CYTOCHROME AND CATALASE PATTERNS DURING GROWTH OF *HAEMOPHILUS PARAINFLUENZAE*

DAVID C. WHITE

The Rockefeller Institute, New York, New York

Received for publication November 2, 1961

ABSTRACT

WHITE, DAVID C. (The Rockefeller Institute, New York, N. Y.) Cytochrome and catalase patterns during growth of *Haemophilus parainfluenzae*. J. Bacteriol. **83**:851–859. 1962.—By following the cytochrome and catalase concentrations during the growth cycle and under various growth conditions in *Haemophilus parainfluenzae*, a rapid increase in the cytochrome oxidases and a large increase in cytochrome c_1 concentration can be demonstrated between log-phase and stationary-phase cells and between vigorously aerated and anaerobic growth conditions.

The three cytochrome oxidases develop differentially under various growth conditions. The principal oxidase formed in vigorously aerated cultures is cytochrome *o*. With limited aeration, maximal development of cytochrome a_2 occurs; with anaerobically grown cells, there is a marked increase in the concentration of cytochrome a_1. With the rapid increase in cytochrome c_1 concentration, soluble, nonenzymatically reducible cytochrome c_1 is also formed, which remains in the bacterial cell sap. From these data it is postulated that the electron-transport system is assembled from individual components which can be modified by the growth conditions. The cytochrome c_1 may be synthesized in the cell sap and then incorporated into the electron-transport system.

Previous studies (White and Smith, 1962) with *Haemophilus parainfluenzae* harvested in the stationary phase showed that the cells respire by way of a cytochrome-linked system composed of six cytochrome pigments. Intact cells are able to oxidize a number of substrates and also the reduced form of diphosphopyridine nucleotide (DPNH) (White and Smith, 1962). These stationary-phase cells contain a relatively large amount of cytochrome c_1, part of which is not reducible enzymatically and which is released into solution when the cells are ruptured (Smith and White, 1962). Additional cytochrome c_1 can be removed from insoluble respiratory particles by washing with buffer; this eventually results in a decrease in the respiratory rate of thees particles. The other cytochromes and flavoproteins remain attached to the particles during the washing. Cytochrome c_1 that remains bound to the particles can be reduced and oxidized during electron transport. Stationary-phase cells contain little or no catalase activity (White and Smith, 1962).

The present work shows the changes in cytochrome and catalase content during the growth cycle and under different growth conditions of *H. parainfluenzae*. Log-phase cells contain lower concentrations of cytochromes and more catalase than stationary-phase cells. There is a relatively greater concentration of cytochromes c_1, a_1, and *o* and lower catalase in cells grown anaerobically than in cells grown with vigorous aeration. With limiting aeration, larger amounts of cytochromes c_1, a_1, and a_2 appear than are present in vigorously aerated cells.

MATERIALS AND METHODS

Growth of bacteria. The bacterial strain and media have been described previously (White and Smith, 1962). In this study, bacteria were grown in a jacketed Woulff bottle with a 240 by 150 mm growth chamber, containing 3 liters of media. A Teflon-covered stirring bar, 75 mm in length and running at a rapid rate, was used to agitate the culture. Air or nitrogen was introduced to the chamber through a cylindrical (45 by 15 mm) coarse-pore sintered-glass filter. Temperature was maintained at 37 C by circulating heated water through the jacket. Under these conditions of growth, the bacteria appeared as discrete coccobacillary forms.

Vigorous aeration was achieved by passing 2.5 liters/min of air through the culture. Limited aeration refers to cultures grown with 250 ml/min of air passed through the culture. Anaerobic growth represents growth where prepurified nitrogen was bubbled through the culture at a rate of 100 ml/min, from a period beginning 2 hr before

inoculation and lasting until the experiment was completed. With this system, the stationary phase could be reached in from 3 to 6 hr for aerated cultures and from 6 to 10 hr for anaerobic cultures, depending on the inoculum size. The usual inoculum was 10^8 stationary-phase organisms.

Bacterial assays. For viable counts, the medium (White and Smith, 1962) containing 1.5% agar, maintained at 45 C, was used in pour plates containing suitably diluted cultures. Bacteria were diluted for cell counts in this medium, containing no diphosphopyridine nucleotide (DPN). Incubation of the dilution tubes can be used as a test of contamination, as *H. parainfluenzae* will not grow without added DPN. Turbidity was measured in a Klett-Summerson colorimeter, using a no. 54 green filter with a band pass between 590 and 490 mμ.

Bacteria were withdrawn aseptically by suction from the growth flask at suitable intervals. The organisms were centrifuged at 8,000 × *g* and washed twice with ½ volume of 0.05 M phosphate buffer (pH 7.0) at 4 C. Spectra were measured in 25% (v/v) glycerin suspensions of this buffer containing between 10 and 20 mg of bacterial protein/ml. The bacteria were resuspended with the aid of a Teflon homogenizer. Spectra were measured with a Cary model 14 spectrophotometer. By using a more intense light source, cultures of this density may be used with band widths of less than 50 A at 700 mμ, and 7 A at 553 mμ. With the 0.1 to 0.2 optical density (OD) slide wire, there is a noise level of ±0.00075 OD units.

Spectra were measured using the difference-spectra methods described by Chance (1954), and were plotted as described previously (Smith and White, 1962). The absorption spectra produced by reduced cytochromes were measured against a similar cell suspension containing oxidized cytochromes. Physiological reduction of the cytochromes was produced by adding 0.1 ml of 0.25 M DPNH (0.006 M final concentration) to the cuvette. The suspension was then left at room temperature for 5 min and measured against cells oxidized by air. Washed stationary-phase cells have an insignificant endogenous respiration. Log-phase cells have a small endogenous respiratory rate after washing. If they are shaken vigorously with air and compared with reduced cells, the time before the level of cytochrome c_1 begins to fall is used as the interval between reoxidations.

The cytochromes may be chemically reduced by adding to the cuvette a few mg of $Na_2S_2O_4$ and bubbling with N_2 to mix. The presence of DPN nucleotidase activity and completeness of reduction may be checked with the oxygen electrode as described previously (White and Smith, 1962).

Carbon monoxide spectra were obtained by bubbling the gas into cell suspensions reduced with either DPNH or $Na_2S_2O_4$ and measuring the spectra against reduced cells. The bubbling was continued until saturation occurred.

To measure nitrate reductase activity, cells reduced with DPNH were compared; then one

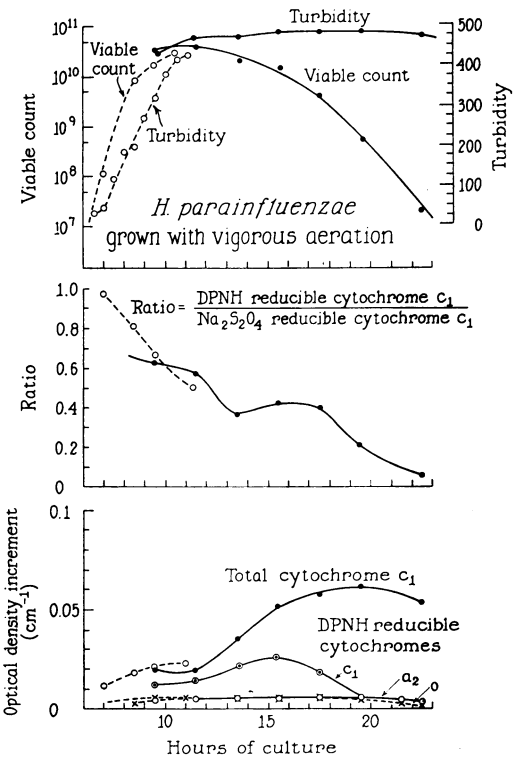

FIG. 1. *Change in cytochromes of H. parainfluenzae during the growth cycle with vigoroue aeration. The bottom graph shows the changes in relative concentrations in whole bacteria measured as described in Methods at a concentration of 10 mg protein/ml per cm of light path. The dashed curve represents one experiment and the smooth curve the second experiment. The middle graph represents the ratio of enzymatically to $Na_2S_2O_4$-reducible cytochrome c_1. The upper graph illustrates the growth phase of the bacteria in terms of viable count and turbidity. Bacteria are suspended in 0.05 M phosphate buffer (pH 7.0) with 20% (v/v) glycerin.*

cuvette was reoxidized by addition of 10^{-2} M nitrate in the absence of oxygen (White and Smith, 1962).

The nomenclature of the cytochromes is that used previously (White and Smith, 1962). Cytochrome c_1 is measured from its maximum at 553 mμ to the trough between 570 and 580 mμ in the oxidized vs. reduced spectra. Cytochrome *o* is

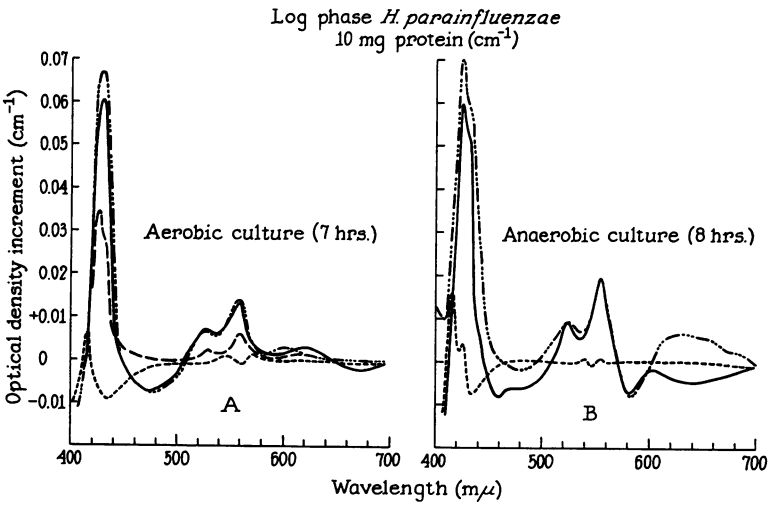

FIG. 2. *Difference spectra of log-phase H. parainfluenzae. Difference spectra of 10 mg bacterial protein/ml per cm light path are shown above. Cells were harvested at a density of 10^8 cells/ml from vigorously aerated (A) and from anaerobic (B) cultures. The spectra are plotted as DPNH reduced vs. oxidized (long dashed line), DPNH-CO vs. DPNH reduced (short dashed line), and DPNH vs. DPNH plus nitrate (dot-dashed line). Bacteria were suspended in 0.05 M phosphate buffer (pH 7.0) with 20% (v/v) glycerin.*

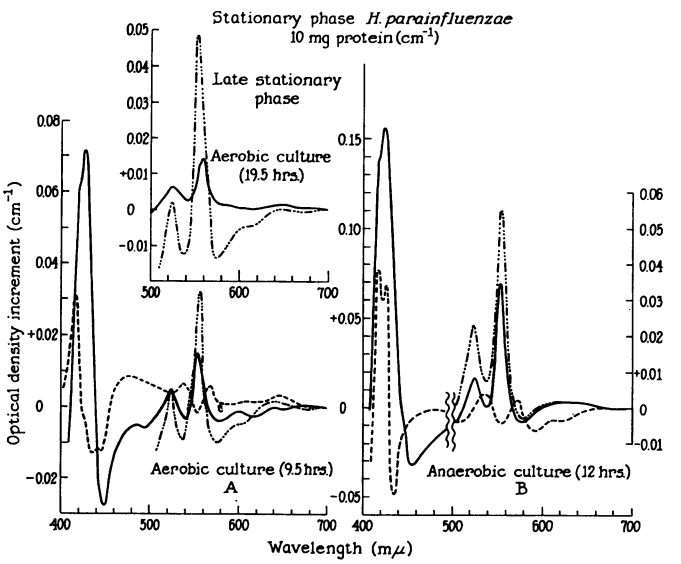

FIG. 3. *Difference spectra of stationary-phase H. parainfluenzae. Spectra represent 10 mg protein/ml per cm light path of aerobic (A) and anaerobic (B) growth. The inset shows late stationary-phase cells grown aerobically. The data are plotted as described in Fig. 2.*

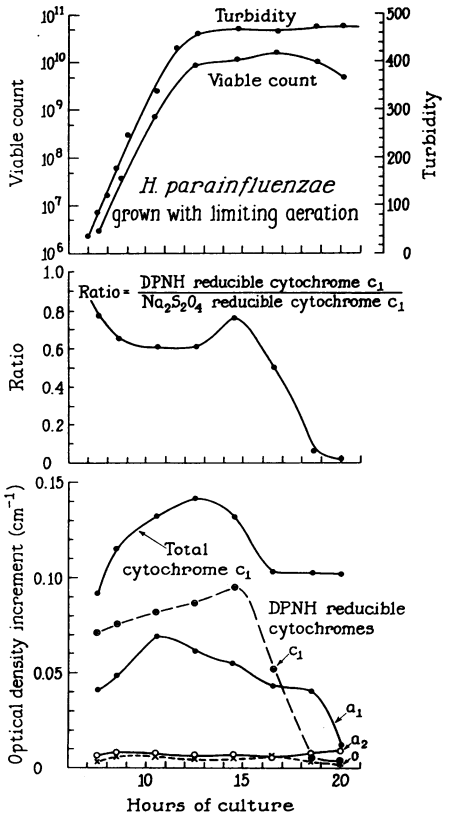

FIG. 4. *Cytochrome patterns of H. parainfluenzae grown with limiting aeration. Data are plotted as described in Fig. 1.*

measured from the peak at 540 mμ to the trough at 500 to 520 mμ in the CO spectra. Cytochrome a_1 is measured where a definite maximum occurs between the maximum at 435 mμ and a line extending from the side of the cytochrome *o* maximum at 416 mμ in the CO spectra. Cytochrome a_2 is measured from its maximum at 640 mμ to the base line at 700 mμ. Cytochrome b_1 is measured between the maximum at 450 mμ and the reference point at 700 mμ in the reduced vs. oxidized spectra. The level of reduction of these last two pigments must be cautiously interpreted in log-phase cells where there is a small endogenous respiratory rate. The difference spectra will give values lower than the true values.

Stationary-phase cells can be washed with buffer six times, as described, without significant change in the ratio of DPNH-reducible to $Na_2S_2O_4$-reducible cytochrome c_1 (ratio = 0.62 ± 0.018). The accuracy of the determination of the $Na_2S_2O_4$-reducible cytochrome c_1 per 10 mg protein of these six preparations was $OD_{(553-575)}$ = 0.128 ± 0.007. The variability was calculated using the expression: value = mean ± $(t_{\alpha/2=0.025})$ (standard deviation/$\sqrt{6}$), where α = the level of confidence.

Protein determinations. Protein determinations were measured by the biuret method (Cornall, Bardawill, and David, 1949).

Catalase. Catalase was measured by the iodometric titration of Herbert (1955). Catalase activity is expressed as per cent bacterial protein assuming a rate constant of 5.3×10^7 M^{-1} sec^{-1} = k_1 and a molecular weight of 2.3×10^5 = mol wt. The crystalline catalases isolated from *Micrococcus lysodeikticus* (Herbert and Pinsent, 1948) and *Rhodopseudomonas spheroides* (Clayton, 1956) have identical first-order rate constants and molecular weights (Chance and Herbert, 1950; Clayton, 1956). Catalase content as per cent of bacterial protein =

$$k_{obs} \times \frac{\text{mol wt}}{k_1} \times \frac{10^2}{\text{mg protein/ml}}$$

where

$$k_{obs} = \frac{1}{t} \times \ln \frac{x_0}{x_1}$$

x_0 = the concentration of H_2O_2 at time 0 (35 μM H_2O_2) and x_t = the concentration of H_2O_2 at time t. The measurements are made at 25 C. The k_{obs} agreed ±5% in four measurements and shows no tendency to decrease at longer times

Reagents. Reagents were as described previously (White and Smith, 1962).

RESULTS

The changes in cytochrome content during the growth phase of vigorously aerated cultures are plotted in Fig. 1. As the stationary phase continues, there is a threefold increase of total cytochrome c_1 and a twofold increase in DPNH-reducible cytochrome c_1. Synthesis of this cytochrome continues into the period of cell death. Log-phase aerobic cultures show a predominant cytochrome b_1 (560 mμ) maximum which hides the cytochrome c_1 in the α region, and a prominent soret maximum at 427 mμ (Fig. 2A). The nitrate reductase system (nitrate oxidation of DPNH-reduced cells) also shows this prominent cytochrome b_1 maximum and a suggestion of a cytochrome a_1 maximum at 600 mμ with a shoulder at 435 mμ. Early stationary-phase spectra show

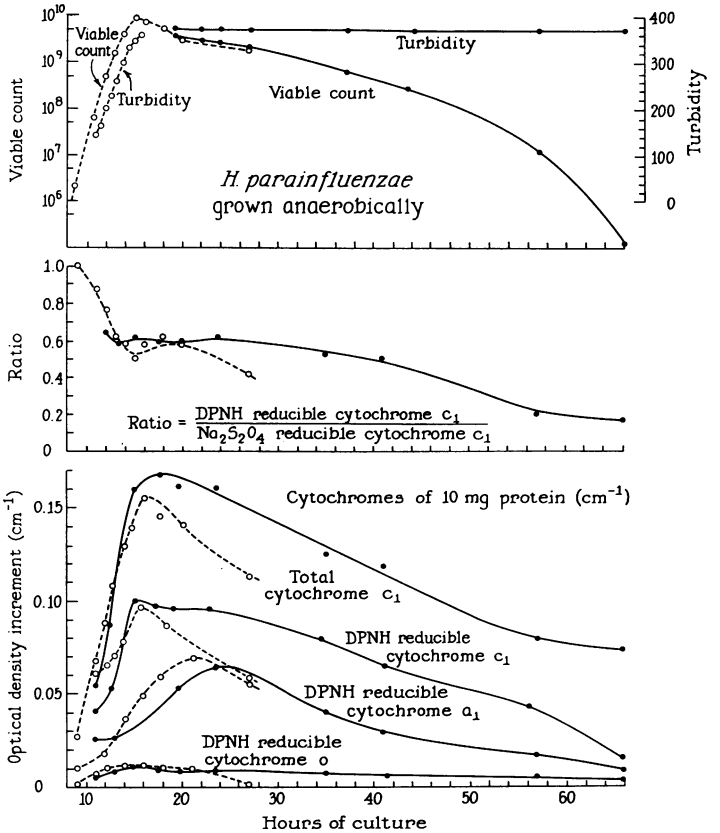

FIG. 5. *Cytochrome patterns of H. parainfluenzae grown anaerobically. One experiment is illustrated with the dashed lines and a second with the smooth curve. Data are plotted as described in Fig. 1.*

half the cytochrome c_1 as DPNH reducible (Fig. 3A). As the aging process continues the DPNH-reducible cytochrome c_1 decreases until the cytochrome b_1 maximum appears again (Fig. 3A, inset).

If the bacteria are grown with limiting aeration, the final cell density reached (470) is nearly equal to that with vigorous aeration (480), and cell death is delayed by 7 hr (Fig. 4). They have a similar increase in cytochrome c_1. In the log-phase spectrum, a prominent shoulder at 561 mμ of cytochtome b_1 can be seen. Certain differences are apparent, however. The total cytochrome c_1 formed is threefold greater than the maximum developed with vigorous aeration. A prominent cytochrome a_1 maximum at 425 mμ in the CO spectrum develops and decays with the onset of cell death. The cytochrome a_1 oxidase reaches its maximum 4 hr before the DPNH-reducible cytochrome c_1 maximum level. The concentration of DPNH-reducible cytochrome a_2 is nearly double that formed with vigorous aeration and does not decay with time. Cytochrome *o* remains at nearly the same level, and follows a similar pattern of development as in vigorously aerated cultures.

Bacteria grown anaerobically develop cytochrome patterns as plotted in Fig. 5. The highest levels of cytochrome c_1 are achieved in this condition. Maximal cytochrome c_1 synthesis begins in early log phase. The cytochrome a_1 develops to a level slightly higher than with limited aeration, and its maximal development appears 2 to 4 hr after the maximal development of DPNH-reducible cytochrome c_1. Essentially no cytochrome a_2 forms. The cytochrome *o* formed is nearly double that formed in the presence of air, and the onset of cell death is delayed 12 hr, as compared with cells grown with limited aeration. Log-phase anaerobically grown cells contain sufficient cytochrome c_1 to mask a cytochrome b_1 maximum (Fig. 2B). The amount of cytochrome

TABLE 1. *Optical density of DPNH-reducible cytochromes formed under various growth conditions*[a]

Growth conditions	c_1	b_1	a_1	a_2	o	Fp
Log phase						
Aerobic	0.012[b]	0.012	0.001	0.001	0.003	0.008
Anaerobic	0.027	—[c]	0.010	0.001	0.003	0.008
Early stationary phase						
Aerobic	0.019	0.014[d]	0.011	0.035	0.006	0.027
Anaerobic	0.039	—[c]	0.049	0.001	0.009	0.032

[a] The Δ OD for each cytochrome was measured as described in Methods, per 10 mg of bacterial protein/ml per cm light path.

[b] Cytochrome c_1 maximum hidden by cytochrome b_1 maximum (Fig. 2A).

[c] Indicates maximum hidden under an adjacent cytochrome maximum.

[d] Seen in DPNH-reduced spectra (Fig. 3B) of very old cells.

TABLE 2. *Maximum cytochromes with various growth conditions*

Growth conditions	Generation time	Maximum turbidity	Maximum viable count	Maximum cytochrome formed				Terminal acceptor (concn)
				c_1[a]	a_1	a_2	o	
	min		*cells/ml*					
Vigorous aeration	20	480	4×10^{10}	0.061	0.005	0.035	0.005	Oxygen (0.20 mM)
Limited aeration	25	470	1.5×10^{10}	0.141	0.068	0.010	0.006	Oxygen, nitrate
Anaerobic	40	370	0.9×10^{10}	0.168	0.070	0.001	0.012	Nitrate (10 mM)

[a] Δ OD of cytochromes measured after $Na_2S_2O_4$ reduction as described in Methods, per 10 mg bacterial protein/ml per cm light path.

c_1 is small enough, however, to allow the shoulder of cytochrome b_1 to be seen in the soret region of the spectrum. The 600 mμ maximum of cytochrome a_1 is easily seen in the physiologically reduced vs. oxidized spectrum. In log-phase cells the amount of cytochrome o is about one-half and the amount of cytochrome a_1 is one-seventh that formed in the stationary phase. However, cells grown under these anaerobic conditions form relatively huge amounts of cytochrome a_1 (Fig. 3B). These difference spectra represent bacteria in the same growth phase as those shown in Fig. 3A.

Through the growth cycle aerobically grown cells contain half the cytochrome c_1 and oxidase o as cells grown anaerobically. Anaerobically grown cells contain between 4 to 7 times the cytochrome a_1 of aerobic grown cells. Cells grown with limiting aeration form twice the cytochrome a_2 of vigorously aerated cells and at least 10 times the concentration found in anaerobically grown cells.

The oxidases a_1 and o increase during the stationary phase under conditions of limited aeration and anaerobiosis. With prolonged incubation, the concentration of DPNH-reducible oxidases a_1 and o gradually decrease. At this time the oxidases are present at their maximal level when measured after chemical reduction.

The relative amounts of DPNH-reducible cytochromes formed under these various growth conditions are presented in Table 1, and the maximal cytochrome formed in Table 2.

Catalase activity was measured under conditions where the disappearance of H_2O_2 with time is constant. There is little if any permeability barrier to H_2O_2, as intact cells are as active as various broken-cell preparations. Catalase activities calculated as per cent of bacterial protein from the rate constant and molecular weight of the two crystalline catalases are plotted in Fig. 6. Log-phase anaerobic cells contain much less catalase activity than cells grown with vigorous aeration. Cells grown with aeration tend to lose catalase activity rapidly during the late stationary phase; anaerobically grown cells lose activity at a much slower rate.

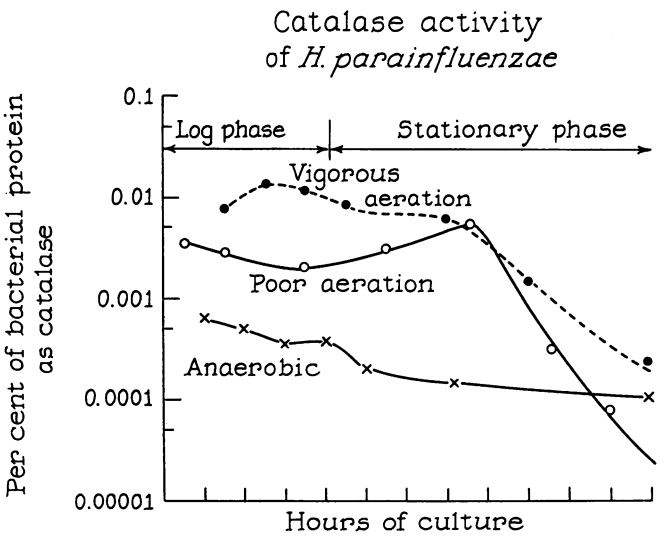

FIG. 6. *Catalase activity of H. parainfluenzae during growth under various conditions. Catalase activity is plotted as per cent of bacterial protein assuming the rate constant and molecular weight of the two bacterial catalases and calculated as described in Methods. The time scale is in hours. To compensate for the longer generation time during anaerobic growth, the anaerobic catalase activity is plotted as one-half actual time (Table 2).*

DISCUSSION

These bacteria grown under these conditions require electron transport through the oxidases for growth, and anaerobic growth is nitrate dependent (White and Smith, 1962).

The order of cytochrome reduction from DPNH to terminal electron acceptor is assumed to be the same as in other cytochrome systems (Fig. 7).

The DPNH oxidase flavoprotein concentration in both phases and between aerobic and anaerobic growth conditions are nearly equal. The cytochrome b_1 seen in the log phase of both aerated growth conditions increases only slightly by the time it can be detected in the stationary phase where most of the cytochrome c_1 is not DPNH reducible. Even with the underestimate inherent in the concentrations of these two pigments from the low log-phase endogenous respiration, their level does not increase remarkably, either with the log to stationary phase or the aerobic to anaerobic growth transitions. The cytochrome c_1 and oxidases, on the other hand, increases 2- to 7-fold between log and stationary phase, and between aerobic and anaerobic growth conditions.

In these bacteria, there is an onset of rapid cytochrome c_1 synthesis which begins earlier in the growth cycle and reaches a higher final level the longer the generation time becomes. Where growth is most rapid (the aerated log phase), the cytochrome c_1 content is so low it is masked

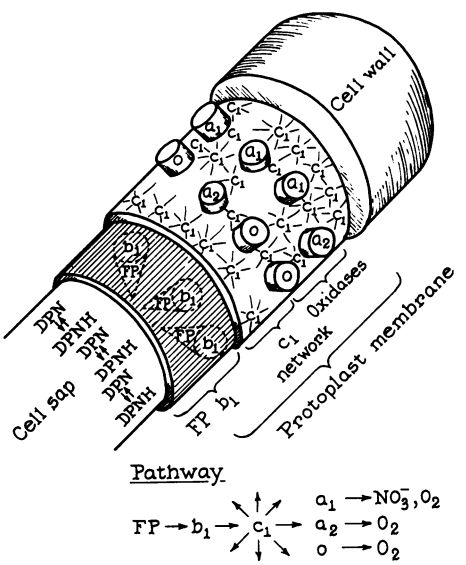

FIG. 7. *Diagram of the electron-transport system of H. parainfluenzae. This represents a hypothesis on the distribution of the cytochromes in the cell membrane. Fp, b_1, c_1, a_1, a_2, and o stand for the flavoprotein and the cytochromes.*

by cytochrome b_1. In addition, the total cytochrome-oxidase concentration increases as the unfavorable growth conditions of stationary phase or of anaerobic growth prevail. Not only is the total oxidase level increased under unfavorable growth conditions, but there seem to be marked changes in the relative levels of the three oxidases. Cytochrome a_2 predominates where there is a limited oxygen supply, and cytochrome a_1 increases greatly as nitrate replaces oxygen as the terminal acceptor of electrons.

As conditions of growth become less favorable, more of the oxidase molecules are formed relative to the DPNH oxidase flavoprotein cytochrome b_1 concentration. This results in an increasingly branching electron-transport system. Concurrent with this increased branching, very large concentrations of DPNH-reducible cytochrome c_1 are formed. The formation of this increased level of cytochrome c_1 begins earlier in the growth phase and reaches a higher level the less favorable the growth condition (measured in terms of the generation time). This increased level of DPNH-reducible cytochrome c_1 is necessary for the enzymatic reduction of the oxidase molecules. With prolonged incubation, both the enzymatically reducible cytochrome c_1 and the oxidases a_1 and o decrease. One can visualize a compensatory mechanism for unfavorable growth conditions as the formation of an increasingly branched electron-transport system involving a network of enzymatically reducible cytochrome c_1 molecules connecting a DPNH oxidase flavoprotein to several oxidase molecules (Fig. 7).

This study indicates that at least a part of the cytochrome c_1 is synthesized in the solute form. Perhaps this is detectable as DPNH non-reducible after saturating its sites of attachment to the membrane. The oxidase molecules are very likely made individually, then assembled into the electron-transport system; the pattern of oxidase concentrations can change remarkably under conditions of little or no cell division.

Where nitrate becomes the terminal acceptor of electrons, cytochrome a_1 is synthesized in largest amounts. This reaction is much less efficient than that of oxygen, in terms of generation time, despite the 50-fold concentration advantage. An additional compensatory mechanism has been detected in the appearance of an unknown system that completely removes nitrite from the media during the stationary phase (White, *unpublished data*). Nitrite is toxic to the respiratory system (White and Smith, 1962).

It is not clear why a pathogen living in the vertebrate nasopharynx should retain an elaborate mechanism to utilize nitrate, unless there is an unknown electron acceptor for anaerobic growth available in the nasopharynx, or there may be an unrecognized saprophytic existence of these bacteria.

Using extinction coefficients and molecular weights of similar cytochromes in other species, it is calculated that there are between 10^3 and 10^5 cytochrome c_1 molecules, about 10^3 molecules of cytochrome b_1, and 10^3 to 10^4 oxidase molecules in a bacterial cell.

The increased level of catalase activity with aeration may result from the mechanism demonstrated by Clayton (1961*a*,*b*) in *R. spheroides*; he proposed aeration-producing H_2O_2, which stimulates an inducer, which in turn calls forth de novo catalase synthesis.

In *H. parainfluenzae*, vigorous aeration induces a 10- to 15-fold greater catalase concentration than found in anaerobically grown cells. Catalase activity is greatest where the cytochrome content is lowest. The function of the catalase is obscure. Growth of this strain of *Haemophilus* is inhibited by 200 μM H_2O_2, which is less than that required to inhibit most other species of bacteria (M'Leod and Gordon, 1923). Perhaps the catalase acts in some unknown manner to potentiate the cytochrome oxidases in the presence of oxygen.

ACKNOWLEDGMENTS

I wish to thank Lucile Smith, in whose laboratory these studies were begun and from whom the techniques and implications of the data had their genesis. Her continued interest and criticism are very gratefully appreciated. I also wish to thank S. Granick and D. Mauzerall for continued encouragement and valuable criticism, and S. S. White for technical assistance.

LITERATURE CITED

CHANCE, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science **120**: 767–776.

CHANCE, B., AND D. HERBERT. 1950. Enzyme substrate complexes of bacterial catalase and peroxidase. Biochem. J. **46**:402–414.

CLAYTON, R. K. 1956. Purified catalase from

Rhodopseudomonas spheroides. Biochim. et Biophys. Acta **36**:40–47.

CLAYTON, R. K. 1961*a*. Physiology of induced catalase synthesis in *Rhodopseudomonas spheroides*. J. Cellular Comp. Physiol. **55**:1–7.

CLAYTON, R. K. 1961*b*. An intermediate stage in the H_2O_2-induced synthesis of catalase in *Rhodopseudomonas spheroides*. J. Cellular Comp. Physiol. **55**:9–14.

CORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.

HERBERT, D. 1955. Catalase from bacteria (*Micrococcus lysodeikticus*), p. 784–788. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 2. Academic Press, New York.

HERBERT, D., AND J. PINSENT. 1948. Crystalline bacterial catalase. Biochem. J. **143**:193–202.

M'LEOD, J. W., AND J. GORDON. 1923. Catalase production and sensitiveness to H_2O_2 amongst bacteria. J. Pathol. Bacteriol. **26**:326–331.

SMITH, L., AND D. C. WHITE. 1962. Structure of the respiratory chain as indicated by studies with *Hemophilus parainfluenza*. J. Biol. Chem. **237**:1337–1341.

WHITE, D. C., AND L. SMITH. 1962. Hematin enzymes of *Hemophilus parainfluenza*. J. Biol. Chem. **237**:1332–1336.