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Electron Transport System Associated with Membranes of *Bacillus cereus* During Vegetative Growth and Sporulation

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Membranes isolated from *Bacillus cereus* ATCC 4342 during vegetative growth and during sporulation contained cytochromes *b*, *c* and $a + a_3$ as well as flavoprotein as determined from reduced-minus-oxidized difference spectra. Although there appeared to be no qualitative change in the cytochromes, there was a significant increase in the amount of cytochromes associated with membranes isolated from sporulating cells. Succinate and nicotinamide adenine dinucleotide (reduced form) (NADH) reduced the same cytochromes indicating similar pathways of electron transport. The electron transport inhibitors—cyanide, azide, 2-heptyl-4-hydroxyquinoline-*N*-oxide, dicumarol and atebrine—were examined for their effect on succinate oxidase (succinate: $[O_2]$ oxidoreductase) and NADH oxidase (NADH: $[O_2]$ oxidoreductase). NADH oxidase associated with vegetative cell membranes was less sensitive to certain inhibitors than was succinate oxidase, suggesting a branched electron transport pathway for NADH oxidation. In addition to electrons being passed to O_2 through a quinone-cytochrome chain, it appears that these intermediate carriers can be bypassed such that O_2 is reduced by electrons mediated by NADH dehydrogenase. Both oxidases associated with sporulating cell membranes were inhibited to a lesser degree than were the oxidases associated with vegetative cell membranes.

A previous report from this laboratory demonstrated a significant increase in the levels of enzymes associated with energy production via an electron transport system during sporulation in *Bacillus cereus* (14). These enzymes included succinate:DCPIP oxidoreductase, succinate: cytochrome *c* oxidoreductase, succinate oxidase, nicotinamide adenine dinucleotide (reversed form) (NADH):DCPIP oxidoreductase, NADH:cytochrome *c* oxidoreductase, and NADH oxidase. Cytochromes of the *a*, *b*, and *c* types were detected in isolated membrane preparations, and there was a marked increase in the level of cytochromes by the end of vegetative growth; these levels remained throughout sporulation. Inhibition of the electron transport energy generating system, by the addition of cyanide to the growth medium, had little influence on growth but strongly inhibited sporulation (14). These results supported the idea that a typical terminal respiratory pathway is not needed for growth but is an absolute requirement for sporulation (8, 12) and supported the work of Szulmajster and Schaeffer (27) who demonstrated increased activity of particulate NADH oxidase during sporulation of a sporogenic strain (Sp^+), whereas in an asporogenic strain (Sp^-) this activity remained low and constant. Transformation of the Sp^- mutant with Sp^+ DNA resulted in increased NADH oxidase activity and the ability to sporulate. NADH dehydrogenase, cytochrome *c* reductase and cytochrome *c* peroxidase were also shown to be higher during sporulation of the Sp^+ strains compared to the Sp^- mutants. In addition, the increased oxygen consumption observed with *B. cereus* T at the time cells have stopped duplication and have begun initial sporulation stages (10) agrees with our earlier data demonstrating an increase in electron transport components and the necessity of this development for sporulation to occur (14).

In the present study, the electron transport system(s) for the oxidation of succinate and NADH was more fully characterized in *B. cereus* during growth and sporulation.

MATERIALS AND METHODS

Growth and harvesting procedures. *B. cereus* was cultured as previously described (under forced aeration; 14). Cells were harvested during vegetative growth (2 h) and during sporulation (8 h). At 8 h, phase-dark forespores were present as seen through a phase-contrast microscope. Cells were rapidly chilled over crushed ice and immediately centrifuged in a Sharples centrifuge; the cell paste was washed with TM buffer [0.05 M tris(hydroxymethyl)aminomethane, pH 7.6, containing 10 mM $MgCl_2$] at 4 C by centrifugation in a Sorvall RC-2B centrifuge.

Preparation of membranes. Membranes were prepared from 2- and 8-h-old cells after disruption in an automatically powered French pressure cell (American Instrument Co.) and were isolated as described previously (14). After being washed, the membranes were suspended in TM buffer to a final concentration of 10 mg of protein per ml and used as required. Membrane protein was determined by the method of Lowry et al. (18). All membrane preparations were used within 5 days and were stored in the cold.

Analysis of cytochromes. Respiratory pigments associated with isolated membranes from vegetative cells and from sporulating cells were identified by reduced-minus-oxidized difference spectra at ambient and liquid nitrogen temperatures employing an Aminco-Chance split-beam, recording spectrophotometer. Estimation of each cytochrome concentration was determined from the ambient temperature dithionite reduced-minus-oxidized difference spectrum employing the following wavelength pairs and millimolar extinction coefficients: cytochrome *a* ($+a_3$), E (603–630) = 24 $mM^{-1}\,cm^{-1}$; cytochrome *b*, E (563–575) = 22 $mM^{-1}\,cm^{-1}$; cytochrome *c*, E (550–540) = 19 $mM^{-1}\,cm^{-1}$, and cytochrome a_3 ($+a$), E (445–460) = 164 $mM^{-1}\,cm^{-1}$ (19, 29, 30). Steady-state reduction levels obtained with succinate and NADH were determined from the succinate reduced-minus-oxidized and the NADH reduced-minus-oxidized difference spectra employing the above wavelength pairs and millimolar extinction coefficients.

Electron transport inhibitors. The effect of electron transport inhibitors on succinate oxidase and NADH oxidase was examined by adding the particular inhibitor to the reaction mixture used to assay the oxidases. Succinate oxidase and NADH oxidase were assayed by using a Clark oxygen electrode (Yellowsprings Instrument Co.). The reaction vessel contained 50 mM phosphate buffer, pH 7.4, membranes (1 to 2 mg of membrane protein) and distilled water to a final volume of 2 ml. The reaction mixture was incubated for 3 min before initiating the reaction with substrate (170 mM succinate or 0.42 mM NADH). Succinate oxidation was measured at 30 C and NADH oxidation was determined at 35 C; reactions were traced for 2 min. Inhibitors were added just prior to the 3-min incubation period and included cyanide (KCN), sodium azide (NaN_3), 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), dicumarol, and atebrine. Cyanide and azide were dissolved in phosphate buffer (0.5 M, pH 7.4) and added at the desired concentrations. Dicumarol was dissolved in 0.03 M potassium hydroxide (KOH), and HOQNO in 0.003 M KOH; the addition of 0.03 M KOH to the reaction mixture had no influence on the assays. Atebrine was added as a water solution.

Catalase associated with whole cells. Catalase was assayed in whole cells by the procedure of Herbert (11). Whole cells were added to a solution of hydrogen peroxide (H_2O_2, 100 μM) and were allowed to incubate for varying lengths of time. The reaction was stopped by the addition of 1 N sulfuric acid, and the amount of H_2O_2 remaining was determined by adding 10% potassium iodide and titrating the liberated iodine with sodium thiosulfate. Cellular protein was determined by suspending the cells in 1 N NaOH and heating in a boiling-water bath for 15 min (21). After centrifugation at 10,000 $\times$ *g*, the supernatant fluid was used to determine cellular protein by using the method of Lowry et al. (18).

All results reported in the present studies were reproducible with different batches of membranes. Individual experiments were done in duplicate and were reported as average values.

Reagents. The sources of the reagents used were as described previously (14) and were of reagent grade.

RESULTS

Growth of the organism. Culture characteristics of *B. cereus* grown in the medium used in this study have been published previously (5, 15). All culture parameters previously reported were reproducible in the present study.

Analysis of cytochromes. Respiratory pigments associated with the membranes of vegetative (2-h old) and sporulating cells (8-h old) were identified from the reduced-minus-oxidized difference spectra determined at ambient and liquid N_2 temperatures. Figure 1 shows the ambient temperature, difference spectra for vegetative cell membranes when dithionite, NADH, or succinate were the reductants. Characteristic absorption maximums of the cytochrome complex, $a + a_3$, were observed at 603 nm and 443 nm with all reductants. Cytochromes *b* and *c* absorbed at 526 nm and 556 nm; in the Soret region of the spectrum, cytochrome *b* was detected at 428 nm. The absorption spectra were generally similar for all three reductants; the one exception was that the relative amount of cytochrome *b* (428 nm) reduced was larger than the amount of cytochrome a_3 (443 nm) reduced when dithionite was used as compared to substrate (succinate and NADH) reduction. Difference spectra of sporulating cell membranes (Fig. 2) also gave absorption peaks characteristic of cytochromes *b*, *c* and $a + a_3$. Dithionite reduction gave a major absorption peak at 552 nm and a shoulder at 556 nm, whereas succinate and NADH reduction resulted in a single absorption peak at

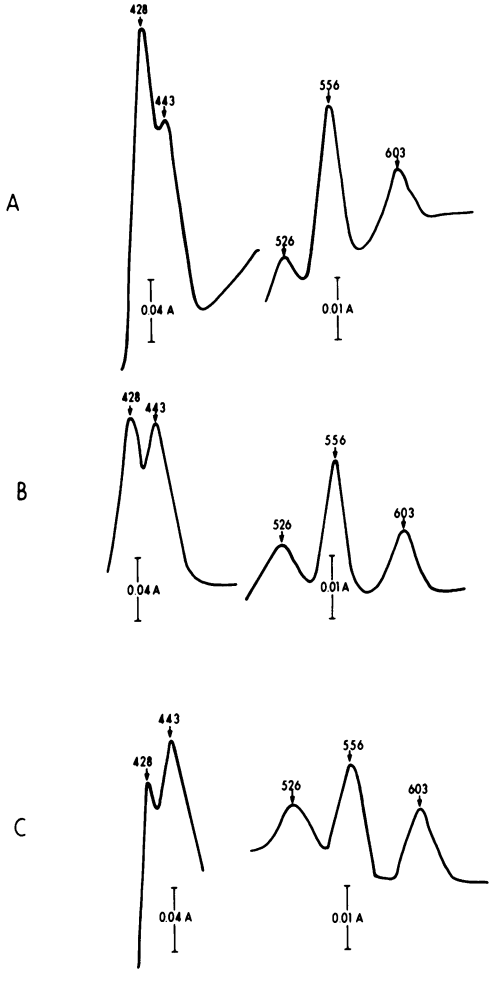

FIG. 1. *Ambient temperature, reduced-minus-oxidized difference cytochrome spectra of vegetative cell membranes. Cuvettes contained 12.5 mg of membrane protein. Reduction was by: dithionite (A), NADH (B), or succinate (C). In this and in subsequent spectra the ordinate scale is absorbancy per centimeter and the abscissa is wave length and the effective band width is 2 to 3 nm.*

550 nm. As with vegetative cell membranes, dithionite reduced relatively more cytochrome *b* (428 nm) than cytochrome a_3 (443 nm) as compared with succinate or NADH reduction. Liquid N_2 temperature, reduced-minus-oxidized, difference spectra of vegetative cell membranes (Fig. 3) agree with the spectra obtained at ambient temperature. Major absorption peaks at 603, 555, 444, 428 nm and a shoulder at 548 nm were observed. Greater resolution of the 548 and 555 nm peaks was obtained with sporulating cell membranes (fig. 4).

The amount of individual cytochromes present in membranes from vegetative and sporulating cells is shown in Table 1. As reported earlier (14), there was an increase in the amount of cytochromes associated with cells undergoing sporulation. Cytochromes a_3, *a*, *c*, and *b* were two, three, five, and six times higher, respectively, in sporulating cell membranes. Steady-state levels of cytochrome reduction in vegetative cell membranes showed cytochrome *a* was reduced 100%; cytochrome a_3, 43%; cytochrome *c*, 12%; and cytochrome *b*, 64%. NADH, at steady state, reduced 100% of cytochrome *a*, 43% of cytochrome a_3, 70% of cytochrome *c*, and 82% of cytochrome *b*. Both succinate and

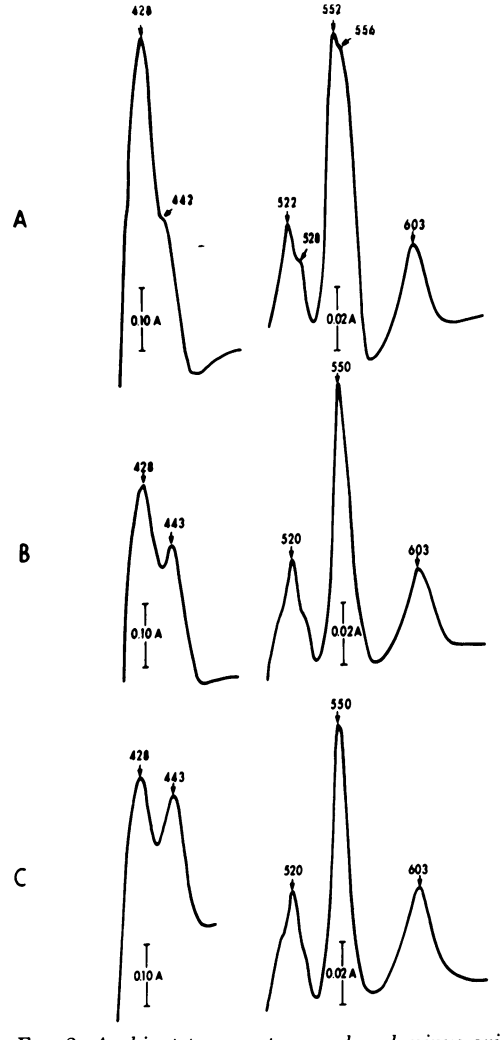

FIG. 2. *Ambient temperature, reduced-minus-oxidized difference cytochrome spectra of sporulating cell membranes. Cuvettes contained 12.5 mg of membrane protein. Reduction was by: dithionite (A), NADH (B), or succinate (C).*

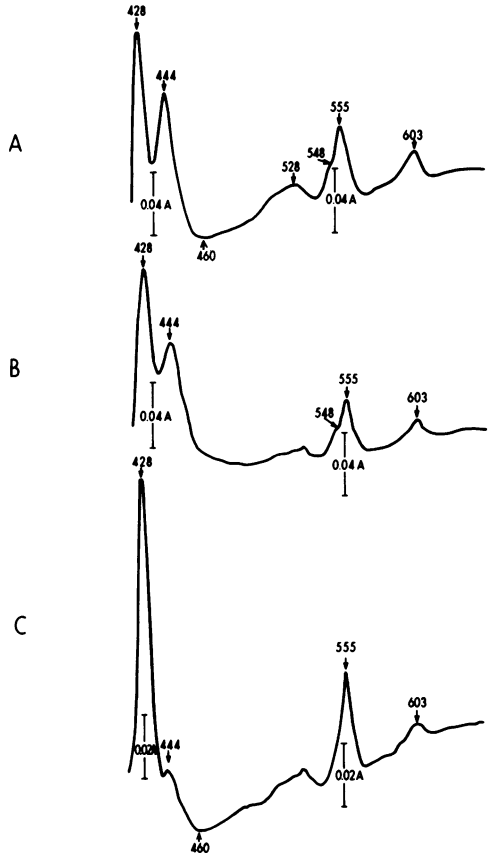

FIG. 3. *Liquid N_2 temperature, reduced-minus-oxidized difference cytochrome spectra of vegetative cell membranes. Cuvettes contained 0.4 ml of membrane preparation (10 mg of protein per ml). Reduction was by dithionite (A), NADH (B), or succinate (C).*

NADH reduced 100% of cytochrome *a* and a_3 and cytochrome *c* at steady state with sporulating cell membranes; cytochrome *b* was reduced 20% by succinate and 14% by NADH.

Electron transport inhibitors. The effect of the electron transport inhibitors, cyanide and azide, on succinate oxidase and NADH oxidase associated with vegetative cell membranes was examined. Cyanide inhibited both oxidases similarly at concentrations ranging from 0.15 to 60 mM. Azide was more effective on succinate oxidase than NADH oxidase at concentrations ranging from 2 to 120 mM; at 20 mM azide, succinate oxidase was inhibited approximately twice as much as NADH oxidase.

Dicumarol and HOQNO were also more inhibitory to the succinate oxidase than to the NADH oxidase of vegetative cell membranes (Table 2). At the lowest concentration of these inhibitors examined succinate oxidase was inhibited 100%, whereas NADH oxidase was inhibited 60% (HOQNO) and 50% (dicumarol). Dicumarol, an inhibitor of electron transport, is an analogue of natural ubiquinones and naphthoquinones (9) and the inhibitor, HOQNO, affects bacterial respiratory systems by preventing electron transfer from cytochrome *b* to cytochrome *c* (17, 28) or by preventing reduction of cytochrome *b* (17).

Since NADH oxidase was less sensitive to inhibitors of electron transport (azide, HOQNO, and dicumarol) it was reasoned that all electrons from NADH were not being transported through a typical respiratory chain. It

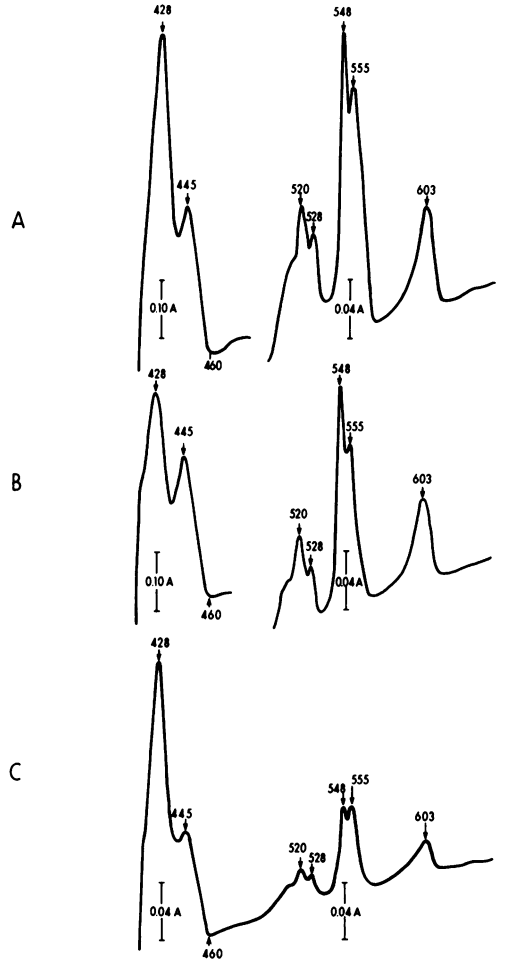

FIG. 4. *Liquid N_2 temperature, reduced-minus-oxidized difference cytochrome spectra of sporulating cell membranes. Cuvettes contained 0.4 ml of membrane preparation (10 mg of protein per ml). Reduction was by dithionite (A), NADH (B), or succinate (C).*

TABLE 1. *Amount[a] of individual cytochromes present in vegetative and sporulating cell membranes*

Cytochrome	Type of cell from which membranes were prepared	
	Vegetative	Sporulating
a	0.06	0.2
a_3	0.14	0.25
c	0.17	0.9
b	0.11	0.65

[a] Expressed as nanomoles of cytochrome per milligram of protein. Values were calculated from the dithionite reduced-minus-oxidized difference spectrum at ambient temperature using the wavelength pairs and millimolar extinction coefficients cited in the text.

TABLE 2. *Effect of 2-heptyl-4-hydroxyquinoline-N-oxide, dicumarol, and atebrine on succinate oxidase and NADH oxidase associated with vegetative cell membranes[a]*

Inhibitor	Concentration (M)	Inhibition (%)	
		Succinate oxidase	NADH oxidase
HOQNO	1.8×10^{-6}	100	60
	3.6×10^{-6}	100	65
	9.0×10^{-6}	100	73
	1.8×10^{-5}	100	78
Dicumarol	2.25×10^{-4}	100	50
	4.5×10^{-4}	100	50
	9.0×10^{-4}	100	50
Atebrine	1.0×10^{-3}	73	47
	2.0×10^{-3}	73	76
	3.0×10^{-3}	73	85
	4.0×10^{-3}	73	90

[a] Two milligrams of membrane protein were used in the assays for the oxidases.

was also suggested from the results with atebrine, an inhibitor of flavine enzymes (Table 2), that the branch-point of electron transport from NADH oxidation occurred after the flavoprotein dehydrogenase but before the quinone component.

Transfer of electrons from NADH dehydrogenase to O_2, bypassing a quinone-cytochrome chain, could result in the production of H_2O_2, necessitating the removal of this toxic product by catalase or peroxidase. Catalase was detected in vegetative cells of the organism.

When the aforementioned inhibitors of election transport were examined using sporulating cell membranes, the results were more complex. Table 3 shows the results of inhibition studies with cyanide and azide. NADH oxidase was more sensitive to cyanide than was succinate oxidase; azide exerted no inhibition on the succinate or NADH oxidase systems. The concentration of cyanide needed to inhibit O_2 uptake by sporulating cell membranes was considerably higher than that required by vegetative cell membranes. Knowing that enzyme activity is masked in sporulating cell membranes and that masking can be released by detergents such as sodium deoxycholate (DOC) or sodium dodecyl sulfate (SDS) (14), the effect of the aforementioned electron transport inhibitors was examined in the presence of detergents. Neither DOC nor SDS had any significant affect on the sensitivity of NADH oxidase and succinate oxidase to cyanide and azide.

Succinate oxidase was more sensitive to HOQNO than was NADH oxidase (Table 4); at 1.8×10^{-5} M, which gave 100% inhibition of succinate oxidase, only 30% of the NADH oxidase was inhibited. Both oxidases associated with sporulating cell membranes were less affected by HOQNO than were the oxidases associated with vegetative cell membranes (Tables 2 and 4).

NADH oxidase was more sensitive to dicumarol than was succinate oxidase in sporulating cell membranes (Table 4). Dicumarol inhibited succinate oxidase greater in vegetative cell membranes, whereas NADH oxidase showed greater inhibition in sporulating cell membranes (Tables 2 and 4).

Unlike results obtained with vegetative cell membranes, atebrine did not inhibit the two oxidases similarly in sporulating cell membranes; NADH oxidase was more sensitive than succinate oxidase (Table 4). The level of inhibition of NADH oxidase in both sporulating and vegetative cell membrane preparations was similar. However, inhibition of succinate oxidase by atebrine was considerably less in sporulating cell membranes (Tables 2 and 4). Malon-

TABLE 3. *Effect of cyanide on succinate oxidase and NADH oxidase associated with sporulating cell membrane preparations[a]*

Inhibitor	Concentration (M)	Inhibition (%)	
		Succinate oxidase	NADH oxidase
KCN	1.5×10^{-4}	11	15
	6.0×10^{-4}	23	19
	1.5×10^{-3}	28	42
	6.0×10^{-3}	45	63
	1.5×10^{-2}	67	75
	6.0×10^{-2}	78	92

[a] Two milligrams of membrane protein were used in the assays for the oxidases.

TABLE 4. *Effect of 2-heptyl-4-hydroxyquinoline-N-oxide, dicumarol, and atebrine on succinate oxidase and NADH oxidase associated with sporulating cell membrane preparations*[a]

Inhibitor	Concentration (M)	Inhibition (%)	
		Succinate oxidase	NADH oxidase
HOQNO	1.8×10^{-6}	67	—
	3.6×10^{-6}	78	—
	9.0×10^{-6}	89	—
	1.8×10^{-5}	100	30
	3.6×10^{-5}	—[b]	37
	7.2×10^{-5}	—	55
	1.4×10^{-4}	—	67
Dicumarol	2.25×10^{-4}	38	49
	4.5×10^{-4}	50	60
	9.0×10^{-4}	—	69
Atebrine	1.0×10^{-3}	25	54
	2.0×10^{-3}	25	68
	3.0×10^{-3}	25	78
	4.0×10^{-3}	25	80

[a] Two milligrams of membrane protein were used in the assays for the oxidases.

[b] Not tested.

ate, a specific inhibitor of succinate dehydrogenase, gave similar results as atebrine (Table 5).

Sporulating cells also contained catalase.

DISCUSSION

Difference spectra of membranes from both vegetative and sporulating cells show the presence of cytochromes *b*, *c* and $a + a_3$, as well as the flavoprotein moiety associated with succinate dehydrogenase and NADH dehydrogenase. Cells undergoing sporulation contain a larger amount of these respiratory chain components than do vegetative cells; it had been reported previously that a functional electron transport system is required for normal sporulation but is not needed for growth (14).

It is assumed that a quinone component is also a constituent of the respiratory chain since dicumarol inhibited the oxidation of succinate and NADH. In general, gram-positive organisms contain naphthoquinones, whereas ubiquinones predominate in gram-negative organisms (4, 16).

Succinate and NADH reduce the same cytochrome components; however, in vegetative cell membranes the response of succinate oxidase and NADH oxidase was different to various inhibitors. Azide, HOQNO, and dicumarol were less effective against NADH oxidase than against succinate oxidase. Cyanide inhibited both oxidases similarly; however, cyanide may not have been acting as a classical terminal oxidase inhibitor. Chelation of metals by cyanide is well known. Chelation has been implicated as a mechanism of inhibition of the electron transport system in *Mycobacterium phlei* (1) and *Nitrobacter agilis* (26) by binding to a nonheme metal in the transport chain. In the present study the metal chelators, 8-hydroxyquinoline, ethylenediaminetetraacetate, and *o*-phenanthroline were examined, but they had no effect on either succinate oxidase or NADH oxidase. The low sensitivity of NADH oxidase to the electron transport inhibitors suggests that for NADH oxidation a branched pathway exists where the branch-point would be at the level of the flavoprotein; atebrine effectively inhibited both oxidases.

A postulated scheme of electron transport with either succinate or NADH as substrate is shown in Fig. 5. Electrons from succinate are passed to a quinone component, probably a vitamin-K derivative, by means of a flavine-associated succinate dehydrogenase. The electrons then move through a cytochrome chain composed of cytochromes *b*, *c* and $a + a_3$ to reduce oxygen. In addition to electrons from NADH being transported through a similar transport system, mediated by flavine-associated NADH dehydrogenase, it appears that electrons can also be passed to oxygen from the flavoprotein, bypassing the quinone-cytochrome chain. What intermediates (if any) are involved in this branched pathway is not known.

This involvement of a flavoprotein oxidase has been detected in other organisms. The functional electron transport system associated with spores of *B. cereus* T contains a soluble flavoprotein oxidase (6); vegetative cells have a typical cytochrome system. A particulate flavoprotein oxidase associated with NADH oxidation in *B. subtilis* has also been postulated (20). A branched electron transport system has been

TABLE 5. *Effect of malonate on succinate oxidase associated with vegetative cell and sporulating cell membranes*[a]

Malonate concentration (M)	Inhibition (%)	
	Vegetative cell membranes	Sporulating cell membranes
4×10^{-3}	19	0
1×10^{-2}	40	13
2×10^{-2}	56	25
4×10^{-2}	70	44

[a] Two milligrams of membrane protein were used in the assays for both vegetative cell and sporulating cell membranes.

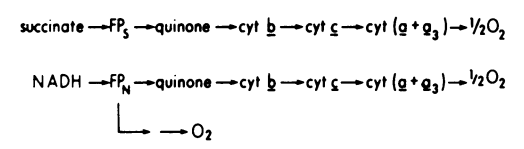

FIG. 5. *Proposed scheme of electron transport from succinate and NADH oxidation in B. cereus. The flavoprotein associated with succinate dehydrogenase is represented by FP_S and the flavoprotein associated with NADH dehydrogenase is represented by FP_N.*

detected in the fungus, *Neurospora crassa* (13), where, in addition to cytochrome *a*, an unidentified alternate terminal oxidase which bypasses the cytochrome chain is operative. This is very similar to a branched electron transport system occurring in some higher plants (2, 7, 22-25).

Of the three electron transport inhibitors studied (azide, HOQNO, and dicumarol) which gave results supporting the idea of a branched pathway involved in NADH oxidation in vegetative cells, only the inhibitor, HOQNO, was less inhibitory to NADH oxidase than succinate oxidase with sporulating cell membranes. The ineffectiveness of azide to inhibit the oxidases associated with sporulating cell membranes may be due to a change in the sensitivity of the terminal oxidase to this inhibitor by an unknown mechanism. Birdsell and Cota-Robles (3) suggested that there were two species of a given cytochrome oxidase present in membranes of *Escherichia coli*. One species was sensitive to low concentrations of inhibitors and the other species required higher concentrations for maximal inhibition. Although the increased cytochrome oxidase level in sporulating cells probably contributes to azide ineffectiveness, its role is considered minor since large concentrations of azide had no effect on the oxidase.

An overall comparison of the effect of inhibitors on the oxidases of vegetative cell membranes to those of sporulating cell membranes is difficult to interpret. Three probable explanations for the decreased inhibitory properties noted in sporulating cell membranes are: (i) an increase in the amount of cytochrome components, (ii) differences in membrane permeability to these inhibitors possibly due to a change in membrane architecture, and (iii) a generalized change in the sensitivity of the inhibitors targets by an unknown mechanism.

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