THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 237, No. 4, April 1962
Printed in U.S.A

Hematin Enzymes of *Hemophilus parainfluenzae**

DAVID C. WHITE† AND LUCILE SMITH‡

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire

(Received for publication, July 18, 1961)

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 are synthesized, particularly one with an absorption spectrum similar to that of cytochrome c_1. Rupture of the stationary phase cells releases considerable quantities of this 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. Leidy (1). They were grown in a medium containing 2% proteose-peptone, 0.5% yeast extract (Difco products), 0.6% NaCl, 0.1% KNO_3, 0.002% $Na_2S_2O_4$, buffered to pH 7.6 with 0.02 M 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 10^7 cells from frozen stock cultures and vigorously aerated during growth. Excessive foaming was prevented by the addition of Dow-Corning Antifoam A. Under these conditions, a yield of about 10 g of cells can be obtained per liter of medium after 36 hours of growth at 37°. 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, as 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°. 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 guaiacol 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 microspectroscope (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 $Na_2S_2O_4$.

Measurement of Oxygen Uptake—Rates of oxygen uptake were measured with a rotating cup oxygen electrode (10). The solutions used were maintained at 25° before addition to the rotating cup, and the measurements were completed in a few minutes. The bacteria were suspended in 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 H_2O_2 (final concentration = 300 μM or 1 mM) was added to the sonic 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).

* This research was supported by a grant from the United States Public Health Service.

† Postdoctoral Graduate Fellow, Rockefeller Institute, New York, New York.

‡ Senior Fellow, United States Public Health Service.

2. There was no change in optical density at 490 mμ 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_2O_2 (600 μM) 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 (13).

The bacteria can respire at a rapid rate; Q_{O_2} 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 mμ, sometimes with a shoulder at about 440 mμ on the large peak at 423 mμ. There is a trough around 460 mμ 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 mμ and absorption peaks at about 645, 565, 532, and 416 mμ, with shoulders around 600 and 430 mμ. The anaerobic minus aerobic difference spectrum of the bacteria cooled in liquid nitrogen shows that the large absorption peak at 553 mμ 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 565 mμ 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 mμ, corresponding to a

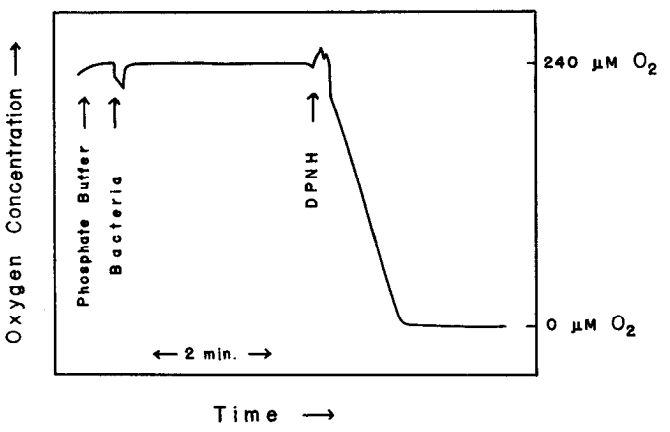

FIG. 1. Tracing of recording from oxygen electrode. The rotating cup contained bacteria washed 3 times suspended in 2 ml of 0.05 M phosphate buffer, pH 7.6. Irregularities in the trace resulted from stirring in bacteria or reagents. The final concentration of DPNH was 0.002 M.

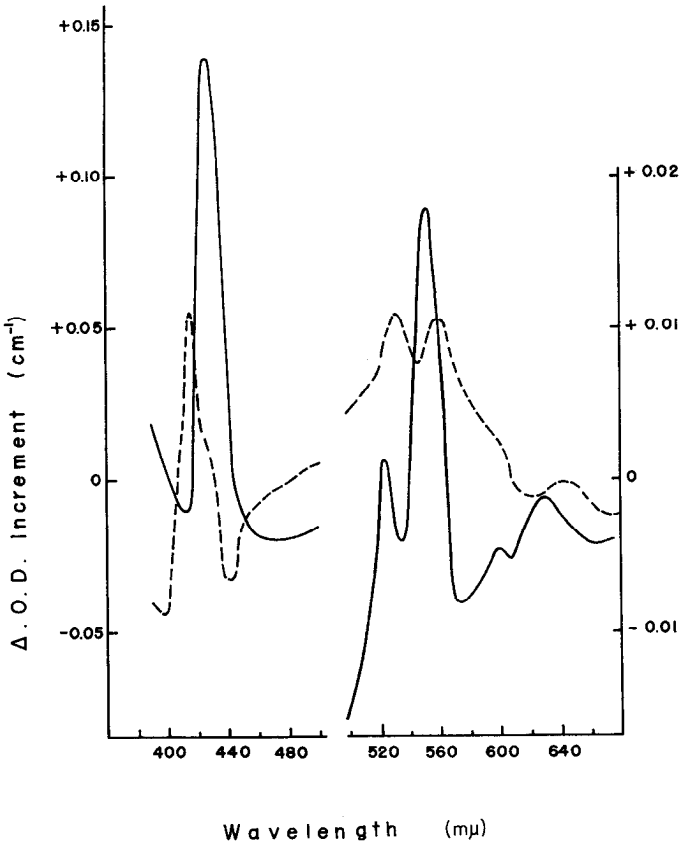

FIG. 2. Difference spectra of intact cells of *Hemophilus parainfluenzae*. *Solid line:* difference in optical density between anaerobic cells (DPNH added) and aerobic cells (no substrate added). *Dashed line:* difference in absorption spectrum between anaerobic cells in the presence and absence of carbon monoxide. The suspension of bacteria contained 7.41 mg of protein per ml. The spectra were measured in the split-beam recording spectrophotometer in the Johnson Research Foundation.

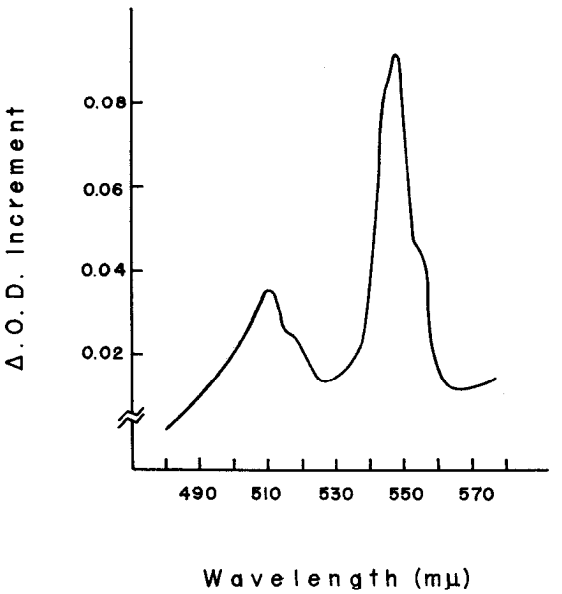

FIG. 3. Low temperature difference spectrum of *Hemophilus parainfluenzae*. The spectrum represents the difference in optical density between anaerobic and aerobic bacteria after cooling in liquid nitrogen. The difference spectrum was obtained by Dr. Walter Bonner in the Johnson Research Foundation. The bacterial suspension was the same as in Fig. 1, but here the light path was 2 mm.

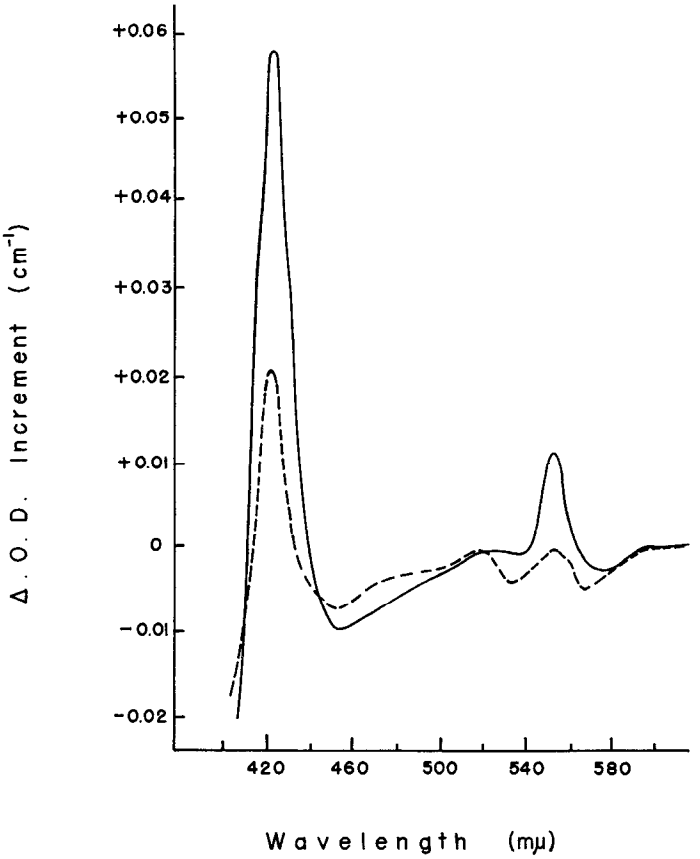

FIG. 4. Difference spectra of respiratory particles of *Hemophilus parainfluenzae*. *Solid line:* difference in absorption spectrum between an anaerobic suspension containing DPNH and an aerobic suspension of particles. *Dashed line:* difference in absorption spectrum between aerobic particles containing 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.

cytochrome *b*, in difference spectra of respiratory particles containing the inhibitor 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, which has been shown to prevent the oxidation of cytochrome *b* in other bacteria (14) (Fig. 4). There is also further evidence for cytochrome *b* on respiratory particles from which some of the c_1-type cytochrome has been removed (see following paper (2)). The only evidence for a *c*-type cytochrome in addition to cytochrome c_1 is the third absorption peak seen in the region around 550 mμ in the low temperature absorption spectrum. The α-absorption band of the soluble cytochrome c_1 isolated from these bacteria did not seem to split when a solution was examined with the visual spectroscope after freezing in liquid nitrogen.

The reduced cytochrome c_1 in the bacteria used to obtain the spectra of Fig. 2 is 1.34% of the bacterial protein, if it is assumed that the molecular weight and the extinction coefficients of the cytochrome c_1 are the same as those of mammalian cytochrome c_1 reported by Sekuzu, Orii, and Okunuki (15). This represents only about ¼ of the cytochrome c_1 present in this culture (2). Thus the total content of cytochrome c_1 can be as high as 5 to 6% of the cell protein. Also, the ratio of the optical density difference at the Soret peak in the anaerobic minus aerobic difference spectrum divided by the dry weight (measured as turbidity) is large compared to the same ratio in bacteria such as *Escherichia coli* or to yeast and is around ¼ to ⅕ the value obtained with heart muscle respiratory particles (16).

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_1, a_2, *o*, and *b* 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_1 is more than 10 times greater than that of the other cytochromes, and the enzymatically reducible cytochrome c_1 is always in considerable excess over the other pigments.

As in a number of other bacteria (18), cytochrome a_2 only seems to appear in older cultures of *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 mμ are often 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 mμ. The only derivative obtained in large amount is that derived from *c*-type cytochrome. A small amount of protohemin pyridine hemochromogen (α-absorption peak at 557 mμ) would be masked by the large absorption peak at 550 mμ. 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.

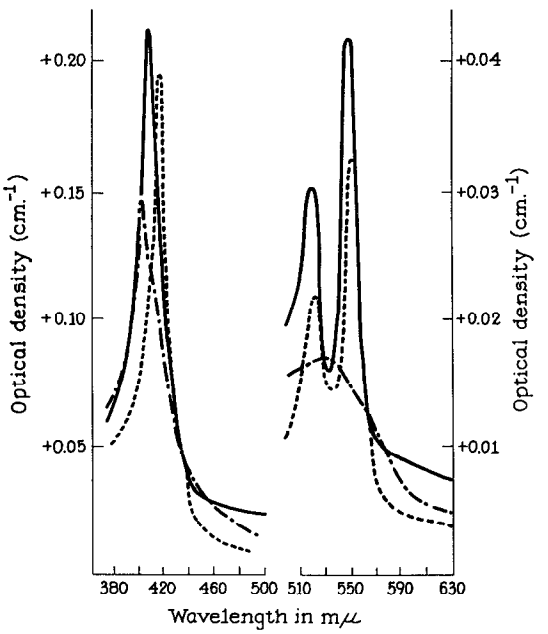

FIG. 5. Absorption spectra of crude oxidized cytochrome c_1 (*dashed curve*) and the cytochrome reduced with $Na_2S_2O_4$ (*solid line*). The *dotted curve* is the absorption spectrum of the pyridine hemochromogen. The spectra were recorded in the Cary spectrophotometer.

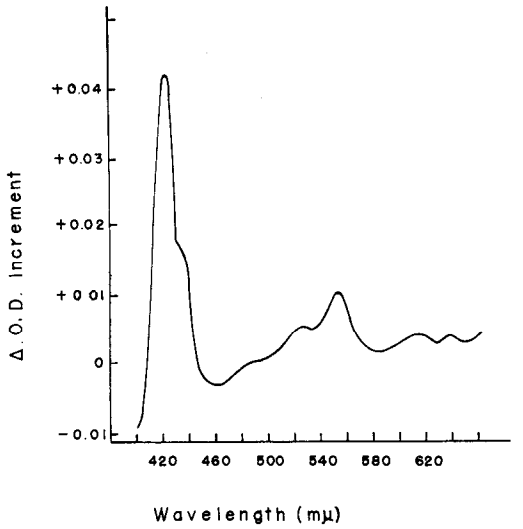

FIG. 6. Difference in absorption spectrum between anaerobic bacteria (DPNH added) and a similar suspension containing 0.01 M KNO_3. The difference spectrum was obtained in the split-beam recording spectrophotometer in the Johnson Research Foundation with the same bacterial suspension used to obtain the data of Fig. 1. Stirring in the same volume of buffer as that of nitrate added did not introduce enough oxygen to give so large a change in absorption spectrum, and the suspension rapidly became anaerobic again.

The c_1-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 *c*-hemin (20). The absorption spectra of this cytochrome are identical with those of heart muscle cytochrome c_1 except for a shift of 2 mμ in the γ-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 mμ, 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 $\frac{1}{4}$ 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 c_1-type cytochrome is reduced to different extents when the bacteria are anaerobic in the presence of reduced diphosphopyridine nucleotide or of succinate, or when $Na_2S_2O_4$ 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.

Acknowledgments—The authors appreciate the hospitality of Dr. Britton Chance and the Johnson Foundation for obtaining some of the difference spectra. Dr. Walter Bonner kindly ran the low temperature difference spectra.

We also wish to express our appreciation for the invaluable technical assistance of Miss Marjorie Krause.

REFERENCES

1. Leidy, G., Hahn, E., and Alexander, H. E., *J. Exptl. Med.*, **104,** 305 (1956).
2. Smith, L., and White, D. C., *J. Biol. Chem.*, **237,** 1337 (1962).
3. Herbert, D., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. II*, Academic Press, Inc., New York, 1955, p. 784.
4. Chance, B., in J. T. Edsall (Editor) *Enzymes and enzyme systems*, Harvard University Press, Cambridge, Mass., 1951, p. 93.
5. Maehly, A. C., and Chance, B., in D. Glick (Editor), *Methods of biochemical analysis, Vol. I*, Interscience Publishers, Inc., New York, 1954, p. 357.
6. Hartree, E. F., in *Modern methods of plant research*, Springer-Verlag, Berlin, 1955, p. 197.
7. Yang, C. C., and Legallais, V., *Rev. Sci. Instr.*, **25,** 801 (1954).
8. Chance, B., *Science*, **120,** 767 (1954).
9. Estabrook, R. W., *J. Biol. Chem.*, **223,** 781 (1956).
10. Davies, P. W., and Brink, F., *Rev. Sci. Instr.*, **13,** 524 (1942).
11. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric techniques*, Burgess Publishing Co., Minneapolis, 1957, p. 73.
12. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, **177,** 751 (1949).
13. Chance, B., *Federation Proc.*, **16,** 671 (1957).
14. Lightbown, J. W., and Jackson, F. W., *Biochem. J.*, **63,** 130 (1956).
15. Sekuzu, I., Orii, Y., and Okunuki, K., *J. Biochem. (Tokyo)*, **48,** 214 (1960).
16. Smith, L., *Arch. Biochem. Biophys.*, **50,** 299 (1954).
17. Smith, L., in I. C. Gunsalus and R. Y. Stanier (Editors) *The bacteria, Vol. II*, Academic Press, Inc., New York, 1961, p. 215.
18. Castor, L. N., and Chance, B., *J. Biol. Chem.*, **234,** 1587 (1959).
19. Moss, F., *Australian J. Exptl. Biol. Med. Sci.*, **34,** 395 (1956).
20. Theorell, H., and Akesson, A., *J. Am. Chem. Soc.*, **63,** 1804 (1941).
21. Sato, R., in W. D. McElroy and B. Glass (Editors) *Inorganic nitrogen metabolism*, Johns Hopkins Press, Baltimore, 1956, p. 163.
22. Gills, M., and Biberstein, E. J., *J. Bacterio l.*, **81,** 380 (1961)