Hematin Enzymes of *Hemophilus parainfluenzae*

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A paste of washed *Hemophilus parainfluenzae* grown to the stationary phase with vigorous aeration appears tan to brown in color. Frozen cells are deep reddish brown and show absorption bands typical of cytochrome pigments when examined with a spectroscope. Studies with these bacteria show that the cytochromes are the major hematin enzymes present and that the respiratory metabolism has the properties of a cytochrome-linked system. There is very little catalase and no peroxidase activity. Under proper growth conditions, unusually large amounts of cytochrome in soluble form. The reduction of nitrate by *H. parainfluenzae* appears to involve reaction with the cytochrome system; this may explain why the organisms are facultative anaerobes.

EXPERIMENTAL PROCEDURE

**Growth of Bacteria**—The bacteria used in this study were a transformable strain of *H. parainfluenzae* ("Bossy No. 7") isolated by G. (1). They were grown in a medium containing 2% proteose-peptone, 0.5% yeast extract (Difco products), 0.6% NaCl, 0.1% KNO₃, 0.002% Na₂SO₄, buffered to pH 7.6 with 0.02 mM Tris. After sterilization, DPN (filtered through a Millipore filter) and sterile glucose were added to give final concentrations of 0.5 μg per ml and 1%, respectively. Two liters of medium were seeded with about 10⁷ cells from frozen stock cultures and vigorously aerated during growth. Excessive foaming was prevented by the addition of Dow-Corning Anti-foam A. Under these conditions, a yield of about 10 g of cells per liter of medium after 36 hours of growth at 37°C. The cells appear in chains of about 10 to 40 units after growth under these conditions.

**Assay of Catalase and Peroxidase**—Broken-cell extracts of the bacteria were prepared either by grinding with alumina, in the preparation of the respiratory particles (2), or by exposing suspensions of bacteria to sonic oscillation in the Raytheon 10 kc. instrument for 10 minutes at about 4°C. Catalase was assayed by the iodometric titration method described by Herbert (3). Peroxidase activity was measured both by testing for the oxidation of reduced cytochrome c as outlined by Chance (4) and by the guscinol reaction (5).

**Measurement of Absorption Spectra**—Absorption spectra of suspensions of bacteria at room temperature and cooled in liquid nitrogen were examined with a Hartree microspectrograph (6) (Beck and Company). Through the kindness of Dr. Britton Chance, difference spectra of intact bacteria were measured using the split-beam recording spectrophotometer (7) in the Johnson Research Foundation of the University of Pennsylvania. The methods for obtaining the difference spectra have been described by Chance (8). Similar difference spectra of suspensions of the respiratory particles (see following paper (2)), which are less turbid, were obtained in the Cary recording spectrophotometer, model 14. Dr. W. Bonner of the Johnson Foundation generously measured some difference spectra of the bacteria cooled in liquid nitrogen; the method has been described by Estabrook (9).

**Pyridine Hemochromogens**—These derivatives of the cytochrome pigments were prepared by adding KOH to a concentration of 0.03 M, then adding 1 volume of pyridine to 3 volumes of the alkaline mixture and finally a trace of solid Na₂S₂O₄. Some difference spectra of the bacteria were measured using 0.05 M phosphate buffer, pH 7.6, and small volumes of strong (1 M) solutions of substrates were added on the tip of a stirring rod. The inhibition of respiration by cyanide was tested with the usual Warburg manometry (11), with cyanide in the center well. Cyanide cannot be used with the oxygen electrode.

**Reagents**—DPN and DPNH were Sigma grade products. The 2-n-heptyl-4-hydroxyquinoline-N-oxide was a gift from Dr. J. W. Cornforth to Dr. Britton Chance. Sodium succinate was recrystallized twice from hot water by the addition of ethanol.

**Protein Determination**—The protein content of whole cells or extracts was measured by the biuret method (12) in the presence of 0.06% sodium deoxycholate.

RESULTS

**Catalase**—With the iodometric titration method using broken-cell extracts of *H. parainfluenzae* (1.32 mg of protein per ml of alumina-ground cell extract in the assay system or 25.8 mg of protein per ml of sonically disrupted cells) or in cells shaken with toluene (20.8 mg of protein per ml), Kat. f. (protein) values of 0.00 to 0.05 were obtained. Thus, in stationary phase cells, the catalase content is very low or absent.

**Peroxidase**—1. Chance (4) has shown that all peroxidases studied so far can oxidize mammalian cytochrome c. No oxidation of reduced cytochrome c was observed when H₂O₂ (final concentration = 300 μM or 1 mM) was added to the sonicate extract (6.45 mg of protein per ml) or to the alumina ground cell extract (0.67 mg of protein per ml) of *H. parainfluenzae* containing 18.5 μM reduced cytochrome c in 0.01 M acetate buffer (pH 4.7) or in 0.01 M phosphate buffer (pH 7.6).
2. There was no change in optical density at 490 nm when guaiacol (final concentration, 20 mM) was incubated with the alumina-ground cell-free extract (4.44 to 20.0 mg of protein per ml) and H$_2$O$_2$ (600 $\mu$M) for 5 minutes. The reagents were mixed in 0.05 M phosphate buffer (pH 7.6) or in 0.01 M acetate buffer (pH 4.7) at 25°.

Respiration—Fig. 1 is a tracing of an oxygen electrode recording of bacteria respiring in the presence of DPNH. With cells washed three times, the respiration with endogenous substrate is quite low. The rate of oxygen uptake with DPNH or with other substrates is constant as the oxygen concentration in solution decreases until a very low oxygen concentration is reached. This shows that the respiratory chain system has a high affinity for oxygen, which is typical of cells respiring via a cytochrome chain (15).

The bacteria can respire at a rapid rate; $Q_0$ values as high as 640 at 25° have been observed. The respiration is inhibited by cyanide, carbon monoxide, and by 2-n-heptyl-4-hydroxyquinoline-N-oxide, but it is not inhibited by azide, even in concentrations as high as 0.01 M. The details of the respiratory metabolism will be reported separately.

Cytochromes—Fig. 2 is a plot of the difference in absorption spectrum at room temperature, between anaerobic cells of $H$. parainfluenzae (DPNH as substrate) and aerobic cells (no substrate added). Absorption peaks of reduced cytochromes are apparent at 635, 600, 553, 523, and 423 $\mu$m, sometimes with a shoulder at about 440 $\mu$m on the large peak at 423 $\mu$m. There is a trough around 480 $\mu$m due to flavoprotein. Fig. 2 also shows the carbon monoxide difference spectrum of a similar suspension of cells; this plots the difference in absorption spectrum between anaerobic cells containing CO and anaerobic cells without CO. There are troughs at 635 and 440 $\mu$m and absorption peaks at about 645, 560, 532, and 416 $\mu$m, with shoulders around 600 and 430 $\mu$m. The anaerobic minus aerobic difference spectrum of the bacteria cooled in liquid nitrogen shows that the large absorption peak at 553 $\mu$m splits into three peaks at the low temperature (Fig. 3). Thus the difference spectra give evidence for an abundance of cytochromes: $a_1$, $a_2$, $o$, $b$ (or $b_1$), $c_1$, and possibly $c$ (9). It is assumed that the three absorption peaks in the region 545 to 555 $\mu$m in the low temperature difference spectrum are related to cytochromes $c$, $c_1$, and $b$. There is clear evidence for an absorption band at 560 $\mu$m, corresponding to a
cytochrome b, in difference spectra of respiratory particles containing the inhibitor 2-n-heptyl-4-hydroxyquinoline-N-oxide and an aerobic suspension containing neither substrate nor inhibitor. The spectra were measured in the Cary spectrophotometer.

The data obtained from the difference spectra do not allow precise calculations of the content of the cytochromes which have only small peaks in the absorption spectra. However, cytochromes a, a_s, b, and c seem to be present in roughly equivalent amounts, if it is assumed that they have the same extinction coefficients as other cytochromes with similar absorption spectra (17). The relative amount of cytochrome c is difficult to assess. But the total amount of cytochrome c is more than 10 times greater than that of the other cytochromes, and the enzymatically reducible cytochrome c is always in considerable excess over the other pigments.

As in a number of other bacteria (18), cytochrome c_1 only seems to appear in older cultures of \textit{H. parainfluenzae} (late log phase). This effect may be related to the oxygen tension during growth (19).

The difference spectra of Fig. 3 of the following paper (2) show that the insoluble respiratory particles have the same cytochrome system as the intact bacteria. Here the shoulders at 560 and 440 m\textmu are more obvious than in the spectra obtained with intact bacteria.

The cytochrome c_1 of both intact bacteria and respiratory particles is reduced to a greater extent when the bacteria become anaerobic in the presence of DPNH than in the presence of succinate. Also in both the addition of sodium dithionite results in still further reduction of the cytochrome c_1 (see Fig. 3 of following paper (2)).

Pyridine hemochromogen derivatives of the cytochromes of the respiratory particles show absorption peaks at 550, 520, and 414 m\textmu. The only derivative obtained in large amount is that derived from c-type cytochrome. A small amount of protohemin pyridine hemochromogen (a-absorption peak at 557 m\textmu) would be masked by the large absorption peak at 550 m\textmu. It is not known whether a-type pyridine hemochromogens would be formed under these conditions. Thus this experiment indicates a large excess of cytochromes with a c-type heme linkage.

![Fig. 4. Difference spectra of respiratory particles of \textit{Hemophilus parainfluenzae}. Solid line: difference in absorption spectrum between an anaerobic suspension containing DPNH and an aerobic suspension of particles. Dashed line: difference in absorption spectrum between aerobic particles containing DPNH and the inhibitor 2-n-heptyl-4-hydroxyquinoline-N-oxide and an aerobic suspension containing neither substrate nor inhibitor. The spectra were measured in the Cary spectrophotometer.](image)

![Fig. 5. Absorption spectra of crude oxidized cytochrome c_1 (dashed curve) and the cytochrome reduced with Na_2S_2O_4 (solid line). The dotted curve is the absorption spectrum of the pyridine hemochromogen. The spectra were recorded in the Cary spectrophotometer.](image)
The c-type cytochrome can be easily extracted from these bacteria in soluble form; this will be described in the following paper (2). Fig. 5 plots the absorption spectra of the unpurified cytochrome, along with the spectrum of the pyridine hemochromogen derived from it. This identifies this cytochrome as one with a e hemin (20). The absorption spectra of this cytochrome are identical with those of heart muscle cytochrome c1 except for a shift of 2 mp in the \(\gamma\)-band of the reduced compound.

**Nitrate Reductase**—The difference spectrum of Fig. 6 shows that the addition of 0.01 m KNO\(_3\) to anaerobic bacteria results in oxidation of the same cytochromes oxidized by oxygen, but with nitrate there is relatively greater oxidation of cytochrome \(a_1\) and flavoprotein, compared to the other cytochromes. The extent of oxidation of cytochrome \(c_1\) in the presence of 0.01 m nitrate is only 25% of that produced by low concentrations of oxygen. The concentration of nitrate must be kept rather low (0.01 m or less), since larger concentrations result in irreversible changes in the bacteria. The same results are obtained with the respiratory particles.

**DISCUSSION**

*H. parainfluenzae* shares with the obligately aerobic Azotobacter vinelandii and *Azotobacter chroococcum* the distinction of having the largest number of respiratory chain cytochromes yet observed in any cell. Like the *Azotobacter*, *H. parainfluenzae* can have a high rate of respiration.

The designation of the cytochromes as \(a, b,\) and \(c\) types is in some instances based only on the position of the bands in the absorption spectra. For example, when we refer to cytochromes \(c\) and \(c_1\), we mean only that two cytochromes are present with separate absorption bands (at low temperature) around 550 mp, and that they seem to have a c-type hemin linkage. The cytochromes of *H. parainfluenzae* seem to fit into the classification given, which is the only one possible at this stage of our knowledge (17).

Not only does *H. parainfluenzae* contain a goodly array of cytochrome types, but the bacteria can synthesize relatively large amounts of these pigments. Cytochrome \(c_1\) can be as much as 6% of the protein of the cells. The cytochrome system has a number of unusual aspects, such as the different extents of reduction of some cytochromes in the anaerobic state by different substrates. This will be discussed in detail in the following paper (2).

Other bacteria have been shown to form cytochrome \(a_2\) only after the logarithmic phase of growth is passed or at low oxygen concentration in solution (18, 19). The cytochrome \(a_2\) of *H. parainfluenzae* seems to behave similarly. Castor and Chance (18) have shown that cytochromes \(a_1, a_2,\) and \(o\) can all react as terminal oxidases and all three have high affinities for oxygen. There is some evidence that in *H. parainfluenzae* the rate of synthesis of cytochromes \(c_1\) and \(o\) is increased with longer periods of growth and in fact increases markedly during the stationary phase. This will be described in detail in a subsequent publication.

Our data indicate that nitrate is reduced by reaction with the cytochrome system of *H. parainfluenzae* and that cytochrome \(a_1\) seems to react more rapidly with nitrate than does cytochrome \(a_2\) (the oxidation of cytochrome \(o\) cannot be distinguished). Since the extent of oxidation of cytochrome \(c_1\) in the presence of nitrate is only about \(\frac{1}{2}\) of that with oxygen, the rate of electron transport with oxygen as the final acceptor must be more rapid. Actually, the generation time of the bacteria growing in air plus nitrate (30 minutes) is greater than that in nitrate under anaerobic conditions (75 minutes). Since the bacteria do not grow readily anaerobically in the absence of nitrate\(^1\) (no growth for 1 week), and anaerobic growth with nitrate is inhibited by cyanide and CO, it seems obvious that anaerobic growth does not involve energy production by fermentation reactions, but rather by oxidative reactions in which nitrate is the final acceptor of electrons in place of oxygen.

Sato, who has summarized some of the evidence for the participation of cytochromes in nitrate reduction in bacteria (21), found in *Micrococcus denitrificans* that an "a-type" cytochrome was oxidized to the same extent by oxygen and by nitrate, whereas the oxidation of the c-type cytochrome was less with nitrate. Since he found that cytochrome \(o\) was also present, the data could be explained by assuming that both cytochromes \(o\) and the "a-type" cytochrome react with oxygen, but only the a-type with nitrate.

Although Gills and Biberstein (22) reported catalase activity in several strains of bacteria like *H. parainfluenzae*, the cytochrome pigments seem to be the major hematin enzymes in stationary phase cells of the strain of bacteria used in our studies.

**SUMMARY**

The major hematin enzymes in stationary phase cells of *Hemophilus parainfluenzae* are cytochrome pigments; there is very little catalase and no peroxidase activity in these cells. There is evidence for six cytochrome pigments, and unusually large amounts of these pigments can be synthesized. A soluble cytochrome can be extracted from broken-cell preparations, which has absorption spectra nearly identical with those of mammalian cytochrome \(c_1\).

\(^1\) D. C. White, unpublished observations.
The bacteria appear to respire exclusively by means of a cytochrome-linked system.

The ct-type cytochrome is reduced to different extents when the bacteria are anaerobic in the presence of reduced diphosphopyridine nucleotide or of succinate, or when Na₂S₂O₄ is added to the anaerobic cells.

The cytochrome system of H. parainfluenzae is oxidized on addition of nitrate, and one of the cytochromes is oxidized to a relatively greater extent than the others.

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REFERENCES