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Characterization of the Electron Transport System in *Brucella abortus*

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The electron transport system in *Brucella abortus* has been characterized. Spectral studies of membrane preparations have indicated the presence of cytochromes $a + a_3$ (maxima at 612 nm), cytochrome *b* (maxima at 560, 530, and 428 nm), cytochrome *c* (maxima at 552 and 522 nm), cytochrome *o* (maxima of carbon monoxide complex at 418 nm), and flavoproteins (minimum at 582 and 450 nm). Cytochromes $a + a_3$ appeared only after cells had reached late log phase, possibly due to lowered oxygen tension in the medium. Dehydrogenases were shown to be present for D-erythritol 1-phosphate, L-lactate, reduced nicotinamide adenine dinucleotide, and succinate. All of the above substrates reduced the electron transport chain and at least some of the flavoproteins, indicating similar pathways of electron transport. *N*-ethylmaleimide, *p*-chloromercuribenzoate, and KCN were the only electron transport inhibitors that blocked electron transport by 100%. The system seemed to be uniquely resistant to other electron transport inhibitors.

The *Brucella* are small, aerobic, gram-negative bacilli with the unique property of survival and growth within mononuclear cells. The rate of multiplication and carbohydrate utilization markedly increases when *Brucella* are grown under vigorous aeration (19), which suggests that some sort of a respiratory chain system coupled to oxygen is important in the physiology of the cell. Thiele et al. (24, 25) showed that *B. abortus* contained ubiquinone 50 (coenzyme Q_{10}), but they were unable to show the presence of menaquinone. Richardson (18) observed the presence of cytochrome oxidase in three species of *Brucella* but did not further characterize the system or primary dehydrogenases coupled to it.

Our experiments on transport systems in *Brucella abortus* prompted us to consider the mechanisms of energization of active transport in this organism. Barnes and Kaback (3) proposed that an intact electron transport system coupled to an active dehydrogenase is necessary for active transport of metabolites by bacteria; however, no matter the mechanism of energization of active transport, the electron transport system of an important aerobic pathogen, *Brucella*, was of interest. We also wanted to know how the respiratory chain of a facultative intracellular parasite might compare with other aerobic organisms (1, 8, 11, 26) and with another small gram-negative rod, *Haemophilus influenzae* (27).

This report deals with the characterization of the respiratory chain of *B. abortus* and identifies some of the dehydrogenases which interact with it.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Brucella abortus*, British strain 19, was obtained from B. L. Deyoe, National Animal Disease Laboratories, Ames, Iowa. Cells were grown on a rotary shaker in 250-ml Erlenmeyer flasks containing tryptose, yeast extract, vitamins, salts, and glucose as described previously (16).

Preparation of membrane fragments. *B. abortus* (400 ml) was harvested at an optical density measured at 620 nm (OD_{620}) of 4 for mid-log cells or 7 for late log cells in a Sorvall refrigerated centrifuge (model RC2B) at 4080 × *g* for 20 min. During mid-log phase growth, one OD unit (Bausch and Lomb Spectronic 20) corresponded to 1.4×10^{10} colony-forming units per ml, or 0.35 mg (dry weight) per ml. The cell pellets were combined and washed once with 0.1 M triethanolamine buffer (pH 7.0) containing 10 mM $MgSO_4$ and resuspended in 30 ml of 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.0) containing 10 mM $MgSO_4$ (HEPES-NH_4OH-Mg). To this suspension was added 2 ml of niacinamide (2 mg/ml) and 2 ml of 2 mM dithiothreitol. The cell suspension was pipetted into an 80-ml glass Bronwill bottle (B. Braun Melsungen Apparatebau) containing 12 g of 0.17- to 0.18-nm glass beads, and the cells were broken for 4 min in a cell homogenizer (Bronwill) as described previously (16). The broken cell suspension was allowed to settle for 30 min at room temperature, and the supernatant fraction was transferred to a

Teflon-glass homogenizer (Tri-R Instruments Co.) to which was added 1 mg each of crystalline deoxyribonuclease (EC 3.1.3.5) and ribonuclease (EC 3.1.4.22). The broken cell suspension was gently homogenized for 30 s at room temperature and centrifuged at 3,020 × *g* for 10 min to remove whole cells and large cell fragments. The supernatant was drawn off and centrifuged in a Beckman refrigerated ultracentrifuge (model L2-65B) at 25,000 rpm for 1 h in an SW27 rotor. The pellet was finally suspended in 12 to15 ml of HEPES-NH_4OH-Mg buffer. This suspension was the crude membrane preparation and was used for all experiments within 8 h after its preparation.

Analytical methods. Protein concentrations were determined by the microbiuret method (2). Ubiquinone was extracted and quantitated by the method of Redfearn (15) from 1 ml of membranes containing 10 mg of protein. Nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide phosphate (NAD-NADPH) transhydrogenase was assayed by the method of Humphrey (10).

Analysis of cytochromes and other respiratory components. For the presence of cytochromes and flavoproteins, spectral assays were performed with a Cary recording spectrophotometer (model 14). Scans were run at room temperature from 650 to 400 nm on a sensitive slidewire (000_{620} of 0 to 0.2) at 2.5 nm/s using a variable slit width. For the reduced plus CO versus reduced spectrum, CO was gently bubbled through a 3-ml cuvette containing 2 ml of reduced membranes with the use of a 20-gauge hypodermic needle. The concentrations of the cytochromes and total flavoproteins were determined using the following wave-length pairs and millimolar extinction coefficients (26): cytochrome $a + a_3$, ϵ (604 to 623 nm) = 16 mM^{-1}; cytochrome *b*, ϵ (562 to 574 nm) = 20 $mM^{-1}cm^{-1}$; cytochrome $c + c_1$, ϵ (552 to 540 nm) = 19 $mM^{-1}cm^{-1}$; cytochrome *o*, ϵ (415 to 480 nm) = 110 $mM^{-1}cm^{-1}$; and flavin adenine dinucleotide, ϵ (450 nm) = 11.3 × 10^3 cm^2/mmol.

When anaerobic spectra were measured, the membranes were deaerated in serum cap-covered cuvettes by gently bubbling helium over the membranes for 15 min. All reagents added to the anaerobic cuvettes were deaerated in the same manner. Baseline spectra were always run before experimental spectra for comparative purposes.

Measurement of respiration and effects of respiratory inhibitors. A Clark oxygen electrode (Yellow Springs Instrument Co.) was used to measure oxygen utilization by *B. abortus* membrane preparations to observe (i) dehydrogenase activity associated with the reduction of the cytochrome chain, and (ii) the effects of inhibitors on electron transport coupled to oxygen utilization. The reaction reservoir contained: substrate (1 to 10 μmol), membranes (0.5 to 1.5 mg of protein), inhibitors when added (0.01 to 10 μmol), and HEPES-NH_4OH-Mg buffer to 3 ml. Reactions were run at 37 C. It was assumed that there was 0.22 μmol (0.45 μg-atom) of dissolved O_2 per ml of reaction mixture (3).

Materials. Antimycin A, atabrine (quinacrine hydrochloride), L-ascorbic acid (sodium salt), phenazine methosulfate, succinate, D,L-lactate, β-OH-butyrate, malate, and coenzyme Q_{10} were obtained from Sigma Chemical Co. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine was purchased from Aldrich Chemical Co. D-Lactate and L-lactate were obtained from California Corporation for Biochemical Research. D-erythritol 1-phosphate were kindly supplied by Jay F. Sperry of this laboratory. All other reagents and chemicals were obtained from sources previously described (16).

RESULTS

Spectrophotometric evidence for cytochromes. Initially, membranes prepared from late-log phase cells (OD_{620} of 7) were used for the spectral observation of cytochromes. Membranes in the sample cuvette were reduced with a few crystals of dithionite, and the reduced versus oxidized spectrum was recorded. To insure that complete reduction of the cytochromes had occurred, scans were run immediately after addition of dithionite and again at 5 min after addition of dithionite. The data in Fig. 1 (solid line) indicate that the following cytochromes were present at their respective absorption maxima: cytochromes $a + a_3$ at 612 nm; cytochrome *b*, α band at 560 nm, β band at 530 nm, and Soret band at 428 nm; and cytochrome *c*, α band at 552 nm and β band at 522 nm.

When the same type of experiment as above was repeated with membrane preparations from mid-log cells (OD_{620} of 4), the results in Fig. 1 (broken line) were observed. All bands for cytochromes *b* and *c* were present, but the band at 590 to 610 nm was absent. This indicated that cytochromes $a + a_3$ were absent or at a very low level in these preparations.

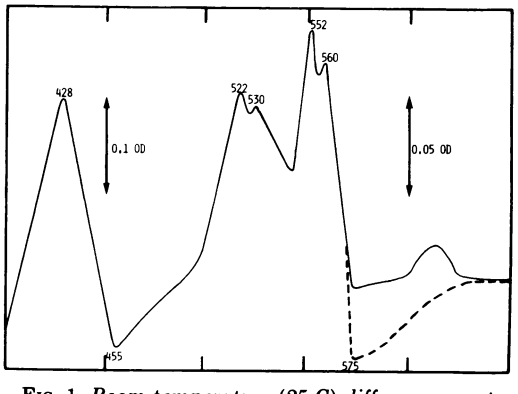

FIG. 1. *Room temperature (25 C) difference spectra (dithionite reduced minus oxidized) of B. abortus membranes prepared from mid-log cells (broken line) and late-log cells (solid line). Baseline spectra were run before addition of dithionite. Each division represents 50 nm. Protein concentration was 12 mg per ml.*

Since cytochromes $a + a_3$ did not appear to be present in the mid-log membranes, and since only a few bacterial systems (7, 9, 12) are known to utilize cytochrome *c* to transfer electrons directly to oxygen, we looked for another cytochrome oxidase in the mid-log membrane preparations of *B. abortus*. Cytochrome *o* has been reported to act as a terminal oxidase in several bacteria (5, 17) and is observable spectrally when reduced membranes are complexed with CO. Cytochromes $a + a_3$ can also be observed when reduced and then complexed with CO, and this fact was used to check for the absence or presence of cytochromes $a + a_3$. Both sample and reference membranes were reduced with a few crystals of dithionite, CO was gently bubbled through the sample cuvette for 5 min, and a reduced plus CO versus reduced spectrum was taken. As seen in Fig. 2, a peak at 418 nm (cytochrome *o*) and a trough at 433 nm (cytochrome $a + a_3$) was observed with late-log membranes (solid line), whereas only the 418 nm maximum (cytochrome *o*) was observed with mid-log membranes (broken line). These experiments indicated that cytochrome *o* was present in both mid-log and late-log membranes and confirmed the data from the reduced versus oxidized spectrum: cytochromes $a + a_3$ were present only in late-log membranes.

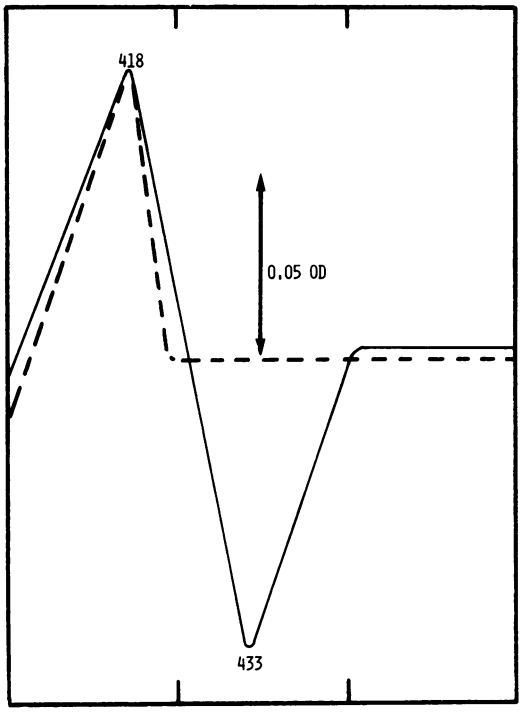

FIG. 2. *Room temperature (25 C) difference spectra (carbon monoxide-dithionite reduced minus dithionite reduced) of B. abortus membranes prepared from mid-log cells (broken line) and late log cells (solid line). Dithionite was added to the sample and reference cuvettes, and the baseline spectra was run. Carbon monoxide was gently bubbled through the sample cuvette for 2 min. Each division represents 25 nm. Protein concentration was 15 mg per ml.*

Polarographic evidence for membrane-bound dehydrogenases. Polarographic studies were performed to identify membrane-bound dehydrogenases associated with the electron transport system. Table 1 contains substrates that were tested for their ability to reduce molecular oxygen (O_2) in the presence of mid-log or late-log membrane preparations. D-Erythritol 1-phosphate, L-lactate, reduced NAD (NADH), NADPH, and succinate were capable of reducing O_2 in the presence of membranes. No differences in O_2 reduction were observed between mid-log and late-log preparations. The rate obtained for NADPH could be caused by either an NADPH dehydrogenase or an NAD-NADPH transhydrogenase; however, no membrane-bound or soluble transhydrogenase could be detected in these preparations. The oxygen utilization observed in all of the above experiments could be due either to membrane-bound dehydrogenases or to membrane-bound oxygenases present for the individual substrates in the membranes. To clarify this point, the reduction of the membranes was observed spectrally instead of polarographically.

Spectral evidence for membrane-bound dehydrogenases. Reduced versus oxidized spectra were run with late-log membrane preparations. To the sample cuvette, containing 2 ml of membranes, was added 0.22 ml of substrate, and to the reference cuvette, containing 2 ml of membranes, was added 0.22 ml of HEPES-

TABLE 1. *Substrates for the electron transport system in B. abortus*

Substrate (mM)[a]	O_2 reduced[b]
Ascorbate-PMS (20:0.1)	—[c]
Ascorbate-TPD (20:0.1)	400–600
D-Erythritol-1-PO_4 (5)	40–60
L-Lactate (10)	30–40
D-Lactate (10)	0
NADH (1)	200–400
NADPH (1)	20
Succinate (10)	90–120

[a] PMS, Phenazine methosulfate; TPD, *N,N,N',N'*-tetramethyl-P-phenylenediamine.

[b] Expressed as nanomoles of O_2 reduced per minute per milligram of protein.

[c] This system was too rapidly auto-oxidized to obtain accurate measurements.

NH_4OH-Mg buffer. Scans from 650 to 400 nm were made at 5 and 10 min after addition of substrate to insure complete reduction of the electron transport system. Reduction of the electron transport system was complete after 5 min. D-Erythritol 1-phosphate, L-lactate, NADH, and succinate completely reduced the electron transport system as indicated by the lack of increased absorbance when dithionite was added 10 min after addition of substrate. The spectra of these experiments were similar to those observed in Fig. 1 (data not shown).

Polarographic studies of the effects of electron transport system inhibitors. Mid-log or late-log membranes were incubated for 5 min with the appropriate inhibitor before the addition of substrate. The data were converted to nanomoles of O_2 reduced per minute per milligram of membrane protein (Table 2). The sulfhydryl inhibitors, *p*-chloromercuribenzoate and *N*-ethylmaleimide, blocked O_2 utilization to different extents for the different substrates tested, which indicated that these inhibitors affected the membrane-bound dehydrogenases, as opposed to other components of the electron transport system.

NADH was the only substrate not affected by atabrine. Of all the electron transport inhibitors used, only two seemed to have had any great effect on O_2 utilization. KCN (10 mM), an inhibitor of cytochrome oxidase, blocked O_2 utilization by 100% with all substrates tested. Dicumarol (1 mM), an inhibitor of electron transfer at the ubiquinone level, appeared to affect the dehydrogenation of D-erythritol 1-phosphate more than the other substrates.

The presence of other electron transport components. The presence of flavins within the electron transport system was indicated spectrally by the minima observed at 575 and 455 nm. (Fig. 1) Ubiquinone was extracted from the membrane preparations, and its concentration was calculated from spectral data (15). The calculated concentrations of all the components of the electron transport system are seen in Table 3. Cytochromes *b* and *c* were present at approximately five times the concentrations of the oxidases, cytochromes *o* and $a + a_3$. Ubiquinone was present at 10 to 50 times the concentration of the cytochromes.

TABLE 2. *Percentage of inhibition of O_2 utilization by electron transport system inhibitors*

Inhibitor (mM)	Substrate			
	D-Erythritol-1-PO_4	L-Lactate	NADH	Succinate
KCN (1)	80	67	ND[a]	47
KCN (10)	100	100	100	100
NEM (0.1)	44	100	8	39
pCMB (1)	100	100	48	100
NaN_3 (10)	44	34	40	29
Atabrine (1)	34	50	0	16
Dicumarol (0.1)	92	46	16	42
HOQNO (0.1)	ND	28	42	20
Amytal (1)	20	11	25	22

[a] ND, Not determined.

DISCUSSION

Membrane-bound dehydrogenases, flavoproteins, coenzyme Q_{10}, and cytochromes (*b*, *c*, $a + a_3$, and *o*) were observed in membrane preparations of *B. abortus* in this study. Membranes prepared from mid-log cells contained cytochromes *b*, *c*, and *o*, whereas membranes from late-log cells also contained cytochromes $a + a_3$. We did not observe a shoulder at 440 nm indicative of cytochrome a_3 (Fig. 1) and have no explanation for the observation. The order of cytochrome reduction from NADH to the terminal acceptor(s) has not been established and is assumed to be the same as observed in other cytochrome systems (22). It appears that the respiratory chain in *Brucella* is complex and branched (28), with both cytochromes $a + a_3$ and cytochrome *o* acting as terminal oxidases; however, it is not possible to determine which oxidases are functional without measuring the carbon monoxide action spectrum which follows the release of inhibition in the presence of exogenous substrate (28). The branched system may be useful to *Brucella* for intracellular survival within phagocytic cells. A nitrate reductase has also been detected (data not shown) in membrane preparations, which is somewhat surprising, since the cells were grown under vigorous aeration.

TABLE 3. *Components of the electron transport system in B. abortus*

Component	Wavelength[a]	ϵ ($mM^{-1}\,cm^{-1}$)	Amount[b]
$a + a_3$	612↑	18	0.028
a_3	433↓	82	0.018
b	560↑	21	0.14
c	552↑	19	0.18
o	418↑	110	0.027
Flavins	460↓	11	0.09
Ubiquinone (extracted)	275↑	12.15	1.75

[a] Arrows indicate maxima or minima.

[b] Expressed as nanomoles per milligram of membrane protein.

Brucella grows to high cell densities in the complex medium used in this study and may lower the oxygen tension to the point where a nitrate reductase is induced due to the lowered redox potential (21). The significance of nitrate respiration in these aerobic bacteria is under investigation. Zobel and Meyer (29) reported nitrate reductase in all 425 strains of *Brucella* which were examined.

Thiele and Kehr identified the quinone extracted from *Brucella* membranes as coenzyme Q_{10} (25). We found no indication of a naphthoquinone in the extract as determined by thin-layer chromatography, which supports the work of Thiele and Hoffmann (24). Most bacteria contain quinones with 6 to 9 isoprenoid units coupled to the ring structure of the vitamin (4); however, CoQ_{10} has also been detected in *Pseudomonas denitrificans* (13), *Agrobacter tumefaciens* (14), and some of the purple sulfur bacteria (6, 23).

The data in Table 3 indicate that the concentrations of cytochromes in membrane preparations are lower than those reported for other bacteria (22), making it possible that a low level of cytochrome a_3 would not have been detected. *Brucella* are difficult to disrupt (20), and breakage using the Bronwill cell homogenizer may solubilize some membrane components. The low levels of cytochromes might explain the long generation of *Brucella* (2.5 to 3.0 h) if the rate of growth is proportional to the rate of energy metabolism. Factors which control cytochrome synthesis in *Brucella* need to be examined.

Brucella membranes were shown to contain a variety of dehydrogenases which acted as donors for the electron transport system. It is interesting that D-erythritol 1-phosphate, an intermediate of the erythritol pathway (J. F. Sperry and D. C. Robertson, submitted for publication), is an electron donor. At least one other intermediate of the erythritol pathway, 3-keto-L-erythrose 4-phosphate, also reduces the complete electron transport system of *B. abortus*. There is apparently very "tight" coupling between the electron transport system, active transport (16), and metabolism in these bacteria.

Polarographic data were equivocal as to points where the dehydrogenases coupled to the electron transport system; however, spectral studies suggested they were located early in the respiratory chain, before cytochrome *b*. Except for KCN, the electron transport of *B. abortus* seemed more refractory to electron transfer inhibitors than has been observed in other systems (8, 9, 11, 26).

Glucose catabolism in *Brucella* occurs via the hexose monophosphate pathway in conjunction with the trichloroacetic acid cycle (19). *Brucella* lacks a functional glycolytic pathway due to the absence of fructose 6-phosphate kinase and fructose 1,6-diphosphate aldolase (20); thus, adenosine-5'-triphosphate synthesis by substrate level phosphorylation is minimal if D-glyceraldehyde 3-phosphate is needed solely as a carrier to maintain the hexose monophosphate cycle. However, NADH derived from the pathway can be oxidized by the electron transport system described herein and supply adenosine-5'-triphosphate for biosynthetic and metabolic needs.

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