (28)
Soluble	1[e]	108	33.1 ± 1.9	3575 (89)
	2	148	40.2 ± 1.1	5950 (86)
	3	162	40.3 ± 0.7	6529 (72)

[a] Total milligrams of protein recovered. Protein in soluble fraction includes the protein recovered in the supernatants of the four washes of the particulate fraction.

[b] Nanomoles of Mn^{2+} released per milligram of protein per hour, measured in flasks shaken at 200 rpm for 3 h at 37 C.

[c] Total protein × specific activity.

[d] Numbers in parentheses refer to percentage of combined activity. Total activity of particulate fraction or total activity of soluble fraction divided by the sum of the total activities of the particulate and soluble fractions.

[e] Figure 2 shows difference spectra of these fractions. Note cytochrome peaks in particulate fraction (b) and lack of cytochrome in soluble fraction (c).

TABLE 4. *Effect of 1 mM cyanide and 0.01 mM p-CMB on ferrocyanide-linked activity[a] in cell fractions of Bacillus 29*

Inhibitor	Fraction[b]	% Inhibition[c]
Cyanide	Soluble	58
	Particulate	70
p-CMB	Soluble	26
	Particulate	27

[a] Measured in flasks shaken at 200 rpm for 3 h at 37 C. Mean specific activity of fractions without inhibitor are given in Table 3, experiment 2, for cyanide, and experiment 3 for *p*-CMB.

[b] Fractionation procedure described in text.

[c] See Table 1.

fractions was investigated by comparing the effects of cyanide and *p*-CMB on the activity of the fractions. Table 4 shows that both fractions were inhibited to nearly the same extent by 1 mM cyanide. The same trend was observed when 0.01 mM *p*-CMB was applied to the fractions (Table 4). On the assumption that different enzymes would be inhibited to very different extents by cyanide and *p*-CMB, these results suggest that the same terminal enzyme was probably functioning in both fractions.

Difference spectra of cell fractions. Dithionite-reduced-minus-oxidized difference spectra of washed particulate fractions showed evidence of *a*-, *b*-, and *c*-type cytochromes (Fig. 2b; peaks at 445 and 600 nm, 428 and 562 nm, and 552 nm, respectively). A depression in the region between 450 and 500 nm of these spectra was gradually removed as the membranes were washed, indicating that flavoproteins associated with the membranes were soluble to some extent. Difference spectra of soluble enzyme fractions (Fig. 2c) were devoid of cytochrome peaks. They showed only a generalized bleaching between 375 and 510 nm after reduction with dithionite, suggesting that flavoproteins and possibly non-heme iron proteins were present in these fractions.

Attachment of *Bacillus* 29 to synthetic Fe-Mn oxide. *Bacillus* 29 cells attached to an amorphous synthetic Fe-Mn oxide were easily embedded in epoxy resin and sectioned by conventional methods. In most instances the electron-opaque mineral was embedded in the matrix of the bacterial cell wall (arrows, Fig. 3), indicating the intimacy of the cell-mineral attachment. Many electron micrographs showing other cells similarly attached to oxide particles were obtained. It may be assumed that *Bacillus* 29 attaches to the synthetic MnO_2 in a similar manner.

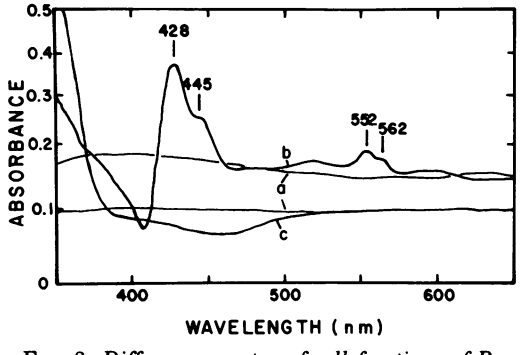

FIG. 2. *Difference spectra of cell fractions of Bacillus 29. (a) Oxidized-minus-oxidized base lines; (b) dithionite-reduced-minus-oxidized spectrum of washed particulate fraction (specific activity = 19.0; Table 3, experiment 1); (c) dithionite-reduced-minus-oxidized spectrum of soluble enzyme fraction (specific activity = 33.1; Table 3, experiment 1). Note lack of cytochrome peaks in (c).*

DISCUSSION

Electron transport components of the MnO_2-reductase system in *Bacillus* 29. The effects of respiratory inhibitors on MnO_2-reductase activity and the results of spectral studies aided in partial identification of components of the reductase system.

Cyanide to concentrations near 10 mM almost completely inhibited all MnO_2-reducing activity of induced and uninduced *Bacillus* 29 (Tables 1 and 2). At 1 mM concentration, cyanide inhibited the ferrocyanide-linked activity of cell fractions (Table 4). Cyanide probably affected a key enzyme in the system, which may have been the terminal reductase itself, or at least a component of the terminal portion of the system. Since cyanide is thought to exert its inhibiting effects by complexing with metals at active sites of metalloenzymes (10), it is likely that the key enzyme is a metalloenzyme.

MnO_2-reductase activity (ferrocyanide-linked activity) in cell fractions was also inhibited by 26% in the presence of 0.01 mM *p*-CMB (Table 4), indicating that the key metalloenzyme contains sulfhydryl groups at its active site (19). In other experiments (Ghiorse, 1972, Ph.D. thesis) the glucose-linked activity of uninduced whole cells was inhibited by 38% in the presence of 1 μM *p*-CMB. This greater sensitivity of whole cell activity to *p*-CMB suggests that other, more sensitive sulfhydryl enzymes, along with the key metalloenzyme, are involved in the glucose-linked MnO_2-reducing enzyme system of *Bacillus* 29.

Azide inhibited MnO_2-reductase activity of induced and uninduced cell extracts but at higher concentrations than cyanide (Tables 1 and 2). Since the mechanism of action of both cyanide and azide are known to be similar, and since higher azide than cyanide concentrations are generally required for the same extent of inhibition of the same metalloenzymes (10), it is probable that 100 mM azide affected the same enzyme which was inhibited by 10 mM cyanide.

The difference in effect of various azide concentrations on either whole cells or cell extracts (Tables 1 and 2; Fig. 1) can be explained on the basis that two different components are affected by this inhibitor. In extracts, one component, probably the cyanide-inhibited terminal MnO_2-reductase, is inhibited by 100 mM azide but not by 10 mM azide. The other component is maximally inhibited by 10 mM azide. This component, presumably another metalloenzyme, is

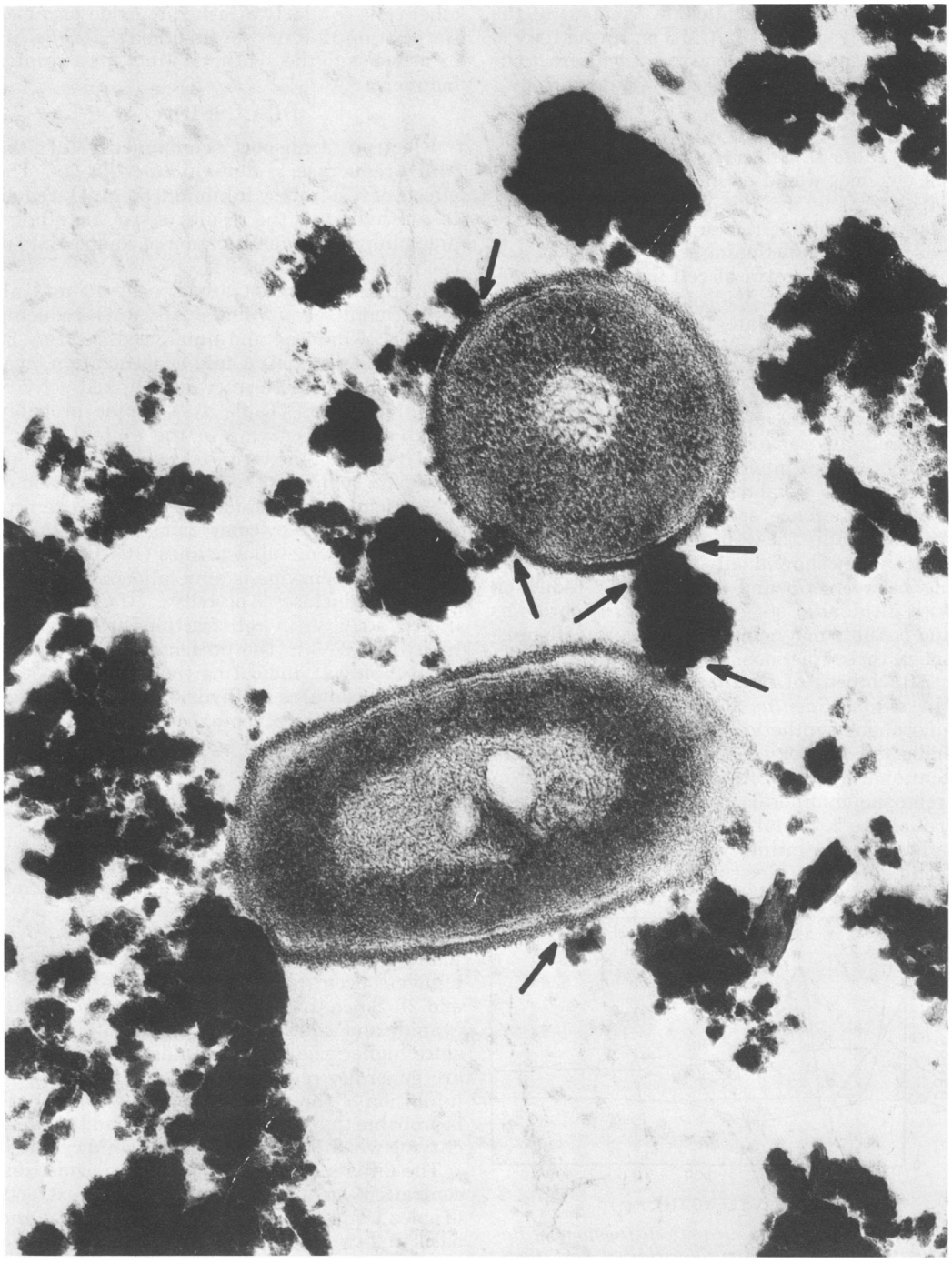

FIG. 3. *Thin sections of two Bacillus 29 cells attached to synthetic Fe-Mn oxide particles. Arrows indicate particles which are embedded in the matrix of the cell walls.* ×60,000.

not involved in electron transport to MnO_2, but may be involved in electron transport to oxygen. It is thought to be located in a branch to the terminal part of the inducible MnO_2-reductase system. Such an inhibition pattern could account for the observed stimulation of the extract activity from induced cells by preventing the loss of electrons to oxygen, thus allowing more electrons from glucose oxidation to be used for MnO_2 reduction. Branched electron transport systems of the type in which different terminal oxidases respond differently to high and low concentrations of cyanide and azide have been demonstrated in other bacteria (20). Although cyanide and azide are both known to bind to metalloenzymes, no concentration of cyanide was found in these experiments that would stimulate MnO_2 reduction. This may have been due to a greater and approximately equal sensitivity to cyanide of the two metalloenzymes.

Although spectral studies showed the presence of *b*- and *c*-type cytochromes in the membrane of *Bacillus* 29 (Fig. 2), negative results with antimycin A and NOQNO, both of which block electron transport between cytochromes *b* and *c* (9), indicate that these cytochromes do not participate together in electron transfer from glucose to MnO_2. The participation of either one alone in the induced system cannot be excluded, however. The finding that soluble enzyme fractions contained no cytochromes, yet were rich in ferrocyanide-linked activity (Fig. 2, Table 3), indicates that cytochromes are not part of the terminal portion of the system in uninduced cells.

The findings with atebrine, a flavoprotein inhibitor, suggest that at least one flavoprotein is involved in the MnO_2-reductase system. Atebrine inhibited the induced but not the uninduced system (Tables 1 and 2), supporting the suggestion by R. B. Trimble (Ph.D thesis, 1969, Rensselaer Polytechnic Institute, Troy, N.Y.) that the inducible portion of the system contains a flavoprotein which is inhibited by atebrine.

Dicumarol, which is thought to inhibit vitamin K-mediated electron transport in bacteria (1, 19), inhibited both induced and uninduced systems in *Bacillus* 29 (Tables 1 and 2). This indicates that a vitamin K-type quinone, which is probably the same in both systems, is involved in the transport of electrons to MnO_2.

TTFA, which inhibits mitochondrial succinic oxidase by chelating iron associated with a non-heme iron protein (13), had no effect on MnO_2-reductase activity in cell extracts (Table 2). Thus, it seems unlikely that such a non-heme iron protein participates in electron transport to MnO_2.

The results from these inhibitor studies and the inferences drawn from them are consistent with an electron transport model for *Bacillus* 29 (Fig. 4). According to this model, electrons derived from glucose oxidation are transported to MnO_2 by one of two pathways, depending on whether the cells are induced or not. In induced cells the natural electron transport system is used. This system includes an inducible flavoprotein (FP_1) which is inhibited by atebrine. It transfers electrons via a constitutive dicumarol-sensitive vitamin K-type quinone to another unknown inducible portion of the system containing a component which is physiologically linked, possibly at the cytochrome *c* level, to a metalloenzyme (ME_2), inhibited by 10 mM azide, as well as to the terminal MnO_2 reductase (ME_1), inhibited by 100 mM azide. The more azide-sensitive metalloenzyme (ME_2) is pictured as not participating in electron transport to MnO_2 but to O_2. In uninduced cells, a system utilizing a constitutive flavoprotein (FP_2) which is not inhibited by atebrine may be used. This system transfers electrons from FP_2 to the dicumarol-sensitive quinone used in the induced system and then to the same cyanide-, azide-, and *p*-CMB-sensitive terminal metalloenzyme (ME_1) used in the induced system, but using the ferri/ferrocyanide couple to bypass the inducible portion of the electron transport system containing the branch point.

The model in Fig. 4 accounts for all the data presented in this paper. In addition, this model predicts that MnO_2 reduction should be stimulated in induced cells and cell-free extracts by the removal of O_2. This prediction is in conflict,

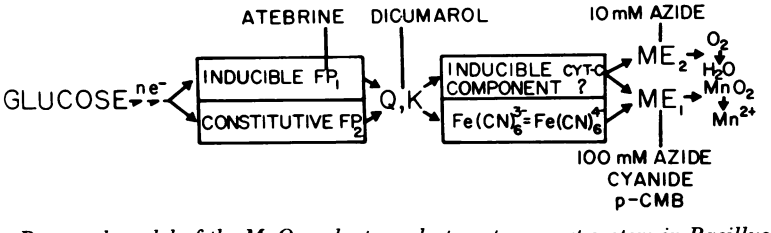

FIG. 4. *Proposed model of the MnO_2-reductase electron transport system in Bacillus 29.*

however, with the results of previous studies (16, 17) which showed that anaerobiosis did not affect the MnO_2-reductase activity of either adapted cells growing on MnO_2 or induced cell-free extracts. This discrepancy remains to be resolved.

Preliminary inhibitor studies with two MnO_2-reducing, bacterial isolates from the Pacific indicate that these organisms use a somewhat different electron transport system than *Bacillus* 29. Their MnO_2-reducing activity was not stimulated by any azide concentration tested, and they were inhibited by NOQNO and antimycin A (Ehrlich, unpublished data).

Location of the terminal MnO_2-reductase system. Cell fractionation showed that 11 to 28% of the terminal MnO_2-reductase (ME_1; Fig. 4, ferrocyanide linked) activity was associated with the washed particulate fraction of *Bacillus* 29. The remainder was found in the soluble enzyme fraction (Table 3). In previous work (5), the terminal portion of the system was found to be associated with large fragments of the cell envelope. This evidence indicates that the terminal reductase is located at the cell surface, probably at the cell membrane. The finding that the major portion of the ferrocyanide-linked activity was contained in the soluble fraction suggests that the attachment of the terminal reductase to the membrane must be rather loose. Such a loose attachment may be necessary to facilitate interaction with an insoluble substance. The solubility of the terminal reductase also suggests that this part of the system is of relatively low molecular weight, perhaps consisting of only one or a few enzymes.

Although it was not possible to test the location of the terminal MnO_2 reductase in induced cells of *Bacillus* 29 because of an inability to test separately its activity and that of the induced component, we believe that the terminal MnO_2-reductase (ME_1) is similarly located in induced cells.

The occurrence of intimate contact with the electron acceptor MnO_2 is suggested by the observation of Fe-Mn oxide particles embedded in the wall matrix of *Bacillus* 29 cells (Fig. 3) after exposing them to the oxide. This observation raises the question of how the terminal reductase of intact cells contacts this solid substrate. It may be that the wall acts as a sort of cement, attaching bacterial cells by electrostatic charges to MnO_2 particles under the influence of the ionic environment (8). Sticky cell wall material, being rather loosely constructed, could allow an uneven MnO_2 surface to penetrate to a level where the membrane-associated terminal reductase would contact the mineral. Thus, the properties of *Bacillus* 29 cell walls could greatly increase the contact between the MnO_2-reductase system and its solid substrate. This increased contact may account for much of the higher specific activity of whole cells over cell extracts (Ghiorse, 1972, Ph.D. thesis).

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