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

# Structure of the Respiratory Chain System as Indicated by Studies with *Hemophilus parainfluenzae**

LUCILE SMITH† AND DAVID C. WHITE‡

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

(Received for publication, July 18, 1961)

Studies on the respiration and the cytochrome system of *Hemophilus parainfluenzae* have been described in the preceding paper (1). Some of the unusual properties of the respiratory chain system of these organisms have afforded a new experimental approach to the nature of this multienzyme system.

*H. parainfluenzae* harvested in the stationary growth phase contains a number of different cytochromes, including a relatively large quantity of a $c_1$-type cytochrome. The extent of reduction of the latter, when the bacteria become anaerobic, is different in the presence of succinate from what it is in the presence of reduced diphosphopyridine nucleotide. A considerable proportion of this cytochrome is not reduced anaerobically in the presence of either substrate. When the cells are broken, the cytochrome $c_1$ which is not reduced enzymatically is found in solution, and further amounts of the particle-bound cytochrome can be extracted by washing with buffer. The respiratory rate of the particles does not decrease until a large amount of the cytochrome $c_1$ has been removed. The cytochrome in solution does not interact with the particulate oxidases and reductases. From this type of observation, inferences can be drawn concerning the structure of the electron transport system.

## EXPERIMENTAL PROCEDURE

*Growth of Bacteria and Preparation of Respiratory Particles*—The methods for culturing the bacteria are described in the preceding paper (1). Respiratory particles are prepared by grinding a paste of washed cells with 3 to 4 times its weight of Alcoa alumina A-301 in a cold mortar, then extracting with about 4 volumes of 0.5 M sucrose containing 0.05 M phosphate buffer, pH 7.6. The alumina is removed by centrifugation at 2,000 × *g* for 15 minutes. Then centrifugation at 6,000 × *g* for 15 minutes removes most of the unbroken cells, and a further centrifugation at 8,000 × *g* carries down the remaining whole cells plus some large particles, as shown by examination with the phase contrast microscope. Most of the insoluble particles containing the respiratory chain enzymes, which are suspended in the supernatant fluid, can be collected by centrifugation at 35,000 × *g* for 30 minutes. All of these particles can be centrifuged down by exposure to 140,000 × *g* for 30 minutes. The insoluble particles can thus be washed with buffer by centrifugation and resuspension in buffer with the aid of a Teflon homogenizer.

*Respiration Measurements*—Respiration of the bacteria or respiratory particles with added DPNH or other substrates was measured with the platinum oxygen electrode, as described in the preceding paper (1). Oxygen uptake of the particles with silicomolybdate as the electron donor was followed similarly, using the reagent described by Jacobs (2) in the presence of 1 mM ascorbate, pH 7.0.

*Difference Spectra*—The difference in absorption spectrum between anaerobic and aerobic suspensions of intact bacteria was measured either in the split-beam recording spectrophotometer in the Johnson Foundation of the University of Pennsylvania, through the courtesy of Dr. Britton Chance, or in the Cary model 14 recording spectrophotometer. When difference spectra of intact bacteria were measured in the Cary spectrophotometer, the bacteria were suspended in 50% glycerol by volume. Difference spectra of broken-cell suspensions or of washed particles in buffer were also measured in the Cary spectrophotometer. Details of the procedures are given in the preceding paper (1). The plotting error in the Cary recordings amounted to 0.0005 in optical density, using the 0 to 0.1 slide wire; the absorption spectra have been traced with a line through the middle of the tracing error.

*Reduction of Ferricyanide*—The rate of reduction of ferricyanide was assayed by measuring the change in optical density at 429 mμ at 25° with a reaction mixture containing: 0.05 M phosphate buffer (pH 7.6), 0.5 mM potassium ferricyanide (freshly prepared), 5 mM potassium cyanide, 0.02 M sodium succinate or other acids tested as substrates or 5 mM DPNH, and bacterial particles containing 0.5 to 1 mg of protein in a final volume of 3 ml.

*Protein Determination*—The protein content of the bacterial or particle suspensions was measured by the Gornall biuret method (3) in the presence of 0.06% sodium deoxycholate.

*Reagents*—All chemicals were reagent grade commercial products, used as obtained, except for sodium succinate, which was recrystallized twice from hot water by the addition of alcohol.

## RESULTS

*Difference Spectra of Intact Cells and Cell-free Extracts*—The *dotted line* of Fig. 1 plots the difference in absorption spectrum between an aerobic suspension of washed *H. parainfluenzae* and some of the same suspension which has exhausted the oxygen in solution on oxidizing succinate (the depletion of oxygen

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

† Senior Fellow, United States Public Health Service.

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

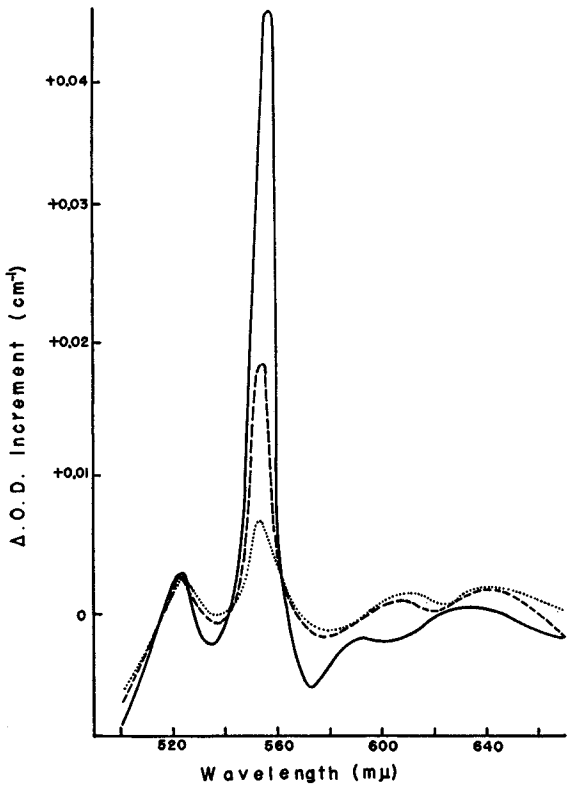


FIG. 1. Difference spectra of intact cells. The suspension of washed bacteria in buffer contained 7.4 mg of protein per ml. In each case the reference cuvette contained aerobic bacteria with no added substrate. For the *dotted curve*, succinate was added to the other cuvette and the suspension allowed to become anaerobic. Then DPNH was added and the *dashed curve* recorded after 9 minutes, when no further change in optical density took place. After the addition of a few grains of $Na_2S_2O_4$, the solid curve was traced. The difference spectra were measured in the split-beam recording spectrophotometer in the Johnson Research Foundation.

being measured by the oxygen electrode). As explained in the preceding paper, $\alpha$-absorption peaks corresponding to cytochrome $a_2$, $a_1$, and $c_1$ are readily distinguished at 630, 600, and 553 m$\mu$, respectively. When DPNH is then added to the anaerobic cells containing succinate, the peak in the absorption spectrum at 553 m$\mu$ increases until it reaches the value seen in the *dashed curve*. Then addition of $Na_2S_2O_4$ immediately results in further reduction of the cytochrome $c_1$ (*solid curve*). The extent of reduction of cytochromes $a_1$ and $a_2$ is essentially the same in all three conditions. Fig. 2 plots similar difference spectra of a culture having higher levels of cytochromes $a_1$ and $a_2$ and shows more clearly that cytochromes $a_1$ and $a_2$ are completely reduced in anaerobic cells containing DPNH. The amount of cytochrome $c_1$ reduced anaerobically with DPNH is 5 to 10 times greater than the amount of cytochromes $a_1$, $a_2$, $b$, or $o$. The excess of cytochrome $c_1$ which can be reduced only with $Na_2S_2O_4$ is variable in different cultures and particularly seems to vary with the age of the culture.

The same extent of reduction of cytochrome $c_1$ is observed in anaerobic cells containing DPNH whether succinate is present or not; that is, the extent of reduction of the cytochrome $c_1$ by succinate and DPNH is not additive. The addition of 1 mM cyanide does not increase the extent of reduction of cytochrome $c_1$ by succinate or DPNH.

The wave lengths of the absorption peaks in the anaerobic minus aerobic difference spectra of broken-cell suspensions or washed particles of *H. parainfluenzae* are the same as those obtained with intact bacteria. And in the cell-free extracts the increase in the reduction of the cytochrome $c_1$ on addition of DPNH to cells anaerobic in the presence of succinate takes place immediately. Fig. 3 plots the difference spectra of particles washed once, from which 53% of the cytochrome $c_1$ of the cell-free extract has been removed (see below). The anaerobic minus aerobic difference spectrum shows evidence for an absorption peak corresponding to a *b*-type cytochrome (absorption peak at 560 m$\mu$). This, plus the absorption spectra of bacteria inhibited with 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (1) form the evidence for the participation of a *b*-type cytochrome in the respiratory chain of these bacteria. In the presence of the large quantity of cytochrome $c_1$ usually present, the absorption peak of cytochrome *b* is masked. The carbon monoxide difference spectrum of the particles shows absorption peaks corresponding to cytochromes *o* and $a_1$. The respiratory particles can only respire in the presence of succinate or DPNH, and the rate of respiration is around 6- to 20-fold more rapid with DPNH than with succinate. The characteristics of the respiratory particles will be described in a separate publication.

*Separation of Soluble Cytochrome $c_1$ from Cell-free Extracts and Removal of Cytochrome from Respiratory Particles*—When a freshly prepared broken-cell extract is centrifuged for 45 minutes at 140,000 $\times$ *g*, some of the cytochrome $c_1$ remains in the

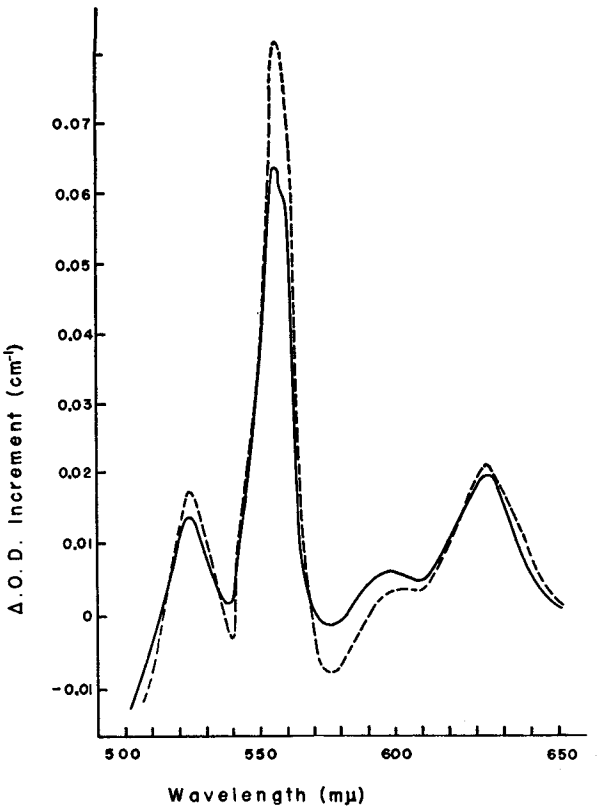


FIG. 2. Same as in Fig. 1; the *solid curve* was obtained with anaerobic cells in the presence of DPNH, then the *dashed curve* was recorded after addition of a small quantity of solid $Na_2S_2O_4$. The absorption spectra were measured in the Cary model 14 spectrophotometer with a strong suspension of bacteria (14.7 mg of protein per ml) in 50% (volume per volume) glycerol.

supernatant fluid. The remainder of the cytochrome $c_1$ and all of the other cytochromes are centrifuged down with the insoluble pellet. The data of Table I show that the cytochrome $c_1$ in the supernatant fluid is exactly equal to the amount of this cytochrome in the whole extract which cannot be reduced enzymatically in the presence of DPNH, but can only be reduced by $Na_2S_2O_4$. In other words, only the particle-bound cytochrome can be reduced enzymatically.

Additional cytochrome $c_1$ is progressively removed from the particles by washing with buffer by centrifugation and suspension; in each case only the cytochrome remaining on the particles can be reduced enzymatically (Table I).

The supernatant fluid from centrifugation of the broken-cell extract and the washings of the particles show absorption spectra typical of cytochrome $c_1$; these plus the absorption spectrum of the pyridine hemochromogen are shown in the preceding paper (1), Fig. 5. The cytochrome $c_1$ can amount to 5 to 6% of the protein of the bacteria, assuming that the molecular weight and extinction coefficients of the cytochrome are similar to those reported for mammalian cytochrome $c_1$.

After considerable cytochrome $c_1$ is removed from the particles by washing, the respiration rate begins to decrease, reaching a low value after 3 to 5 washings (Table II), but some ability to respire remains. In one experiment, it was found that freezing and thawing of the washed particles resulted in a further release of cytochrome $c_1$ and a complete loss of the ability to oxidize succinate or DPNH with oxygen.

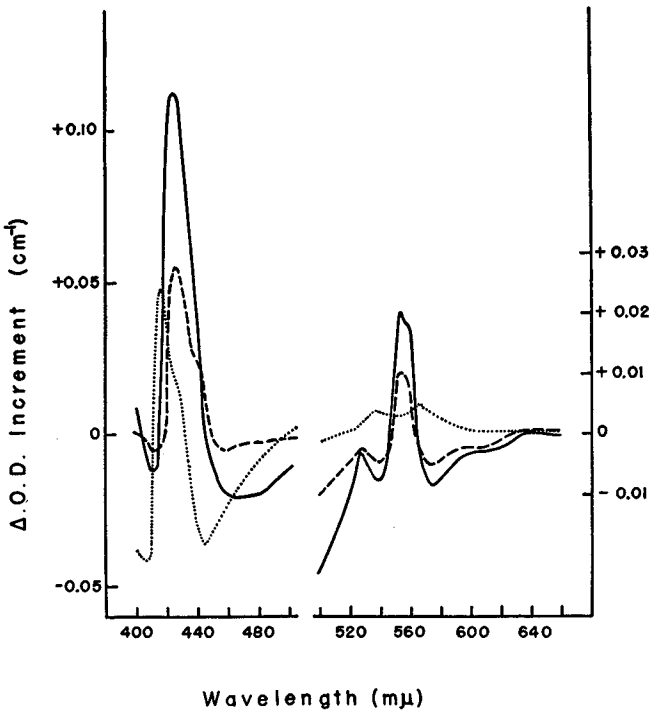


FIG. 3. Difference spectra of respiratory particles. *Dashed line:* difference in absorption spectrum between anaerobic particles containing succinate and aerobic particles (no substrate added). *Solid line:* difference in absorption spectrum between anaerobic particles in the presence of DPNH and aerobic particles. *Dotted line:* difference in absorption spectrum between anaerobic particles (plus DPNH) in the presence and absence of carbon monoxide. The perparation was a once-washed particle suspension containing 7.0 mg of protein per ml. The absorption spectra were recorded in the Cary spectrophotometer.

TABLE I

*Removal of cytochrome $c_1$ from respiratory particles by washing*

The broken-cell extract containing 4.3 mg of protein per ml was centrifuged at 140,000 × *g* for 30 minutes, and the pellet resuspended in 0.05 M phosphate buffer, pH 7.6. Subsequent washings were carried out similarly. The cytochrome $c_1$ in the suspensions was assayed by measuring the increase in optical density on addition of DPNH, then the further increase on addition of $Na_2S_2O_4$. The cytochrome $c_1$ in the supernatant fluids was measured after addition of $Na_2S_2O_4$. $\Delta OD_{553\ m\mu}$ refers to the difference in optical density at this wave length between the oxidized and the reduced forms of the cytochrome.

| Preparation | Cytochrome $c_1$ ($\Delta OD_{553\ m\mu}$) | | |
|---|---|---|---|
| | Reduced by DPNH | Reduced by $Na_2S_2O_4$ | In supernatant after high speed centrifugation |
| Centrifuged cell-free extract | 0.028 | 0.077 | 0.081 |
| Once washed particles | 0.0127 | 0.0112 | 0.0097 |
| Twice washed particles | 0.0041 | 0.0024 | 0.0022 |

TABLE II

*Removal of cytochrome $c_1$ from respiratory particles*

The broken-cell extract (8.2 mg of protein per ml) was centrifuged at 30,000 × *g* for 30 minutes; then the pellet was resuspended in 0.05 M phosphate buffer, pH 7.6, to the volume of the original suspension. Subsequent washings were carried out in the same manner. The cytochrome $c_1$ removed by the washings was assayed by measuring the increase in optical density in the supernatant fluids on addition of $Na_2S_2O_4$.

| Preparation | Cytochrome $c_1$ removed, $\Delta OD_{553\ m\mu}$ | Respiration, $\mu M\ O_2\ sec^{-1}$ | |
|---|---|---|---|
| | | + Succinate | + DPNH |
| Broken-cell extract | | 0.17 | 0.73–1.20 |
| Particles washed once | 0.087 | 0.21 | 1.90 |
| Particles washed 2 times | 0.014 | 0.19 | 1.19 |
| Particles washed 3 times | 0.004 | 0.067 | 0.19 |
| Particles washed 4 times | 0 | 0.065 | 0.20 |

The composition of the medium in which the particles are suspended seems to make no difference in the amount of cytochrome $c_1$ which can be washed off; phosphate buffer from 0.05 to 0.5 M and various sucrose concentrations were tested.

Fig. 3 shows the difference spectra of particles washed once. After the particles are washed about 3 times, the anaerobic minus aerobic difference spectra show an additional absorption peak at 390 mμ; the nature of the compound responsible for this absorption peak is not known.

*Addition of Soluble Cytochrome $c_1$ to Washed Particles*—The low rate of respiration of the severalfold washed particles is not increased by the addition of the supernatant fluid obtained on centrifugation of the broken-cell extract. The soluble cytochrome $c_1$ which was concentrated and slightly purified by precipitation with neutralized ammonium sulfate, then dialysis against cold water, also had no effect on the respiration of the washed particles. The soluble cytochrome $c_1$ was not reduced by the particles in the presence of DPNH and cyanide, although ferricyanide was rapidly reduced (see following section).

*Reduction of Ferricyanide and Oxidation of Silicomolybdate by*

TABLE III

*Ferricyanide reduction by repeatedly washed respiratory particles*

The washed particles were prepared as described in Table I. For each assay, 0.2 ml of particles was added to make a total volume of 3 ml.

| Preparation | Ferricyanide reduction with DPNH first order rate constant $sec^{-1}$ |
|---|---|
| Particles washed once | 0.0063 |
| Particles washed 2 times | 0.0094 |
| Particles washed 3 times | 0.0107 |
| Particles washed 4 times | 0.0125 |

*Respiratory Particles*—The respiratory particles show the ability to reduce ferricyanide in the presence of succinate or DPNH, but not with acetate, citrate, malate, or glucose. The reaction was found to be first order with respect to the concentration of ferricyanide; thus the relative rates are expressed as the first order rate constants. With a number of different preparations the rate constant with DPNH was between 10 and 18 times greater than that with succinate. Table III summarizes the observations of ferricyanide reductase activity of a suspension of progressively washed particles. This activity does not decrease on washing, and in fact increases slightly. The ability to oxidize silicomolybdate also remains associated with the particles after several washings. In the mammalian respiratory chain system, silicomolybdate reacts with the cytochrome oxidase part of the chain, and cytochrome *c* is required for the reaction (4). The data show that both the oxidase and reductase ends of the respiratory chain system of *H. parainfluenzae* remain attached to the particles during repeated washings.

## DISCUSSION

Some of the unusual properties of the respiratory chain system of *H. parainfluenzae* give indications about the structure of this complicated multienzyme system, which is attached in some way to a lipoprotein membrane within the cell:

1. There can be a large excess of one cytochrome ($c_1$-type) over the others, yet all of the cytochrome $c_1$ that is particle bound can become oxidized and reduced during electron transport. Once the cytochrome is removed from the particles into solution, it can no longer interact with the particulate oxidases and reductases; this appears to be true both with intact bacteria and with isolated respiratory particles. Thus the loss of ability to interact does not result from the extraction of an additional essential factor from the particles.

2. A considerable proportion of the cytochrome $c_1$ can be removed from the respiratory particles before a decrease in respiration rate is observed.

3. More of the cytochrome $c_1$ is reduced in the absence of air when the system is oxidizing DPNH than when succinate is the substrate oxidized, but cytochromes $a_1$ and $a_2$ are completely reduced under both conditions. The reduction of the cytochrome $c_1$ with DPNH is the same as it is in the presence of both DPNH and succinate. The rate of reduction of ferricyanide by the respiratory particles plus DPNH can be as much as 20 times greater than the rate of reduction of succinate, although the extent of reduction of the cytochrome $c_1$ by DPNH in the absence of air is usually only twice that with succinate.

4. Although a large proportion of the cytochrome $c_1$ can be removed from the respiratory particles by washing, the other cytochromes remain firmly attached, as do the oxidase and the DPNH ferricyanide reductase activities.

The observations summarized render untenable the concept of assemblies of respiratory pigments consisting of 1 molecule of each type. The data can only be explained in terms of a 3-dimensional array in which electrons can be transferred through any number of pigments attached to the structure, so that loss of some of the pigments does not result in a decrease in the over-all rate of electron transport. The data also require that the DPNH reductase system have more points of attachment to the respiratory chain system than the succinate reductase system. These reductases cannot be attached to separate pathways, since the extents to which they reduce cytochrome $c_1$ are not additive.

Fig. 4 is a schematic representation of the ideas expressed above. The usual sequence of electron transport from *b*-type to *c*-type cytochromes to the oxidases is assumed by analogy with other systems studied (5–7). It is possible that the sequence is maintained by the spatial arrangement of the pigments. There is some evidence that the same sequence is not always maintained when the insoluble respiratory chain particles are broken down into derivative pieces (8, 9).

In the mammalian respiratory chain system, cytochrome *c* is attached to the structure in a manner that allows its removal from intact mitochondria or from muscle mince, but not from the isolated respiratory particles, by treatment with relatively high concentrations of salts (10, 11). Cytochrome $c_1$ can be separated from the mammalian structure only by more drastic treatment, such as mixtures of detergents and salts, with or without organic solvents or digestive enzymes (12–15). A $c_1$-type cytochrome can be removed from *Azotobacter vinelandii* by shaking with butanol (16). The attachment of the cytochrome $c_1$ of *H. parainfluenzae* to the insoluble structure containing the respiratory chain appears to be different from any of these, since it is removed from the structure simply by suspending the particles in aqueous solutions and the ionic composition of the medium appears to have little effect on this. There seems to be a kind of equilibrium between the particle-bound cytochrome $c_1$ and that in solution, since a certain amount will come off of the particles when they are suspended in buffer, then more is removed by centrifuging them down and resuspending in fresh buffer. The cytochrome goes from the particles into solution even at relatively high concentrations of cytochrome $c_1$ in solution. In most other bacterial systems that have been investigated, all of the cytochromes are resistant to removal from the insoluble respiratory particles; the cytochromes of *H. parainfluenzae* other than the cytochrome $c_1$ remain firmly bound.

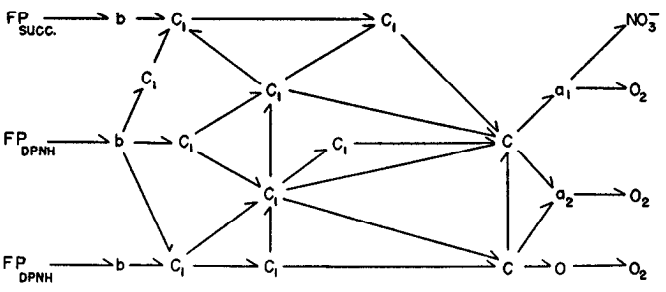


FIG. 4. Schematic representation of the structural arrangement of the cytochrome pigments in the respiratory chain system. The letters *c*, $c_1$, *b*, $a_1$, $a_2$, and *o* refer to cytochromes, *FP* to flavoprotein.

The soluble cytochrome $c_1$ of *H. parainfluenzae* does not interact rapidly with the oxidases or reductases of the respiratory particles. It seems highly unlikely that in this case the lack of reaction could be a result of a "denaturation" of the cytochrome $c_1$. This could mean that the oxidation-reduction reactions require some spatial arrangement of the pigments within the lipoprotein membrane.

Widely varying observations have been made of the reactions of isolated $c_1$-type cytochromes from mammalian sources or from other bacteria with particulate oxidases or reductases. One kind of mammalian preparation was neither oxidized nor reduced by enzymes on heart muscle respiratory particles (14), whereas another preparation was reported to be enzymatically oxidized and reduced by a heart muscle fraction, but it is not clear how rapid these reactions were (13). Both a *c*-type and a $c_1$-type cytochrome isolated from *A. vinelandii* were oxidized and reduced by particulate enzymes from these bacteria, but not by those of other bacteria or of mammalian heart muscle (16).

The observations on the cytochrome system of *H. parainfluenzae* have some implications concerning cytochrome synthesis. It appears that the synthesis of one of the cytochromes continues until there is a large excess of this pigment, then finally continues after the bacteria can incorporate it into the membrane containing the electron transport system. The data suggest that the cytochrome is synthesized in soluble form, then incorporated into the membrane. Further studies are in progress on this subject.

## SUMMARY

The $c_1$-type cytochrome of stationary phase cells of *Hemophilus parainfluenzae* is only partly reduced when the bacteria are anaerobic in the presence of succinate. Further reduction of cytochrome $c_1$ is observed on addition of reduced diphosphopyridine nucleotide to the anaerobic suspension, and still further reduction follows addition of $Na_2S_2O_4$. Cytochromes $a_1$ and $a_2$ are completely reduced in all three conditions.

When the cells are broken, cytochrome $c_1$ is released into solution in amount equal to the portion in the bacteria which cannot be reduced enzymatically. The remainder of the cytochrome $c_1$ and the other cytochromes remain bound to insoluble particles. More of the cytochrome $c_1$ can be removed from the particles into solution by washing with buffer. The respiration rate of the particles does not change until considerable cytochrome $c_1$ has been removed by washing.

The cytochrome $c_1$ in solution is neither oxidized nor reduced by the oxidases or reductases of the respiratory particles. It does not increase the respiration rate of particles which have been depleted of cytochrome $c_1$ by repeated washing.

Washing does not remove the ability of the particles to reduce ferricyanide in the presence of reduced diphosphopyridine nucleotide or succinate or to oxidize silicomolybdate.

These observations lead to hypotheses about the structure of the insoluble respiratory chain system.

## REFERENCES

1. White, D. C., and Smith, L., *J. Biol. Chem.*, **237,** 1332 (1962).
2. Jacobs, E. E., *Biochim. et Biophys. Acta*, **22,** 583 (1956).
3. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, **177,** 751 (1949).
4. Jacobs, E. E., and Sanadi, D. R., *Biochim. et Biophys. Acta*, **38,** 12 (1960).
5. Keilin, D., and Slater, E. C., *Brit. Med. Bull.*, **9,** 89 (1953).
6. Chance, B., and Williams, G. R., in F. F. Nord (Editor), *Advances in enzymology vol. 17*, Interscience Publishers, Inc., New York, 1956, p. 65.
7. Smith, L., *The bacteria, Vol. II, Ch. 7*, Academic Press, Inc., New York, 1961.
8. Chance, B., *Nature*, **169,** 215 (1952).
9. Estabrook, R. W., *J. Biol. Chem.*, **227,** 1093 (1957).
10. Tsou, C. L., *Biochem. J.*, **50,** 493 (1952).
11. Jacobs, E. E., and Sanadi, D. R., *J. Biol. Chem.*, **235,** 531 (1960).
12. Keilin, D., and Hartree, E. F., *Nature*, **176,** 200 (1955).
13. Sekuzu, I., Orii, Y., and Okunuki, K., *J. Biochem. (Tokyo)*, **48,** 214 (1960).
14. Green, D. E., Jarnefelt, J., and Tisdale, H. D., *Biochim. et Biophys. Acta*, **31,** 34 (1959).
15. Bernstein, E. H., and Wainio, W. W., *Arch. Biochem. Biophys.*, **91,** 138 (1960).
16. Tissières, A., and Burris, R. H., *Biochim. et Biophys. Acta*, **20,** 436 (1956).