

# Factors Affecting the Affinity for Oxygen of Cytochrome Oxidases in *Hemophilus parainfluenzae*

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The rate of oxygen utilization by the electron transport system at low oxygen concentrations has been difficult to explain mechanistically (1-3). As in most enzymatic reactions the rate of oxygen utilization is independent of oxygen concentration when the oxygen concentration is high, but becomes dependent upon the oxygen concentration at some critical value of the oxygen tension. Chance (4) has provided evidence from spectrophotometric studies that the rate of oxygen uptake is proportional to the rate of production of fumarate from succinate and to the ratio of reduced cytochrome oxidase to oxidized cytochrome oxidase. By use of an analogue computer, Chance (4) has explained the behavior of this system and accounted for both the influence of the oxygen tension and the influence of the reduced oxidase concentration on the rate of oxygen utilization.

Certain characteristics of *Hemophilus parainfluenzae* permit the study of the effect of the composition of the electron transport system on the rate of oxygen utilization at low oxygen concentrations. The intact bacteria are permeable to NADH which reacts rapidly with the electron transport system. The electron transport system of these bacteria contains flavoproteins, cytochromes  $b_1$ ,  $c$ , and  $c_1$ , and cytochrome oxidases  $a_1$ ,  $a_2$ , and "o" (5). The proportions of the intermediate cytochromes to the oxidases in the electron transport chain can be changed markedly by varying growth conditions (6). Several substrates react directly with the isolated electron transport system to reduce all the cytochrome oxidases but different proportions of cytochrome  $b_1$  (7). This study indicates that the concentration at which the oxygen tension affects the rate of oxygen utilization can be raised by reducing the proportion of cytochrome oxidase to cytochrome  $b_1$ , by using submaximal amounts of substrate, or by using respiratory inhibitors. The critical oxygen concentration is a function of the rate of electron transfer to the oxidases as well as of the rate of reoxidation of the oxidases by oxygen.

## EXPERIMENTAL PROCEDURE

**Growth of Bacteria**—The bacterial strain used in this study was a rapidly growing mutant (7) of *H. parainfluenzae* grown in a proteose-peptone medium (5). Aerated cultures were grown in 100 ml of medium in 1-liter Erlenmeyer flasks. During growth, the flasks were shaken on a rotary shaker at 200 r.p.m. Cultures grown with poor aeration were incubated in stationary 2.5-liter low form Erlenmeyer flasks containing 400 ml of medium. The cultures were incubated for 12 hours at 37° and bacteria were

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harvested by centrifugation, washed once in 0.05 M phosphate buffer pH 7.6, and resuspended in the same buffer as described previously (8). Under these growth conditions, cells were collected in early stationary growth phase.

**Oxygen Uptake**—Oxygen uptake was measured with an oxygen electrode (8).

**Difference Spectra**—The difference in absorption spectrum between anaerobic and aerobic suspensions of bacteria were made by use of the technique developed by Chance (9) as described previously (6, 8). The relative concentrations of cytochromes were estimated from difference spectra of a suspension containing about 10 mg of bacterial protein per ml in the presence of saturating concentrations of NADH and in the absence of any substrate. Under these conditions the dispersion of the Cary model 15 is 1  $m\mu$  at 550  $m\mu$ . Estimation of the relative concentration of cytochrome  $b_1$  was made from the absorbance difference between 561  $m\mu$  and 570  $m\mu$ ; cytochrome  $a_1$  from the difference in absorbance at 600  $m\mu$  and 620  $m\mu$ ; and cytochrome  $a_2$  from the difference in absorbance at 635  $m\mu$  and 660  $m\mu$ . The relative concentration of cytochrome  $o$  was estimated from similar difference spectrum in which the spectrum of a bacterial suspension containing NADH and saturated with carbon monoxide in the test cuvette was measured against a similar suspension containing only NADH in the blank cuvette. The absorbance difference between 410  $m\mu$  and 432  $m\mu$  was used as a measure of the relative concentration of cytochrome  $o$ . The natures of these spectra are shown in Fig. 1.

**Reagents**—NADH supplied by Sigma Chemical Corporation was dissolved immediately before use in 0.05 M Tris pH 8.6. Other reagents were as described elsewhere (5-8, 10).

**Protein Determination**—Protein determination involved a modified biuret reaction (5).

## RESULTS

The respiratory pigments of *H. parainfluenzae* grown with vigorous aeration are cytochrome  $b_1$  and cytochrome  $o$  as seen in Fig. 1A. The same bacteria grown under conditions of poor aeration form an electron transport system seen in the difference spectrum to consist of a NADH oxidase flavoprotein, cytochromes  $c_1$  and  $b_1$ , and the oxidases  $a_1$ ,  $a_2$ , and  $o$  as indicated in Fig. 1B. The differences in number and concentration of respiratory pigments found in cells grown with poor aeration represent an increased branching of the electron transport system (6). Cells grown with poor aeration produce approximately 4.6 times the cytochrome  $b_1$ , 11 times the cytochrome oxidase  $o$ , at least 5 times the cytochrome oxidase  $a_1$ , and at least 6 times the cyto-

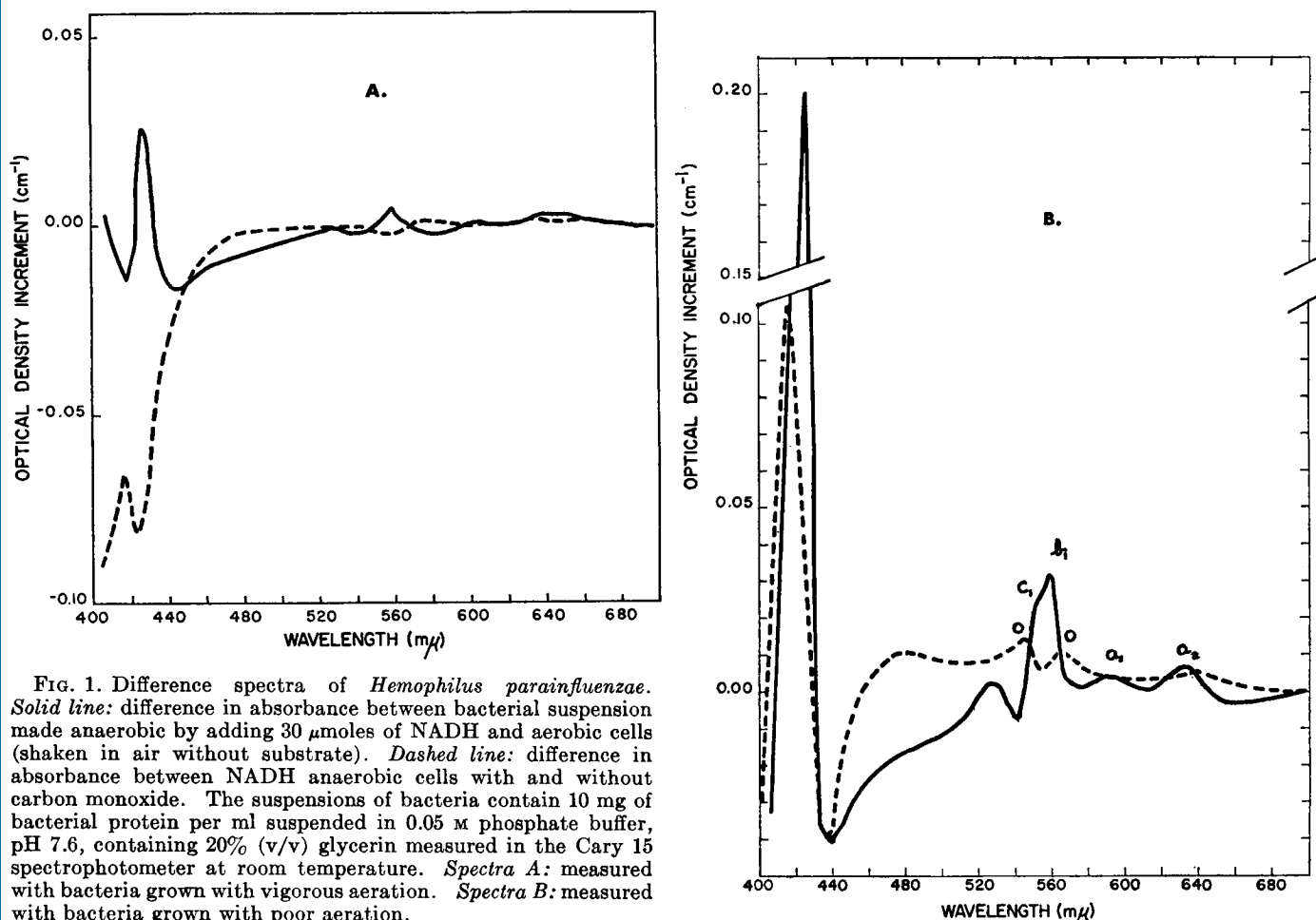


FIG. 1. Difference spectra of *Hemophilus parainfluenzae*. *Solid line*: difference in absorbance between bacterial suspension made anaerobic by adding 30 μmoles of NADH and aerobic cells (shaken in air without substrate). *Dashed line*: difference in absorbance between NADH anaerobic cells with and without carbon monoxide. The suspensions of bacteria contain 10 mg of bacterial protein per ml suspended in 0.05 M phosphate buffer, pH 7.6, containing 20% (v/v) glycerin measured in the Cary 15 spectrophotometer at room temperature. *Spectra A*: measured with bacteria grown with vigorous aeration. *Spectra B*: measured with bacteria grown with poor aeration.

chrome oxidase *a*<sub>2</sub>. Thus, there is half the relative concentration of oxidase *o* and somewhat more than half as much oxidase *a*<sub>1</sub> and *a*<sub>2</sub> per cytochrome *b*<sub>1</sub> as in bacteria grown with poor aeration. Doubling the concentration of a bacterial suspension similar to that used for Fig. 1A confirms the absence of detectable cytochrome *c*<sub>1</sub> and the low levels of cytochromes *a*<sub>1</sub> and *a*<sub>2</sub> shown in Fig. 1A.

The rate of oxygen uptake of *H. parainfluenzae* grown under

the two different growth conditions is shown in Fig. 2. The absence of an endogenous respiratory rate validates the use of difference spectra as a measure of relative cytochrome concentrations. With bacteria grown with poor aeration as seen in Fig. 2B, there is a rapid zero order reaction rate of oxygen uptake. The points at which the reaction is no longer zero order, indicated by the arrows in Fig. 2, represent the critical oxygen concentrations. The value of the critical oxygen concentration is higher in bacte-

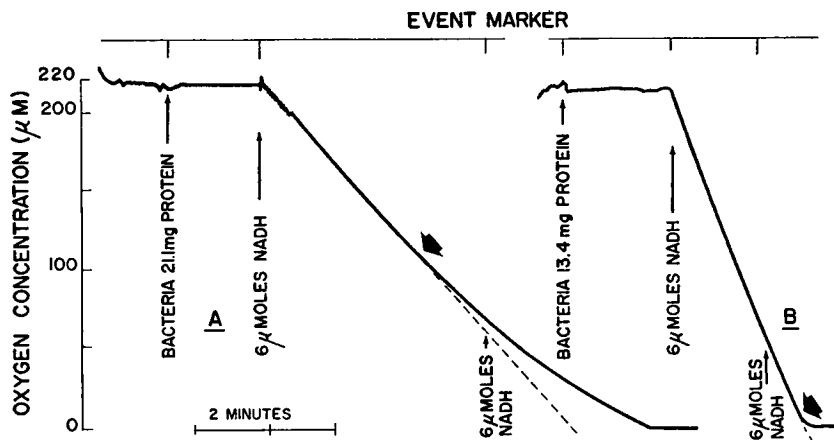


FIG. 2. Tracing of recording from oxygen electrode. The chamber contained 2.7 ml of 0.05 M phosphate buffer, pH 7.6, saturated with air at 30° to which 0.3 ml of bacterial suspension grown with vigorous aeration (A) and poor aeration (B) was added. The trace irregularities resulted from adding bacteria or 0.03 ml of 0.2 M NADH. The arrows indicate the critical oxygen concentrations.

TABLE I  
Effect of growth condition of *Hemophilus parainfluenzae* on critical oxygen concentration, apparent  $K_m$ , and rate of oxygen uptake

Growth condition	Critical O <sub>2</sub> concentration	$K_m^*$		Rate of oxygen uptake†	
		A	B	Per 10 mg protein	Per equal changes in absorbance of cytochrome $b_1$
	$\mu M$	$\mu M$ O <sub>2</sub>		$\mu moles$ O <sub>2</sub> /sec	
Vigorous aeration.....	110	11.1	12.1	0.91	14.0
Poor aeration.....	4.5	2.2	1.8	4.16	13.9

\* Calculated: A, from Lineweaver-Burk (11) plot (Fig. 3) and approximated as half-maximal rate; B, from Walker-Schmidt (12) plot (Fig. 4).

† Rates of oxygen uptake normalized to 10 mg of protein and to a change in absorbance of 0.10 for cytochrome  $b_1$ .

ria grown with vigorous aeration as seen in Fig. 2A. The rate of oxygen uptake per unit of cytochrome  $b_1$  is equal for bacteria grown under both conditions as seen in Table I. In terms of bacterial protein the rate of oxygen uptake is 4.5 times faster in bacteria grown with poor aeration.

A Lineweaver-Burk (11) plot of the reciprocal of the rate of oxygen utilization against the reciprocal of the oxygen concentration for oxygen concentrations less than the critical oxygen tension is shown in Fig. 3. Fig. 3 inset was plotted from a 10-fold magnification of the tracing made with the Nikon optical comparator model 4 between 22  $\mu M$  and zero  $\mu M$  oxygen. Estimates of the oxygen concentrations at which the rate of oxygen utilization is half the maximal rate agrees with the  $K_m$  values calculated from these plots. The lower  $K_m$  value approximates that reported for mitochondria by Chance (4).

A Walker-Schmidt (12) plot of the integrated Michaelis-Menten expression made with the data of Fig. 2A and a 10-fold magni-

fication of Fig. 2B, illustrates linearity between 77  $\mu M$  and 7.0  $\mu M$  oxygen in cells grown with vigorous aeration (Fig. 4A) or between 2.5  $\mu M$  and 0.8  $\mu M$  oxygen in cells grown with poor aeration (Fig. 4B). With this type of treatment of the data, lower values of substrate concentration are the most subject to calculation errors (13). The agreement between observed and extrapolated values of the maximal velocity (the abscissal intercept) is illustrated in Fig. 4. The  $K_m$  values calculated from this plot, shown in Table I, are similar to those predicted by the Lineweaver-Burk method.

It has been previously observed that in the presence of nitrate *H. parainfluenzae* grows anaerobically with concomitant stimulation of the formation of cytochrome oxidase  $a_1$  and to a lesser extent cytochrome oxidase  $o$  (5, 6). As shown in Fig. 3, nitrate is a competitive inhibitor for oxygen in bacteria grown with vigorous aeration. Lines of slope equal to  $(1 + I/K_I)K_m/V_{max}$  for 0.02 M and 0.06 M nitrate in Fig. 3 were drawn over the experimentally determined points using  $K_I = 9.2 \times 10^{-3}$  M calculated for 0.06 M nitrate by the method of Dixon and Webb (14). In cells grown with poor aeration, nitrate in concentrations as large as 0.1 M has no effect on the rate of oxygen utilization.

The effect on the critical oxygen concentration of lowering the rate of electron transport or of competing with the oxidases is seen in Fig. 5. The lower the concentration of NADH added to the bacteria, the higher the critical oxygen concentration. The addition of 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide, which interferes with electron transport, before the addition of NADH results in the reduction of the cytochromes  $b_1$  and  $c_1$ . However, less than 1% of the cytochrome oxidases are reduced. The reduction of the oxidases is measured by their ability to form carbon monoxide complexes. Adding increasing concentrations of the quinoline-*N*-oxide derivative increases the critical oxygen concentration. The same result is found for nitrate.

When carbon monoxide, which reacts with the reduced oxidases, is bubbled into a suspension of bacteria grown with poor aeration until the oxygen concentration is reduced to 110  $\mu M$  and NADH is then added, the resulting rate of oxygen uptake resembles that shown in Fig. 2A. It appears that inhibition of a

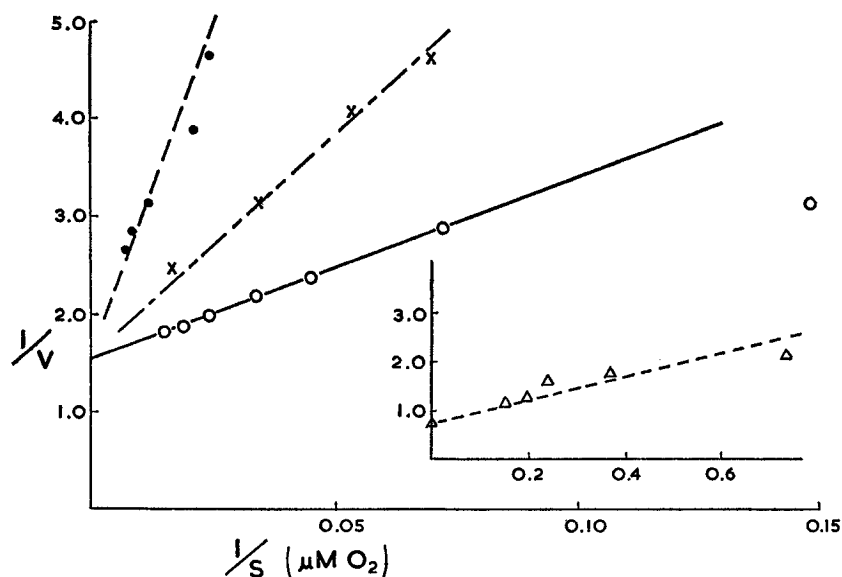


Fig. 3. Lineweaver-Burk (11) plots of oxygen versus oxygen uptake measured below the critical oxygen concentration.  $\circ$ — $\circ$  refers to rate of oxygen uptake measured from Fig. 2A,  $\times$ — $\times$  and  $\bullet$ — $\bullet$  refer to measurements made in the presence of 0.02 M and 0.06 M nitrate and NADH. The inset figure is for data taken from a 10-fold magnification of Fig. 2B.

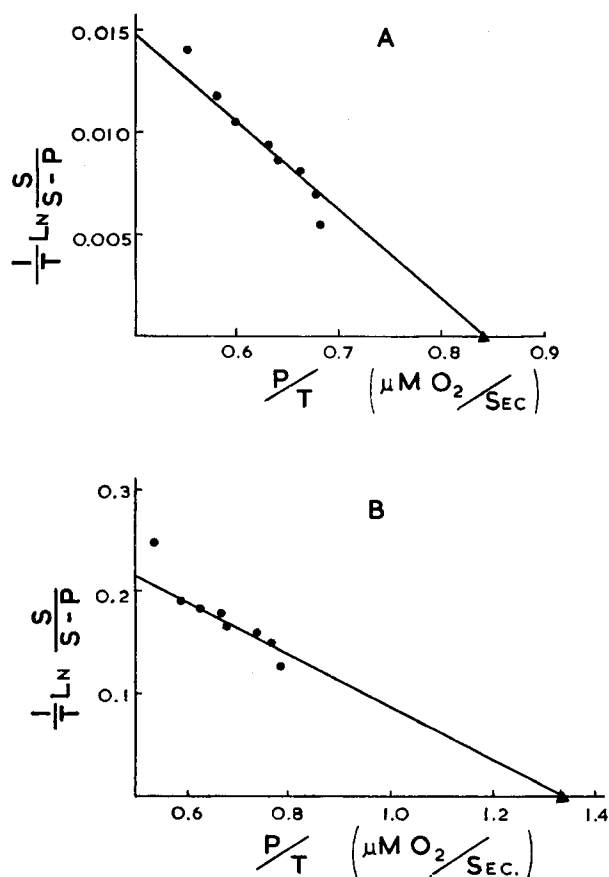


FIG. 4. Rate of oxygen utilization versus oxygen concentration obtained by the integrated rate expression of Walker-Schmidt (12) for: A, bacteria grown with vigorous aeration taken from Fig. 2A; B, bacteria grown with poor aeration taken from a 10-fold magnification of Fig. 2B.  $\blacktriangle$  indicates the observed maximal rate of oxygen utilization expressed in  $\mu\text{M O}_2$  per second.  $S$  represents the oxygen concentration at zero time and  $S - P$  the concentration at time  $T$ .

portion of the cytochrome oxidases produced by growth under conditions of poor aeration causes the behavior of these organisms to resemble that of bacteria grown with good aeration.

The electron transport system of *H. parainfluenzae* can be directly reduced by some substrates other than NADH. These substrates reduce different amounts of cytochromes  $b_1$  and  $c_1$  but all completely reduce the cytochrome oxidases with either intact bacteria or cell-free electron transport preparations (7, 10). These substrates that reduce a lesser proportion of the cytochrome  $b_1$  would be expected to produce a higher critical oxygen concentration and lower rates of oxygen utilization. This is indeed the case as seen in Table II. Cytochrome  $a_2$  is conveniently used as a measure of reduction of the oxidases as its  $\alpha$ -maximum is far from those for interfering cytochromes.

If the critical oxygen concentration is a reflection of the Michaelis constant of the reduced oxidases for oxygen, the critical oxygen concentration should be independent of the amount of bacteria added so long as the concentration of oxidases is much less than that of dissolved oxygen. To have measurable rates of oxygen uptake at higher bacterial densities, succinate must be used as substrate. By use of sufficient succinate to saturate the electron transport system, the critical oxygen concentration is

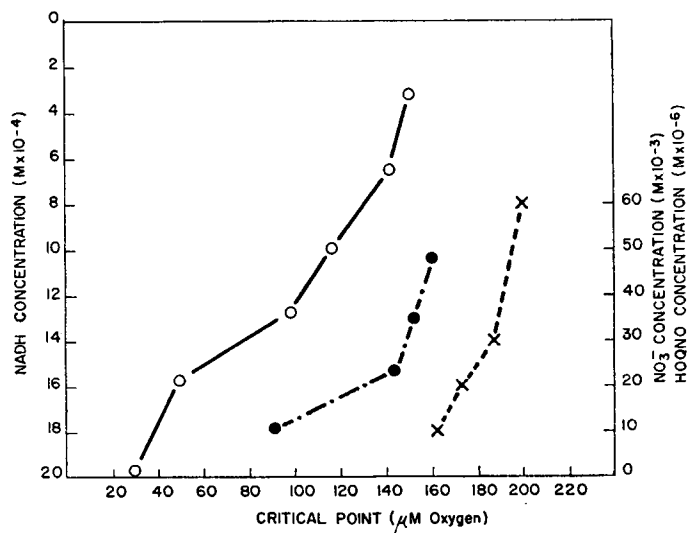


FIG. 5. Effect of NADH (O—O) and respiratory inhibitors on the critical oxygen concentration measured as in Fig. 2 on bacteria grown with poor aeration after adding 25.8 mg of bacterial protein to the oxygen electrode. The 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (●—●) and nitrate (X—X) were added to similar bacterial suspensions before the addition of 6  $\mu\text{moles}$  of NADH. Note that the direction of the ordinates is not the same.

TABLE II

Effect of different substrates which reduce different amounts of cytochrome  $b_1$  on the critical oxygen concentration with *Hemophilus parainfluenzae*

Experiment*	Substrate	Absorbance		Initial rate of oxygen uptake per 10 mg protein†	Critical oxygen concentration
		Cytochrome $b_1$ reduced	Cytochrome $a_2$ reduced		
1	Formate	0.032	0.006	16.7	1.4
2	NADH	0.030	0.006	4.16	4.5
3	L-Lactate	0.026	0.006	3.74	78.0
4	D-Lactate	0.024	0.006	2.80	138.0
5	Succinate	0.024	0.006	1.99	165.0

\* Experiments 2 and 5 are with the same bacterial suspension used in Fig. 1; Experiments 1, 3, and 4 are with a similar bacterial suspension grown with poor aeration.

† Difference in absorbance per 10 mg of protein measured as described in "Experimental Procedure" and between identical bacterial suspensions oxygenated with air and at zero oxygen tension after addition of saturating substrate concentrations.

‡ Measured at saturating substrate concentrations.

TABLE III

Variation of critical oxygen tension with bacterial concentration of *Hemophilus parainfluenzae*

Oxygen uptake in the presence of 0.03 M succinate measured as in Fig. 2A with bacteria grown with vigorous aeration.

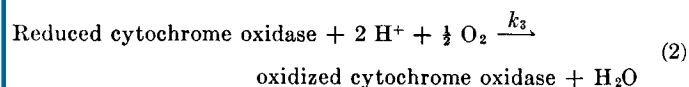
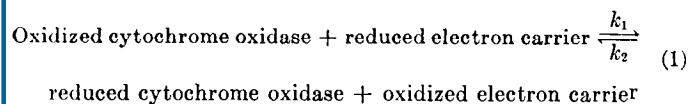
Bacterial protein	Critical O <sub>2</sub> concentration	Initial rate of oxygen uptake
mg/3 ml	$\mu\text{M O}_2$	$\mu\text{moles O}_2/\text{sec}$
65.1	132	0.99
130.2	128	1.99
195.3	127	3.09



little affected by a 3-fold increase in cell density, although the rate of respiration is tripled as shown in Table III.

#### DISCUSSION

The linear plot of the integrated Michaelis-Menten expression suggests that the interaction of reduced cytochrome oxidase and oxygen at low oxygen concentrations implicates two sequential reactions involving the formation and breakdown of an enzyme-substrate complex. This can be formulated as follows.



$K_m$  in this system equals  $(k_2 + k_3)/k_1$ . Since reactions at the oxidase end of the electron transport system are essentially irreversible,  $K_m$  can be approximated as  $k_3/k_1$ . In enzyme systems with this simple type of kinetics, 90% of the maximal velocity is achieved at a substrate concentration equal to 10 times  $K_m$  (15). The critical oxygen point, the oxygen concentration giving maximal velocity is thus a measure of  $K_m$ . When the oxygen electrode is used as described in this study, the errors in estimation of oxygen concentration and rates of oxygen utilization are larger the lower the oxygen tension. Consequently, the estimation of  $K_m$  directly or from the critical oxygen point is more subject to error the lower the value.

This study shows that decreased electron flow to the oxidases raises the critical oxygen concentration and thus the  $K_m$ . Reducing the value of  $k_1$  without affecting  $k_3$  by using suboptimal substrate concentrations or substrates which do not reduce all the intermediate electron carriers raises the critical oxygen concentration. Inhibiting electron transport between cytochrome  $c_1$  and the oxidases with 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, as well as decreasing the proportion of cytochrome electron carriers to cytochrome oxidases by vigorous aeration during growth, decreases  $k_1$  and raises the critical oxygen concentration. Adding inhibitors which are competitive with oxygen for reduced cytochrome oxidase such as nitrate or carbon monoxide produces similar effects.

*H. parainfluenzae* cannot grow without forming an electron transport system (5). When grown under conditions of poor aeration, it forms new oxidases  $a_1$  and  $a_2$  and expands and branches its electron transport system by increasing the cytochrome  $b_1$  concentration and greatly increasing the oxidase and cytochrome  $c_1$  concentrations (6). This produces an electron transport system with a  $K_m$  typical of that for mitochondrial cytochrome oxidase (1-4). Bacteria grown with vigorous aeration, reducing  $k_1$  by decreasing the proportion of cytochrome oxidase to cytochrome  $b_1$ , produce an electron transport system with a poor affinity for oxygen. Increasing the proportion of cyto-

chrome oxidases to cytochrome  $b_1$  and forming new oxidases to compensate for growth at low oxygen tensions allows *H. parainfluenzae* the maximal rate of oxygen uptake at oxygen tensions just slightly above the concentration at the half maximal respiratory rate.

#### SUMMARY

The electron transport system of *Hemophilus parainfluenzae* can be used to demonstrate that the proportion of the oxidases maintained reduced determines the oxygen concentrations at which the rate of oxygen uptake becomes dependent upon the oxygen concentration. Factors that reduce electron flow to the oxidases such as: reducing only part of the cytochrome  $b_1$ , submaximal substrate concentrations, electron transport inhibitors, or growth conditions that reduce the amount of oxidases relative to the cytochrome  $b_1$ , raise the threshold below which the rate of oxygen uptake is a function of the oxygen concentration. Lowering the effective concentration of oxygen with competitive inhibitors of cytochrome oxidase such as carbon monoxide and nitrate also raises the critical oxygen tension. These studies suggest that the oxygen *versus* oxygen uptake relationship at low oxygen concentrations behaves as a typical enzymatic reaction dependent upon the formation of an enzyme-substrate complex. This bacterium enlarges and branches its electron transport system when grown at low oxygen tension to maintain the maximal respiratory rate at low oxygen concentrations as a compensatory mechanism. The electron transport system formed during growth with vigorous aeration has a higher  $K_m$  for oxygen than the system that appears during growth with poor aeration.

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