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Electron Transport in *Bacillus popilliae*[1]

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ABSTRACT

PEPPER, ROLLIN E. (Michigan State University, East Lansing), AND RALPH N. COSTILOW. Electron transport in *Bacillus popilliae*. J. Bacteriol. **89**:271-276. 1965.—*Bacillus popilliae* was found to be unique among aerobic microorganisms in that it was deficient in a hydrogen peroxide-scavenging system. Neither catalase nor peroxidase was found. At the same time, a system for producing hydrogen peroxide during oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) was consistently present in the soluble fraction of extracts of cells from older cultures. Cells harvested from 9-hr cultures did not produce a significant amount of peroxide. The soluble $NADH_2$ oxidase was apparently a flavoprotein, since it was stimulated by flavin nucleotides, insensitive to cyanide and azide, and inhibited by Atabrine. Also, difference spectra demonstrated the presence of a reducible flavin in the soluble fraction of cell extracts. The particulate fraction of cell extracts was shown by difference spectra to contain cytochrome b_1; the strong inhibition of $NADH_2$ oxidation by cyanide, azide, and carbon monoxide indicated that a terminal cytochrome oxidase was also present. This system was also flavin-dependent, since it was strongly inhibited by Atabrine. The specific activity of the $NADH_2$ oxidase in the particulate fraction was lower in extracts of cells from older cultures than in those from exponentially growing cultures. Cytochrome *c* was not found in extracts of these cells. It is believed that the increased participation of the hydrogen peroxide-generating $NADH_2$ oxidase in cells of older cultures may be responsible for the rapid loss in cell viability noted in stationary-phase cultures.

Bacillus popilliae was reported by Dutky (1940) as preferentially anaerobic. This was based on growth tests with an agar medium, and the method used to exclude oxygen for anaerobic incubation was not described. However, more recent studies (Steinkraus, 1957; Pepper and Costilow, 1964; Costilow et al., *unpublished data*) have demonstrated that this organism responds greatly to forced aeration of broth cultures. It requires the presence of a carbohydrate for significant growth in artificial media, and it is unable to ferment glucose in the complete absence of oxygen. Thus, *B. popilliae* appears to be a strict aerobe.

One of the primary differences found between this species and other aerobic microorganisms is its failure to produce catalase (Steinkraus, 1957). This was thought to be a possible reason for the rapid loss in cell viability which occurs very soon after cultures of *B. popilliae* attain the stationary phase of growth in artificial media. Furthermore, it was thought that perhaps the failure of this species to sporulate in vitro could be related to the failure to provide some unique factor for electron transport, which could be present in the hemolymph of Japanese beetle larvae in which extensive sporulation occurs. This reasoning prompted the prevent investigation of electron transport in *B. popilliae*.

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MATERIALS AND METHODS

Culture used and extract preparation. B. popilliae NRRL B-2309-P-A was used in all studies. This is a mutant strain of NRRL B-2309-P originally obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. The mutant differs from the parent culture in that it oxidizes acetate (Pepper and Costilow, 1964). It has been tested at the Northern Utilization Research Division and found to produce typical "milky disease" in Japanese beetle larvae.

The *Streptococcus faecalis* culture used as a control in some experiments was from the culture collection maintained in this laboratory. It was maintained in Trypticase Sugar Agar (BBL), and cells were produced in a broth of similar composition incubated at 30 to 32 C on a rotary shaker.

Methods of maintaining and culturing *B. popilliae*, as well as the preparation of extracts with a Servall omnimixer (Ivan Sorvall, Inc., Nor-

walk, Conn.), were as described by Pepper and Costilow (1964). The Nossal cell disintegrator (McDonald Engineering Co., Cleveland, Ohio) was used to prepare some of the cell extracts. A 2-g amount of cells was introduced into a precooled 18-ml capsule with 10 g of no. 100 Superbrite glass beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) and water to capacity. The capsule was shaken for a total of 2 min at 30-sec intervals, with cooling effected by a jet of compressed carbon dioxide.

Particulate- and soluble-extract fractions were obtained by centrifuging an extract at 110,000 × *g* for 3 hr in the Beckman model L preparative ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, Calif.). The soluble fraction was decanted, and particles were resuspended in distilled water. The protein content of extracts was estimated by the method of Lowry et al. (1951).

Analytical methods. Catalase activity was tested for by (i) dropping H_2O_2 on agar colonies, (ii) tipping H_2O_2 into buffered cell extracts in a Warburg cup and measuring oxygen release, and (iii) the iodometric titration method of Herbert (1955) in which residual H_2O_2 is determined after incubating with cell extract. Peroxidase assays were conducted by the procedure of Dolin (1957). Hydrogen peroxide production was determined by allowing cell extracts to oxidize reduced nicotinamide adenine dinucleotide ($NADH_2$) in a Warburg vessel, and then measuring the oxygen return on tipping in a solution of catalase.

Standard Warburg techniques (Umbreit, Burris, and Stauffer, 1957) were used to measure oxygen-uptake rates.

Oxidation of $NADH_2$ was assayed manometrically by determining oxygen uptake with a Warburg respirometer, and spectrophotometrically by following the decrease in optical density (OD) at 340 mμ with a Beckman model DU spectrophotometer. Spectrophotometric assays at 340 mμ were also conducted for reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) oxidase, and for $NADPH_2$-nicotinamide adenine dinucleotide (NAD) transfer enzyme. In the latter assay, the cuvettes contained 0.5 μmole of $NADPH_2$, 10 μmoles of NAD, and cell extract in 3 ml of 0.03 M phosphate buffer (*p*H 7.3). Diaphorase activity was determined by a decrease in OD at 600 mμ in reaction mixtures containing cell extract, $NADH_2$, and dichlorobenzenone indophenol as the electron acceptor. A Spectronic-20 colorimeter (Bausch & Lomb Inc., Rochester, N.Y.) was used for measurement of OD at 600 mμ.

$NADH_2$ cytochrome *c* reductase and succinic cytochrome *c* reductase assays were conducted by methods described by Dowler, Shaw, and Gottlieb (1963).

The concentration (per cent by volume) of carbon monoxide and oxygen and of helium and oxygen in the atmospheres in Warburg vessels was adjusted by the use of flow raters (The Manostat Corp., New York, N.Y.). A control containing an atmosphere of air indicated that the atmosphere containing helium and oxygen did not significantly inhibit oxygen uptake, and a control run with an extract of *S. faecalis* cells indicated that oxygen was not limiting in the atmosphere with carbon monoxide.

Difference spectra of cell extracts were determined with a Cary model 15 spectrophotometer (Applied Physics Corp., Monrovia, Calif.). A few crystals of dithionite were added to cell extract in a sample cuvette and compared with untreated extract in a reference cuvette.

RESULTS

The routine diagnostic test for catalase activity in microorganisms was negative for *B. popilliae* as reported previously. Therefore, more sensitive tests for this enzyme were run. No oxygen evolution occurred when 20 μmoles of H_2O_2 were tipped into a Warburg vessel containing *B. popilliae* cells. Similarly, there was no evidence of H_2O_2 breakdown in cells from either logarithmic- or stationary-phase cultures as determined by iodometric titration (Table 1).

Tests were then conducted for a peroxidase system in these cells. Extracts of *B. popilliae* cells from logarithmic- and stationary-phase cultures oxidized $NADH_2$ rapidly as determined spectrophotometrically, but the rate of oxidation was not stimulated by the addition of H_2O_2. An extract of *S. faecalis* cells was used as a positive control in this test.

The absence of an H_2O_2 scavenging system could be very critical if the cells generated H_2O_2 during respiration. Thus, experiments were conducted to determine whether this compound was produced during $NADH_2$ oxidation by crude cell extracts and soluble and particulate fractions thereof. When a solution of catalase was tipped into reaction mixtures after oxidation of $NADH_2$ in a Warburg vessel, only 2% of the oxygen taken up was released in the vessel with the whole extract and none with the particulate fraction; there was 30% oxygen return with the soluble fraction (Fig. 1). Thus, there appeared to be at least two distinct $NADH_2$ oxidase systems operating, one of which produced H_2O_2 as the primary product.

This organism also produces $NADPH_2$ during glucose oxidation (Pepper and Costilow, 1964). However, no $NADPH_2$ oxidase or $NADPH_2$-NAD transfer enzyme was found in cell extracts as measured spectrophotometrically by loss in OD at 340 mμ.

Further evidence of two distinct electron-transport systems was obtained with the use of inhibitors. Cyanide, azide, and Atabrine almost completely inhibited oxygen uptake by the

particulate fraction of extracts of cells from a 24-hr culture with $NADH_2$ as substrate; cyanide and azide failed to influence the system operating in the soluble fraction; and Atabrine only partially inhibited the uptake (Fig. 2). In similar experiments with other extracts, cyanide was found to inhibit oxygen uptake by the soluble fraction to variable extents, but it never approached the inhibition levels noted with particulate fractions. When catalase was tipped into the Warburg cups after $NADH_2$ oxidation, the oxygen return noted previously was observed with the soluble fraction. Cyanide did not inhibit this return appreciably, but azide inhibited the catalase action as determined experimentally.

The dependence of the soluble oxidase system on flavin nucleotides was further indicated (Table 2) by the stimulation noted upon addition of either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). There was about a 3× increase in the specific activity of this fraction upon addition of either of these. They not only failed to stimulate the oxidase activity in the particles, but FAD strongly inhibited the activity. Dipicolinic acid has been shown to stimulate the soluble $NADH_2$ oxidase of *B. cereus* spores (Doi and Halvorson, 1961*b*), but it had no significant effect on the oxidase from *B. popilliae* cells.

Inhibitor studies indicated that a cytochrome oxidase-independent electron-transport system was operative in intact resting-cell suspensions. The inhibition of oxygen uptake during glucose oxidation by intact cells of *B. popilliae* was only about 30% with either 0.01 M cyanide or azide. Also, the inhibition of glucose oxidation was far from complete in an atmosphere of about 80% CO. The degree of participation of the CO-sensitive electron-transport system was related to the age of the culture at the time of cell harvest. Thus, oxidation by cells from an 18-hr culture was inhibited 37%, but it was inhibited only 24% with cells from a 68-hr culture (Table 3). Oxidation by the *S. faecalis* control was not inhibited by the CO in these atmospheres.

The effect of culture age on the participation of the different types of $NADH_2$ oxidases was substantiated by studies on the specific activities of the oxidases in the soluble and particulate fractions of cell extracts. The specific activity in the soluble fraction was about the same in extracts of cells from 9-, 18-, and 72-hr cultures, while that of the particulate fraction declined with culture age (Table 4). The activity of the particulate oxidase from cells harvested after 72 hr was only about 20% that from a 9-hr culture. There was apparently more than one type of oxidase activity in the soluble fraction of extracts of cells from 9-hr cultures, since cyanide (0.01 M)

TABLE 1. *Determination of catalase activity in crude extracts prepared from log- and stationary-phase cells of Bacillus popilliae**

Reaction time	Amt of 0.0128 N thiosulfate used		
	9-hr cell extract	18-hr cell extract	72-hr cell extract
min	*ml*	*ml*	*ml*
—†	0.0	0.0	0.0
0.00	8.0	7.9	8.0
0.25	7.8	7.3	7.9
0.50	8.0	8.0	8.0
0.75	8.1	8.3	7.9
1.00	8.1	7.8	7.8

* Reaction mixtures contained 5.0 ml of 0.01 M H_2O_2 in 0.01 M phosphate buffer (pH 6.8) to which 4 mg of extract protein were added. Reactions were run at 25 C. Reactions were stopped with 1.0 ml of 1 N H_2SO_4. Residual H_2O_2 was determined by iodometric titration.

† Blank, no H_2O_2.

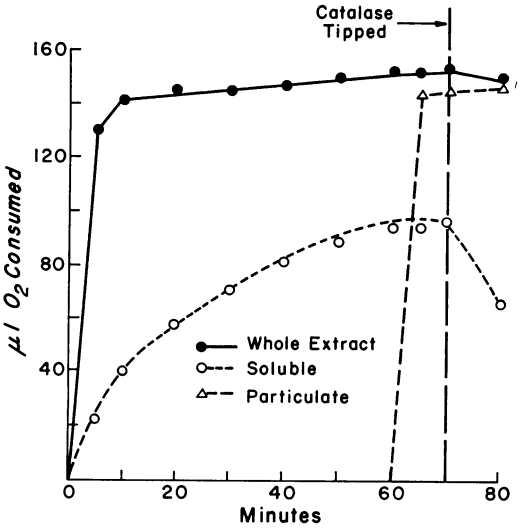

FIG. 1. *Oxygen uptake by cell extracts with $NADH_2$ as substrate, and oxygen return upon adding catalase after the oxidation was completed. Reaction mixtures in Warburg vessels contained 20 μmoles of $NADH_2$, 0.5 ml of 0.2 M phosphate buffer (pH 7.6), and the cell extract indicated. The extract protein levels present were as follows: whole extract, 15 mg; soluble fraction, 1.2 mg; and particulate fraction, 21 mg. A sidearm contained 0.5 ml of a catalase solution, and the center well, 0.2 ml of 20% KOH. The liquid volume was 3 ml. The atmosphere was 100% O_2. No substrate was tipped into the particulate fraction until 10 min before catalase was tipped.*

inhibited $NADH_2$ oxidation by about 40 to 50% as measured spectrophotometrically. The cyanide inhibition of the activity in the particulate fraction was essentially complete.

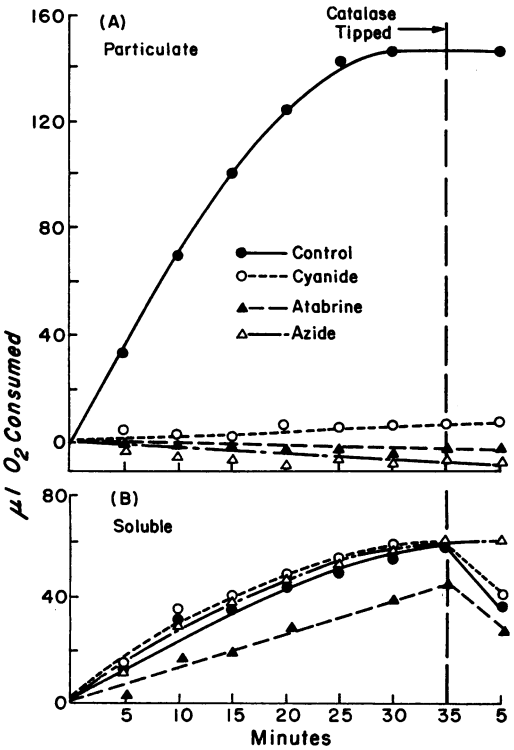

FIG. 2. *Effect of cyanide, azide, and Atabrine on oxygen uptake by cell extracts with $NADH_2$ as substrate. Reaction mixtures in Warburg cups contained the cell extract indicated (1.2 mg of soluble fraction protein, or 4.2 mg of particulate fraction protein), 20 μmoles of $NADH_2$, and 0.5 ml of 0.2 M phosphate buffer (pH 7.6). Cyanide, azide, or Atabrine (30 μmoles) were present where indicated. A sidearm contained 0.5 ml of a catalase solution, and the center well, 0.2 ml of 20% KOH. The liquid volume was 3 ml. The atmosphere was 100% O_2.*

The culture age at cell harvest had a pronounced effect on the amount of H_2O_2 produced during $NADH_2$ oxidation by the soluble fraction of cell extracts (Table 5). Extracts of cells from 9-hr cultures produced practically no H_2O_2, but the H_2O_2 accounted for at least 60% of the oxygen consumed during oxidation by the soluble fraction of extracts of cells from 24- and 72-hr cultures. Cyanide (0.01 M) inhibited oxygen uptake by the soluble $NADH_2$ oxidase from 9-, 18-, and 72-hr cells by 74, 52, and 26%, respectively. No H_2O_2 was generated by the particulate fraction, irrespective of culture age at cell harvest, and 0.01 M cyanide inhibited completely.

Diaphorase activity with dichlorobenzenone indophenol as the electron acceptor was demonstrated in both fractions of cell extracts. The specific activity of the particulate extract was 1.5 to 2.0 (ΔOD at 600 mμ per minute per milligram of protein), as compared with 0.9 to 1.1 for the soluble fraction. There was no appreciable change with culture age up to 72 hr.

To characterize more specifically the types of electron-transport systems operative in *B. popilliae* cells, difference spectra were determined on extracts of cells from 24-hr cultures. An extract of *S. faecalis* cells was used as a control, since this species is known to be free from cytochrome. These gave positive evidence of cytochromes in the particulate fraction (Fig. 3). Spectra of these fractions of a number of extracts all showed peaks at 423 to 426, 528 to 530, and 558 to 560 mμ. These are all indicative of cytochrome b_1 (Smith, 1961). The identity of cytochrome b_1 was substantiated by heat-tolerance studies; exposure to 70 C for 10 min had no effect on the peak at 558 to 560 mμ. Cytochrome *b* is destroyed by this treatment (Smith, 1954). With some of the extracts studied, peaks characteristic of cytochromes a_1 and a_2 were observed at 588 to 590 and 625 to 635 mμ, respectively. The flavoproteins in the soluble fraction of *B. popilliae* cell extract and in the *S. faecalis* extract were demonstrated by

TABLE 2. *Effect of flavin nucleotides and dipicolinic acid (DPA) on $NADH_2$ oxidases**

Compound	Concn (final)	Particulate fraction		Soluble fraction	
		Activity†	Ratio to control	Activity†	Ratio to control
	M				
Control	—	0.555	—	0.057	—
FMN	2.0×10^{-4}	0.530	0.96	0.170	3.00
FAD	2.0×10^{-4}	0.170	0.30	0.180	3.16
DPA	2.5×10^{-3}	0.500	0.90	0.050	0.87

* Cuvettes contained 0.5 μmole of $NADH_2$; 300 μmoles of phosphate buffer, *p*H 7.3; extract; and water to 3.0 ml. Additions were made to the final concentrations noted above.

† ΔOD at 340 mμ per minute per milligram of protein.

the valley at 450 mμ. Apparently a small amount of cytochrome b_1 was solubilized, since small peaks were observed at 423 and 558 mμ in the spectra of soluble fractions.

TABLE 3. *Inhibition of glucose oxidation by carbon monoxide**

Cells	Inhibition
	%
Bacillus popilliae, 18 hr	37
B. popilliae, 68 hr	24
Streptococcus faecalis	0

* Each Warburg vessel contained 58 mg (dry weight) of cells and 33 μmoles of glucose in 3 ml of 0.03 M phosphate buffer (*p*H 7.3). A 0.2-ml amount of 20% KOH was introduced into the center well to absorb CO_2. Control atmospheres were ca. 80% helium and 20% oxygen, treated atmospheres ca. 80% CO and 20% O_2.

TABLE 4. *Activity of $NADH_2$ oxidases in extracts of Bacillus popilliae cells harvested at various culture ages**

Culture age at cell harvest	Specific activity†	
	Soluble fraction	Particulate fraction
hr		
9	0.041	0.86
18	0.048	0.57
72	0.035	0.18

* Cuvettes contained 0.27 μmole of $NADH_2$ and cell extracts in 3 ml of 0.03 M phosphate buffer (*p*H 7.3).

† ΔOD at 340 mμ per minute per milligram of extract protein.

TABLE 5. *Hydrogen peroxide production by extracts of cells of Bacillus popilliae**

Culture age at cell harvest	Per cent oxygen return by catalase	
	Soluble fraction	Particulate fraction
hr		
9	0–1	0
18	20	0
24	30	—
72	29	0

* Each Warburg flask contained 5 mg of soluble extract or 2 mg of particulate extract protein, and 20 moles of $NADH_2$ in 3.0 ml of 0.03 M phosphate buffer (*p*H 7.3). The atmosphere was oxygen and the temperature 30 C. A solution of catalase was tipped into the reaction mixture when $NADH_2$ oxidation had essentially stopped.

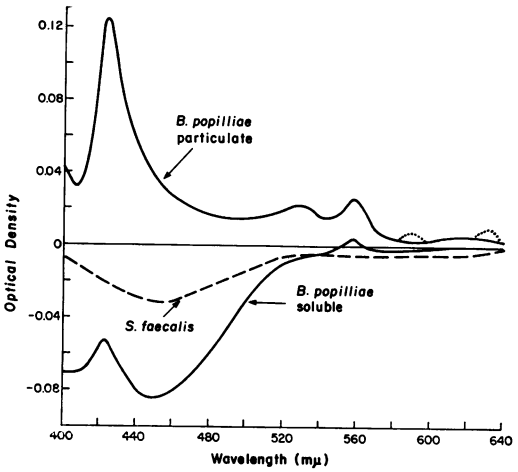

FIG. 3. *Difference spectra of oxidized vs. reduced pigments in the particulate fractions of Bacillus popilliae cell extracts, and in a whole Streptococcus faecalis extract. The reference and sample cuvettes contained the extracts in water solution. A few crystals of dithionite were added to the sample cuvette as the reducing agent. The peaks indicated by the dotted lines at 588 to 590 and 625 to 635 mμ for the particulate fraction were found in some extracts, but were not present in all extracts studied.*

No absorption peaks for cytochrome *c* were found. The absence of cytochrome *c* was further indicated by the failure to find any evidence of a $NADH_2$ cytochrome *c* reductase or of a succinic cytochrome *c* reductase in crude cell extracts of *B. popilliae*.

DISCUSSION

B. popilliae cells contain both a cytochrome and a direct flavin link for the electrons from $NADH_2$ to oxygen. As with *B. cereus* (Doi and Halvorson, 1961*a*), the activity of the cytochrome-dependent system is localized in the particulate fraction and the cytochrome-independent in the soluble fraction of cell extracts; the $NADH_2$ oxidase activity in the particles is much greater than in the soluble system. The soluble $NADH_2$ oxidase was greatly stimulated by the addition of FMN or FAD, but the activity in the particles was greatly inhibited by FAD. Also, *B. popilliae* was comparable to *B. cereus*, because diaphorase activity was found both in the particulate and soluble fractions of cell extracts at about equal levels. However, the cytochrome system in these cells was more similar to those observed in *Aerobacter aerogenes*, *Escherichia coli*, and *Proteus vulgaris* (Smith, 1954) than to aerobically grown *B. cereus*. The difference spectra and the absence of a cytochrome *c* re-

ductase indicated that these cells were devoid of cytochrome *c*. In fact, only cytochrome b_1 was consistently demonstrated by difference spectra of the particles from cells. In this respect, they resembled anaerobically grown *B. cereus* (Schaeffer, 1952). There can be little doubt, however, that there was an active cytochrome-dependent transport system in *B. popilliae*, since it was inhibited almost completely by cyanide and azide, and partially by carbon monoxide. There appeared to be a flavin-dependent step in this sequence, since Atabrine also inhibited it strongly.

The production of hydrogen peroxide by the soluble $NADH_2$ oxidase from cells harvested from stationary-phase cultures, combined with the reduced activity of the particulate $NADH_2$ oxidase in such cells and the absence of catalase and peroxidase, is of much interest. Cells of *B. popilliae* lose viability rapidly once in vitro aerated cultures have attained this phase of growth. Also, Costilow, Sylvester, and Pepper (*unpublished data*) have shown that the viability of cells in a broth culture may be extended greatly by reducing the availability of oxygen when the stationary phase is attained. Thus, peroxide production may be at least one of the primary reasons for the death of cells. Since *B. popilliae* grows to extremely high populations in Japanese beetle larvae and produces endospores after 2 to 3 weeks, the cells obviously retain viability for an extended period. If peroxide production is a primary cause of cell death in vitro, it appears possible that the in vivo environment contains an alternate electron acceptor or provides a peroxide scavenging system.

Attempts to stabilize cells by addition of catalase to cultures have been unsuccessful (Steinkraus, 1957; Costilow, Sylvester, and Pepper, *unpublished data*). However, exogenous catalase could not be expected to protect cells from peroxide produced intracellularly. The amount of hydrogen peroxide produced by intact cells would undoubtedly be small, since the specific activity of the particulate electron-transport system was much higher (5×) than the soluble system, even in extracts of log-phase cells. However, *B. popilliae* cells are very sensitive to low levels of peroxide added to cultures (Costilow, Sylvester, and Pepper, *unpublished data*), and, therefore, would be expected to be sensitive to much lower total levels produced intracellularly during respiration.

The failure of the soluble $NADH_2$ oxidase from log-phase cells to produce a significant amount of peroxide is not resolved. Such extracts were more sensitive to cyanide than those from cells harvested from older cultures, so there may have been a greater participation of an alternate system. The amount of peroxide production was never equal to the theoretical, even in extracts of the older cells, and difference spectra of soluble extracts indicated the presence of some cytochrome b_1. Thus, solubilized cytochromes may have been active to variable extents in the soluble fraction.

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