Biochimica et Biophysica Acta, 449 (1976) 37–47

BBA 47175

OXIDATION AND REDUCTION OF MEMBRANE-BOUND CYTOCHROME *c* IN *HEMOPHILUS PARAINFLUENZAE*

REACTION WITH OXYGEN, HYDROGEN PEROXIDE AND NITRATE

PETER R. SINCLAIR*, DAVID C. WHITE** and LUCILE SMITH

Department of Biochemistry, University of Kentucky, Lexington, Ky. 40506 and Department of Biochemistry, Dartmouth Medical School, Hanover, N.H. 03755 (U.S.A.)

(Received February 16th, 1976)
(Revised manuscript received June 14th, 1976)

SUMMARY

Cytochromes of the *a*-, *b*-, *c*- and *d*-type become reduced when intact cells of *Hemophilus parainfluenzae* have become anaerobic following respiration with substrates such as formate or succinate, as shown previously (J. Biol. Chem. (1970) 254, 5096–5100). In the presence of formate after depletion of O_2, there is an unusual two-step time course of reduction of the membrane-bound cytochrome *c*. The proportion of the cytochrome *c* which is reduced during the second stage is oxidizable by either nitrate or H_2O_2 and is reduced again when the nitrate or H_2O_2 have been depleted. We conclude that the observed two-stage reduction of cytochrome *c* results from the presence of an oxidant, probably H_2O_2, produced by reaction of formate dehydrogenase with O_2. This was shown by the effects of cyanide, catalase and O_2. In addition, no evidence for the production of the oxidant is seen when succinate is the substrate oxidized. Although measurements of absorption spectra indicated only one species of cytochrome *c*, kinetic evidence is presented for some separation of the cytochrome *c* into more than one electron transport pathway.

INTRODUCTION

Previous spectrophotometric studies [1] showed that the cytochromes (*a*-, *b*-, *c*-, *d*- and *o*-types) of intact substrate-free *Hemophilus parainfluenzae* were rapidly reduced to the aerobic steady state on addition of substrates such as succinate, then were further reduced on exhaustion of oxygen. The cytochromes were then rapidly reoxidized upon addition of oxygen, and all followed the same time course of oxidation and reduction, even when the organisms contained widely varying proportions of the

* Present address: MRC Toxicology Laboratories, Woodmansterne Rd., Carshalton, Surrey, U.K.
** Present address: Department of Biological Sciences, Florida State University, Tallahassee, Fla. 32306

different cytochrome pigments [2]. These previous studies were carried out with a rapid-mixing apparatus over time periods lasting around 2 s; they implied that all of the cytochromes participated similarly in the rapid oxidation-reduction reactions of of the respiratory chain system.

In this paper, we show an unusual time course of reduction of cytochrome *c* when formate is oxidized. In contrast to the kinetics observed when succinate is the substrate, part of the cytochrome *c* remains oxidized for a period after O_2 is exhausted. This delay in reduction of part of the cytochrome *c* appears to result from production of an oxidant, most likely H_2O_2, during the aerobic oxidation of formate. Nitrate can oxidize a similar proportion of the cytochrome *c*. These observations have implications about the localization of cytochrome *c* in the membrane.

METHODS

Growth of bacteria. The strain of *H. parainfluenzae*, the growth medium, the determination of purity and the conditions of culture have been described previously [3, 4]. The relationship between dry weight and absorbance at 750 nm established by White [5] was used for measurement of the dry weight of the cell suspensions. For most experiments, the bacteria were washed free of substrates with 50 mM phosphate buffer, pH 7.6 [3].

Spectrophotometry. Difference spectra of cell suspensions (the difference in absorbance between cells anaerobic with substrate and cells aerobic without substrate) were measured with a Cary 14-CM Spectrophotometer equipped with a high-intensity light source, the scattered transmission accessory and the 0–0.2 slidewire, as described previously [4]. Levels of the different cytochromes in the suspension were calculated from the difference spectra measured at room temperature, corrected for overlap of absorption peaks of *b*- and *c*-type cytochromes [4].

Difference spectra at the temperature of liquid nitrogen were measured with apparatus similar to that of Estabrook [6], adapted for use with the Cary Spectrophotometer. Lucite cuvettes with a 3-mm light path containing the cell suspension in phosphate buffer were lowered into liquid nitrogen in the bottom of an unsilvered Dewar flask. The cuvette was then raised into the light path for recording the spectrum. The α-absorption peak of reduced horse heart cytochrome *c* (Sigma Type III) was resolved into two peaks at 548 and 544 nm, even without the 50 % glycerol found to be necessary for adequate resolution of the peaks by Estabrook [6].

The kinetics of oxidation and reduction of cytochromes were followed using an Aminco-Chance Dual Wavelength Spectrophotometer (American Instrument Co.) connected to a Honeywell 906C Visicorder. Cytochrome *c* changes were measured at 552 minus 539 nm, cytochrome *b* at 560 minus 539 nm and cytochrome *d* at 631 minus 650 nm. The slit preceding the monochromator was set at 0.26 mm for all measurements. Small samples of concentrated suspensions of bacteria were added to buffer at room temperature in cuvettes having a 9-mm light path to make a final volume of 8.0 ml; then the reaction was initiated by rapid addition of 0.05 ml of substrate on a polyethylene rod and mixing for at least 3 s. This amount of stirring was found to be sufficient for complete mixing of a dye with this volume. The kinetic measurements were made at room temperature (23 °C). Fig. 1 illustrates such a recording at 552 minus 539 nm (predominantly cytochrome *c*) after addition of formate to the cell

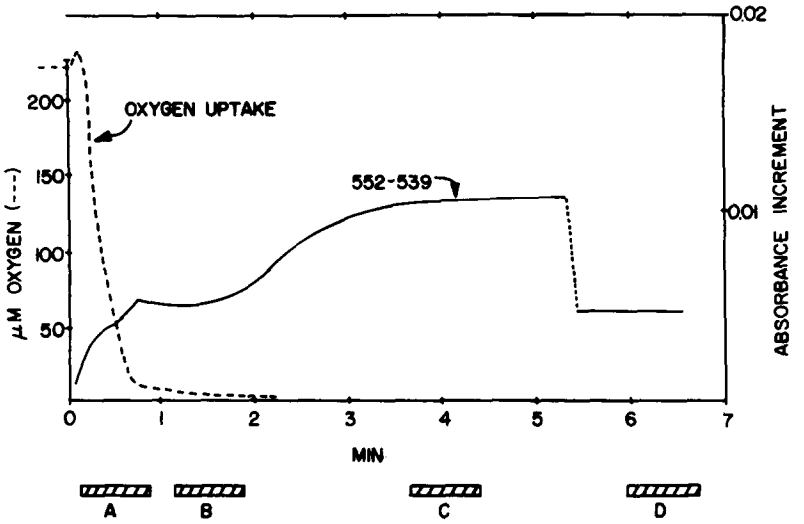

Fig. 1. Reduction of oxygen and cytochrome *c*. Oxygen uptake was measured with a vibrating platinum electrode in the dual wavelength spectrophotometer simultaneously with the absorbance changes. Reduction was initiated by the addition of 6 mM formate to a cell suspension containing 0.76 mg (dry wt.) per ml. Period D represents the time interval after the addition of 17 mM nitrate. Cells were grown aerobically to early stationary phase in the presence of nitrate.

suspension. Similar kinetic changes were observed at 552 minus 575 nm. Negligible absorbance changes were seen with the wavelength pairs 552–552, 560–560 or 539–575 nm.

Assay methods. Oxygen concentration in the cuvettes in the experiments with the Dual Wavelength Spectrophotometer was measured with the vibrating platinum electrode supplied by the manufacturer. The rate of vibration of the electrode was adjusted so that turbulence neither contributed significant noise to the recording nor stirred appreciable oxygen into the sample.

H_2O_2 concentration was assayed spectrophotometrically, using a millimolar extinction coefficient of 61 at 230 nm [7].

Chemicals. The inhibitor sodium secobarbital, sodium 5-allyl-5-(1-methyl) barbiturate (Eli Lilly and Co., USP Grade) was added as a solution in ethanol. Sodium cyanide (Fisher Scientific Co., Analytical Grade) was freshly prepared in buffer before use. Dibasic potassium phosphate, 50 mM, adjusted to pH 7.6 with HCl was used as the buffer throughout. Oxygen was 99.5 % USP Grade (Liquid Carbonic Co.). Nitrogen free of oxygen was prepared by passing nitrogen gas over heated copper as described by White [5]. Carbon monoxide (Matheson Co.) was added as a 1 mM solution, produced by gassing buffer for one minute at room temperature. Other chemicals were all of analytical grade, unless otherwise stated.

RESULTS

Observation of a delay in reduction of cytochrome c after anaerobiosis

Fig. 1 shows simultaneous tracings of changes in O_2 concentration and in absorbance at the wavelength pair adopted for measuring reduction of cytochrome *c*

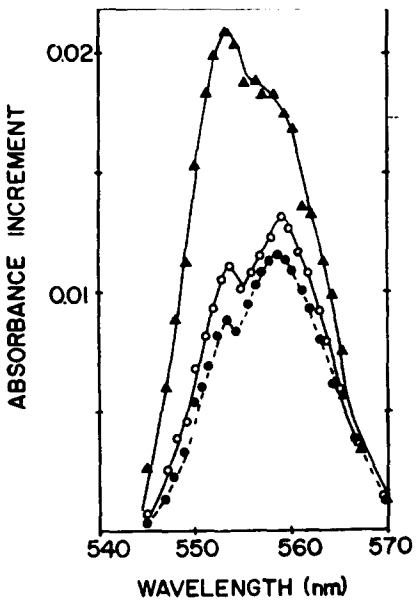

Fig. 2. Spectra of the steady states of cytochrome *b* and *c* during reduction by formate. The absorbance differences between the wavelengths on the abscissa and 539 nm were obtained from the steady states during the periods marked B (○ – ○), C (▲ – ▲) and D (● – ●) shown in Fig. 1.

following addition of formate (6 mM), then finally nitrate (17 mM). The cytochrome is reduced to a slight plateau during the period of rapid O_2 uptake, then is further reduced to a constant level for about a minute after the suspension becomes anaerobic, after which there is a slow reduction of additional pigment. When this is complete, addition of nitrate lowers the absorbance to the value recorded in the initial anaerobic steady state. This component is reduced again when the nitrate is depleted (not shown). We shall subsequently refer to the period labeled B on the figure as the lag period. Similar recordings were made at numerous wavelengths between 545 and 570 nm; the absorbance changes recorded during periods, B, C and D are plotted in Fig. 2. The increased absorption after the lag period appears to result from further reduction of cytochrome *c* and a small amount of cytochrome *b*. Interpretation of the data of Fig. 2 is complicated by the overlapping absorbance of the peaks of cytochromes *b* and *c*, which may be corrected using the formulae previously established [4]:

cytochrome *c* absorption increment $= 1.37\,A_{552} - 0.62\,A_{560}$
cytochrome *b* absorption increment $= 1.07\,A_{560} - 0.15\,A_{552}$

The corrected data are plotted in Fig. 3. They show that no cytochrome *b* is reduced after the lag period; only cytochrome *c* is reduced. Fig. 3 also shows the changes in absorbance at wavelengths between 615 and 648 nm related to 650 nm as reference, indicating the reduction of cytochrome *d*. The maximum at 631 nm and the width at half peak height were similar for measurements made during partial reduction and after complete reduction, suggesting that only one component is responding in this wavelength interval.

The O_2 electrode tracings of Fig. 3 show that the formate oxidase activity was

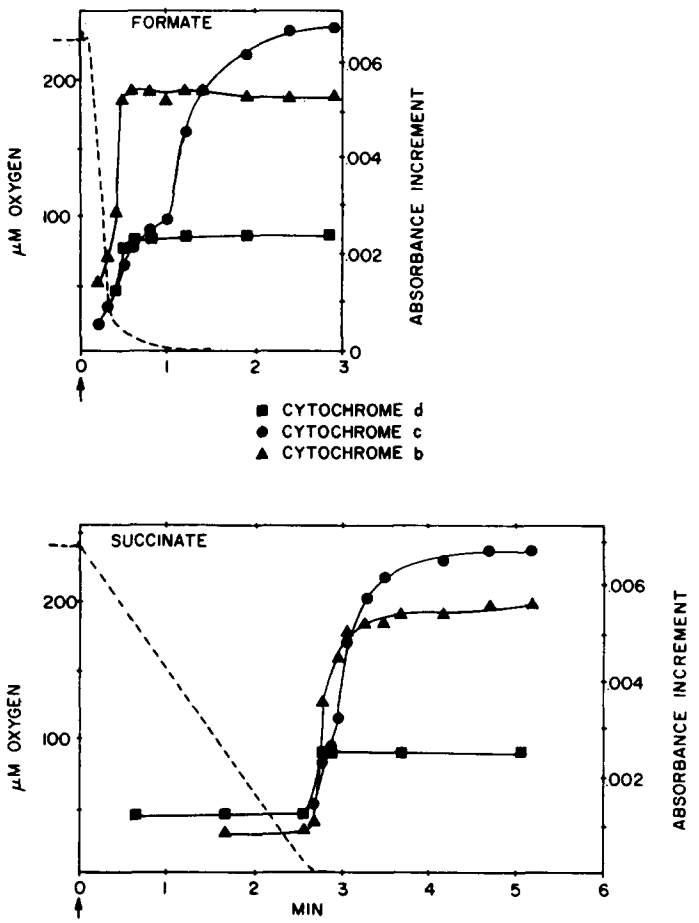

Fig. 3. Reduction of cytochromes *b*, *c* and *d* after addition of formate or succinate. The reduction was recorded as in Fig. 1, using the wavelength pairs 552–539 nm for cytochrome *c* (● — ●), 560–539 nm for cytochrome *b* (▲ — ▲) and 631–650 nm for cytochrome *d* (■ — ■). The absorption differences for cytochrome *b* and *c* were corrected for overlap using the formulae in ref. 4. Oxygen was measured as in Fig. 1 (– – –). The suspension contained 1.16 mg (dry wt.) per ml; 17 mM substrate was added at the time indicated by the arrow. The cells were grown aerobically to early stationary phase.

about 10-fold greater than the succinate oxidase of these cells. As expected, there is less reduction of the cytochromes during the aerobic steady state in the presence of succinate than in the presence of formate; the period is so short during oxidation of this concentration of formate that the exact level is difficult to distinguish. The corrected absorbance data of the figure show that all of the *d*-, *b*-, and *c*-cytochromes are rapidly reduced without lag when the oxygen is exhausted by cells oxidizing succinate. In contrast, as described above, when formate is the substrate there is a lag in the reduction of some of the cytochrome *c* after anaerobiosis is reached. The extent of the lag period is not related to the time required to reach anaerobiosis. This was varied by (1) decreasing the initial formate concentration from 6.3 mM to 0.63 mM (which doubles the time required to exhaust the O_2) or (2) by inhibiting the formate

dehydrogenase with 4 mM secobarbital [8] (which caused a 10-fold increase in the time required to reach anaerobiosis). In neither case was the duration of the lag period affected. Identification of the reason for the lag will be given later.

The data of Figs. 1 and 2 show that only the amount of cytochrome *c* which is reduced after the lag period in cells metabolizing formate is reoxidized on addition of nitrate. This was found to represent about 50 % of the total reducible cytochrome *c* in the cells; at least 90 % of the total reducible cytochrome *b* remains reduced in the presence of nitrate. Addition of nitrate also results in a slow change in absorption of the cytochrome *d* (compared to the rate of oxidation of cytochrome *c*), but only if the bacteria had been grown in the presence of nitrate, when nitrate or nitrite reductase is synthesized [4]. A similar phenomenon was also observed in *Klebsiella* (*Aerobacter*) *aerogenes* [9]. Meyer [10] has reported that NO produced by many organisms can combine with cytochrome *d*, which makes the observations of absorbance changes difficult to interpret.

One interpretation of the two phases of reduction of cytochrome *c* (Fig. 1) is that they represent reactions of two distinct molecular species of cytochrome *c* in these bacteria. This possibility was investigated by examining the absorption spectra

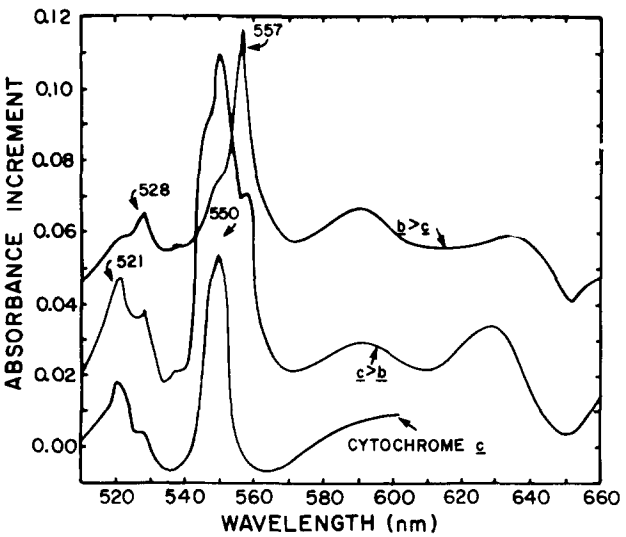

Fig. 4. Low temperature (−196 °C) difference spectra of whole cells of *H. parainfluenzae* and of cytochrome *c* extracted from the cells. The top spectrum represents that of the cells in which the cytochromes of sample cuvette were reduced with formate (17 mM) and then nitrate added (17 mM). The reference cuvette was untreated. After sufficient time for the suspension to have become anaerobic, the cuvettes were plunged into liquid nitrogen then raised into the light path in the apparatus described in Methods. The middle spectrum is of a separate batch which had more cytochrome *c* than *b* and in which the cytochromes were reduced with formate only. The bottom spectrum is of the dithionite-reduced supernatant prepared from cells used for recording the middle spectrum. The cells were broken by sonic oscillation and centrifuged at 25 000 × *g* for 30 min. Fine particles were removed by ammonium sulfate precipitation (45 % saturation) and the supernatant was dialysed overnight against the phosphate buffer. Cuvettes were 3 mm in path-width. Samples were frozen in liquid nitrogen and then raised into the light path. Cell concentrations were: top, 10 mg (dry wt.) per ml; middle 5 mg (dry wt.) per ml. The cytochrome *c* solution contained 1 mg protein per ml.

at the temperature of liquid N_2 (−196 °C) of the cells in states B, C and D of Figs. 1 and 2 and of extracted and partially purified cytochrome *c* (see legend to Fig. 4). As observed with cytochrome *c* from other species [6], the α-absorption peak splits at

TABLE I

RATIOS OF ABSORBANCES OF CYTOCHROMES *c* AND *b* AT −196 °C IN INTACT CELLS

The reduced minus oxidized difference spectra of intact cells at −196 °C were measured as described in Methods. The spectrum of the steady state during the "lag period" was measured after plunging the cuvettes into liquid nitrogen at a time after adding formate to the sample cuvette (30 s) known to reach the "lag period" (B, Fig. 1). The spectrum of completely reduced cells was measured after 5 min incubation of the sample cuvette with formate (C, Fig. 1). The spectrum of the steady state during nitrate reduction was obtained by adding sodium nitrate (17 mM) to the sample cuvette containing cells completely reduced with formate; after 30 s the cuvettes were plunged into liquid N_2 (D, Fig. 1). Cell concentrations were 5 mg (dry weight) per ml.

Steady state	Absorbance 547 nm / Absorbance 550 nm	Absorbance 557 nm / Absorbance 550 nm
During lag period	0.86	1.03
Complete reduction	0.86	0.41
Anaerobic nitrate reduction	0.87	1.00

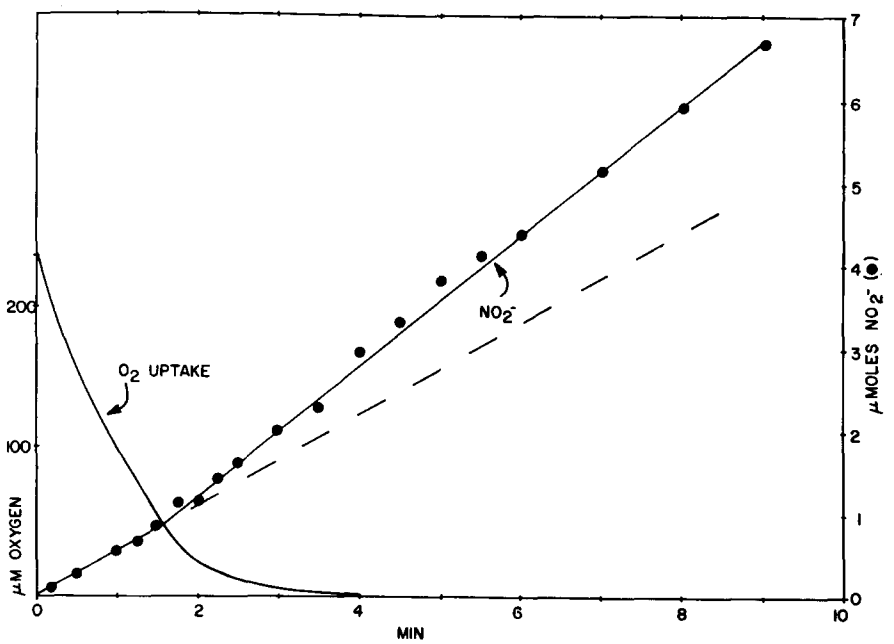

Fig. 5. Simultaneous reduction of oxygen and nitrate. The oxygen uptake of a cell suspension (0.25 mg (dry wt.) per ml) in the presence of 6 mM formate and 6 mM nitrate was measured as in Fig. 1 at 23 °C. At intervals, small aliquots were removed and assayed for nitrite by the Griess-Ilosvay colorimetric procedure [4]. Nitrite was calculated as total produced in the reaction mixture (8 ml). Cells were grown anaerobically with fumarate to early stationary phase.

low temperature into peaks at 550 and 547 nm. These peaks are also observed in intact anaerobic bacteria, in addition to an absorption peak at 557 nm attributable to cytochrome *b*.

The data of Table I show that the ratios of absorbance at 547–550 nm remained constant in three steady state conditions during which the extent of reduction of the *b*- and *c*-type cytochromes varied considerably. These observations argue against differential reduction of separate species of cytochrome *c*.

Fig. 5 shows that O_2 and nitrate are reduced simultaneously from the time of addition of formate, the rate of O_2 reduction being four times greater in terms of electron flux. The change in the rate of nitrate reduction occurs at the time when cytochrome *b* becomes fully reduced (i.e. O_2 is exhausted).

The lag period was only seen when O_2 was present during the oxidation of formate; it was abolished by prior removal of O_2 (Fig. 6), but reappeared upon flushing the suspension with O_2. With a given suspension of cells, the extent of the lag period was inversely proportional to the number of cells in the suspension. In Fig. 6, a more dilute suspension of bacteria was used than that in Fig. 1 and the cells had a high ratio of cytochrome *c* to cytochrome *b*. This emphasized the effects of N_2 and O_2, but at the low absorbance measured, the initial kinetics to the aerobic state are not clear.

Evidence was obtained that the lag period resulted from the accumulation of H_2O_2. Addition of catalase prior to formate nearly abolished the lag (data not shown; they are the same as those of Fig. 6 in the presence of N_2). Then addition of H_2O_2

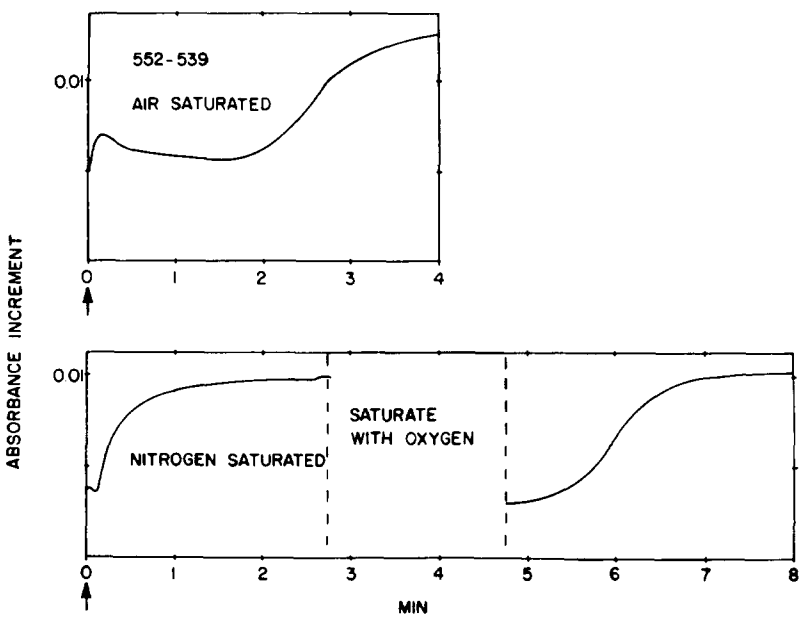

Fig. 6. Reduction of cytochrome *c* in the presence or absence of oxygen. Reduction was measured as in Fig. 1 in the presence of 6 mM formate with cells suspended in air-saturated buffer (upper curve), in buffer deoxygenated by bubbling with nitrogen for 5 min and after the reduced suspension was bubbled with oxygen for 2 min (lower curve). The cuvette contained 0.48 mg (dry wt.) per ml of cells and formate was added as indicated by the arrows. Cells were grown anaerobically with nitrate to early stationary phase.

oxidized cytochrome *c* as in the lag period. The cytochrome *c* was eventually reduced, presumably after cellular metabolism of the added H_2O_2. The duration of the lag period was increased by increasing the amount of H_2O_2 added. The cell suspension used in Fig. 1 removed 1.5 mM added H_2O_2 in 3.2 min; from this it can be estimated that the lag period resulting from aerobic oxidation of formate resulted from the accumulation of 35 μM H_2O_2.

Neither the initial rate of O_2 uptake nor of nitrate reduction to nitrite was inhibited by 5 μM KCN; 1 mM KCN was required to obtain 50 % inhibition of these two reactions. However, 5 μM KCN doubled the usual lag period seen with bacteria metabolizing formate as well as the lag resulting after addition of 35 μM H_2O_2. This can be explained as due to inhibition of the cellular activity which removes the H_2O_2.

The lag period observed in cells oxidizing formate appears to result from the accumulation of an oxidant, possibly H_2O_2, which can oxidize some of the cytochrome *c*; the cytochrome *c* becomes reduced again when this oxidant is removed by the cellular metabolism. Since the lag period was prolonged in cells grown in nitrate (as compared to those grown in its absence), the possibility was suggested that accumulated nitrite may inhibit the intracellular activity which normally removes the oxidant. To test this possibility, cells grown with fumarate as electron acceptor were washed, then incubated with nitrite for 30 min at 25 °C (11.0 mg cells dry wt. per ml). The cells were washed again and resuspended in buffer to a concentration of 2.4 mg dry weight per ml. At nitrite concentrations in the preincubation medium above 1 mM the lag period was increased from 0.2 min in untreated cells to 1.5 min in the cells incubated with nitrite.

DISCUSSION

Our previous studies of the reactions of intact *H. parainfluenzae* on rapid mixing with substrates and O_2 [1] implied that the kinetics of oxidation and reduction are consistent with the sequential arrangement usually proposed for the respiratory chain system of mitochondria and bacteria. The studies reported here, measured on a slower time scale, have revealed some unusual aspects of the electron transport chain of *H. parainfluenzae*.

We have studied the kinetics of oxidation and reduction of *c*-, *b*- and *d*-cytochromes. Thus, it was essential to delineate the changes of absorbance of each cytochrome type. The data would have been misleading if corrections had not been made for the overlapping absorbance peaks of *b*- and *c*-type cytochromes. For instance, in Fig. 2 it would not have been evident that the cytochrome *b* was already reduced at the end of period A. These precautions have been ignored by many investigators.

In these bacteria, during the oxidation of formate by O_2, a substance accumulates which oxidizes part of the cytochrome *c*, even after anaerobiosis is reached and all of the *b*- and *d*-type cytochromes are reduced. After a so-called lag period, during which the accumulated substance is removed by cellular metabolism, the oxidized cytochrome *c* becomes reduced. Evidence that the oxidant produced is H_2O_2 is the following: (1) it is produced only in the presence of O_2 (Fig. 6); (2) addition of H_2O_2 gave a similar oxidation, and the extent of the lag period was proportional to the

amount of H_2O_2 added; and (3) prior addition of catalase to the suspension eliminated the lag period, presumably by destroying the H_2O_2 as it was formed.

The production of H_2O_2 appeared to be exclusive to formate oxidation, since no lag was seen when succinate was the electron donor (Fig. 3). Even when the rate of formate oxidation was decreased in the presence of secobarbital, about the same lag period was observed, showing that the relatively low rate of succinate oxidation could not be the reason for the lack of lag period on oxidation of succinate.

H_2O_2 seems to be produced in a direct reaction of formate dehydrogenase with O_2. This would agree with an earlier observation by one of the authors [8] of reduction of O_2 by formate in excess of the equivalent amount of ferricyanide that was reduced by the formate dehydrogenase. It is possible that superoxide anion (O_2^-) is the initial product of the reaction, by analogy with xanthine oxidase [12], since molybdenum and non-heme iron are present in purified *Escherichia coli* formate dehydrogenase [13]. All aerobic bacteria are reported to contain superoxide dismutase [14]; thus the O_2^-, if formed, would be rapidly converted to H_2O_2. Evidently, the system described in this paper could be useful as a model for study of the formation and metabolism of H_2O_2 in intact cells.

As described above, the length of the lag period is a function of the concentration of H_2O_2 present. Thus, the lag was increased by inhibition of the intracellular enzyme(s) responsible for its destruction, for example, by addition of 5 μM KCN. The enzyme is most probably catalase, which has been previously reported in this organism [15]. The extended lag period observed in cells grown in nitrate can be attributed to an irreversible inhibition of catalase by the significant amounts of nitrite accumulated in the growth medium.

Our data indicate that the nitrate reductase system reacts with membrane-bound *c*-cytochromes, in contrast to the well-established pathway in *E. coli* which involves one or more *b*-cytochromes [16, 17].

An interesting observation is the oxidation of only part of the cellular cytochrome *c* on addition of either nitrate or H_2O_2 to cells with the cytochromes reduced via formate dehydrogenase. Since under these conditions at least 50 % of the cytochrome *c* becomes oxidized, but less than 10 % of the cytochrome *b* (Fig. 3), the data suggest two pools of cytochrome *c*, only one of which can react with the nitrate reductase or with H_2O_2 (or a peroxidase). No evidence was found by spectrophotometry at −196 °C for more than one species of cytochrome *c*. However, this is not conclusive evidence that only one molecular species is present, since many species have similar absorption spectra. The different reactivity of the two portions of cytochrome *c* could result from different localization within the cellular membrane.

To sum up, the data show that about 50 % of the cytochrome *c* can be oxidized by nitrate or H_2O_2 regardless of the rate of electron flow (formate in the presence or absence of secobarbital). These data are difficult to explain as a steady state resulting from varying oxidase and reductase activities. On the other hand the cytochrome *b* level in the aerobic steady state does vary according to the rate of flux from the respective dehydrogenases. We hypothesize that there are two pools of cytochrome *c*, which can interact. The existence of the two pools only becomes evident in the presence of an oxidant of one pool, such as nitrate or H_2O_2. The details of the reaction between these two pools have yet to be explored.

ACKNOWLEDGEMENTS

This research was supported by grant GB-4795 from the National Science Foundation and grant GM-10285 from the National Institutes of Health (to D.C.W.) and by grant GM-06270 from the National Institutes of Health (to L.S.). P.R.S. had a Doctoral Year Fellowship from the University of Kentucky Graduate School.

REFERENCES

1 Smith, L., White, D. C., Sinclair, P. and Chance, B. (1970) J. Biol. Chem. 254, 5096–5100
2 White, D. C. and Sinclair, P. (1971) Adv. Microbiol. Physiol. 5, 173–211
3 White, D. C. and Smith, L. (1962) J. Biol. Chem. 237, 1332–1336
4 Sinclair, P. and White, D. C. (1970) J. Bacteriol. 101, 365–372
5 White, D. C. (1966) Antonie van Leeuwenhoek J. Microbiol. Serol. 32, 139–158
6 Estabrook, R. W. (1956) J. Biol. Chem. 223, 781–794
7 McEwen, C. M. (1971) Method Enzymol. XVII B, 686–692
8 White, D. C. (1964) J. Biol. Chem. 239, 2055–2060
9 Knook, D. L., van 't Riet, J. and Planta, R. J. (1973) Biochim. Biophys. Acta 292, 237–245
10 Meyer, D. J. (1973) Nature New Biol. 245, 276–277
11 Chance, B. (1952) J. Biol. Chem. 194, 471–491
12 McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055
13 Enoch, H. G. and Lester, R. L. (1975) J. Biol. Chem. 250, 6693–6705
14 McCord, J. M., Keele, B. B., Jr. and Fridovich, I. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1024–1027
15 White, D. C. (1962) J. Bacteriol. 83, 851–859
16 Ruiz-Herrera, J. and DeMoss, J. A. (1969) J. Bacteriol. 99, 720–729
17 MacGregor, C. H. (1975) J. Bacteriol. 121, 1111–1116