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Localization of the Enzymes That Catalyze Hydrogen and Electron Transport in *Hemophilus parainfluenzae* and the Nature of the Respiratory Chain System*

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Hemophilus parainfluenzae possess a respiratory chain system with many characteristics similar to those of the mammalian system (1) and of a number of other bacteria (2): it is composed of typical flavoprotein dehydrogenases and six cytochrome pigments, three of which appear to be oxidases (3–5). These pigments are bound to particles which presumably are derived from the cytoplasmic membrane. Unusual aspects of the respiratory chain of *H. parainfluenzae* are the accumulation, under some conditions of growth, of large amounts of one cytochrome that is not reducible in the presence of substrates (4, 6, 7), and the variability of the proportions of the different pigments observed under different growth conditions and in different phases of growth (5–7). Further studies, reported here, show that bacteria having widely varying proportions of the different cytochromes can have respiration rates that are similar with a number of substrates.

These bacteria are readily permeable to substrates and pyridine nucleotides (8). Thus it is possible to compare the reactions of the membrane-bound system in intact bacteria and in the small membrane fragments derived from it upon rupture of the cells. Such studies show little modification of the reactivity immediately after preparation of the membrane fragments, but upon standing some pigments become dissociated from the membrane. As long as the pigments are associated with the membrane in proper orientation, electron transport can proceed rapidly, and the cytochromes and flavoproteins can be seen to undergo oxidation and reduction during electron transport. In either intact cells or particles, the over-all rate of electron transport is always limited by the reaction of the membrane-bound flavoprotein dehydrogenases with the appropriate substrate. Pyridine nucleotide is reduced by dehydrogenases which are not membrane-bound, and the rates of reduction of diphosphopyridine nucleotide are low.

Taken together, all of the studies on the respiratory chain system of *H. parainfluenzae* show that its properties resemble those of mammalian mitochondria in most aspects. However, there is compelling evidence that in these bacteria the system is not composed of fixed units of pigments with a definite "stoichiometry" of the components.

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EXPERIMENTAL PROCEDURE

The bacteria used in most of the experiments are a mutant of the strain of *H. parainfluenzae* (Boss No. 7) previously studied with respect to the cytochrome system (3, 4). The mutant, which appeared spontaneously during transfers of the parental type, has the same cytochrome components as the parental type as shown by measurements of difference spectra (7). However, cytochrome c_1 is synthesized at a lower rate by the mutant, with the result that cytochrome b_1 predominates in the late log phase cells under the growth conditions used. In contrast, when grown under the same condition, the parental type contains a large amount of cytochrome c_1 that is not reducible with substrate and is not membrane-bound (3, 4). The effect of DPN added to intact bacteria in increasing the rate of respiration in the presence of substrate is much more pronounced with the parental type. An advantage of working with the mutant is that the respiration rate shows less variability with the growth phase than does the parent type. The data of Table I, Part A, show that the respiration rates of parent and mutant bacteria are comparable with a number of substrates. The extent of reduction of the predominant cytochrome (c_1 in the parent strain, b_1 in the mutant) is also similar with the different substrates. About 50% of the cytochrome c_1 of the parent strain cannot be reduced by substrates and is reduced only by $Na_2S_2O_4$. The bacteria were grown in 2500-ml Parrot (low form Erlenmeyer) flasks with 400 ml of proteose-peptone medium (3) containing 0.02 M glucose or gluconate (autoclaved separately). After incubation for 14 hours at 37° without agitation, the cells were harvested by centrifugation in the cold, washed once with cold 0.05 M phosphate buffer, pH 7.6, then suspended in buffer to a final concentration of about 100 mg of bacterial protein per ml. The suspension was stored in an ice bath and usually used within 4 hours after harvesting. Contamination of the stock culture was checked (3). These conditions of growth yield bacteria containing high concentrations of the cytochrome pigments (6) as well as flavoprotein dehydrogenases (5).

Suspensions of insoluble respiratory particles were prepared by grinding the bacteria with Alumina A-305 (generously supplied by the Aluminum Company of America) as described (4).

The content of respiratory pigments was estimated from the anaerobic minus aerobic difference spectra (3, 6) in Cary spectrophotometers, models 14CM and 15.

Oxygen uptake was measured polarographically with the Clark electrode (Yellow Springs Instrument Company) in the

TABLE I

Comparison of respiratory rates produced by adding D-lactate, L-lactate, succinate, DPNH, and formate to H. parainfluenzae which formed principally cytochrome b_1 with bacteria which formed primarily cytochrome c_1

Oxygen uptake was measured polarigraphically after addition of approximately 10 mg of bacterial protein and substrates at concentrations of 10 mM, except for DPNH (5 mM), at 30° in 50 mM phosphate buffer, pH 7.6, as reported (9), and is expressed as millimicromoles of O_2 per second per 10 mg of protein. Cytochrome b_1 was estimated as the difference in absorbance between 561 and 575 mμ in anaerobic (in the presence of the substrate) minus aerobic difference spectrum; cytochrome c_1 was estimated similarly at 553 and 575 mμ per 10 mg of protein.

Substrates	Mutant (cytochrome b_1 predominant)			Parental type (cytochrome c_1 predominant)		
	Rate of oxygen uptake	Rate expected if additive	Cytochrome b_1 reduced	Rate of oxygen uptake	Rate expected if additive	Cytochrome c_1 reduced
A. Added singly						
D-Lactate	2.23		0.028	0.67		0.048
L-Lactate	4.60		0.033	3.68		0.053
Succinate	1.48		0.026	2.12		0.064
DPNH	1.77		0.032	2.95		0.064
Formate	43.5		0.034	46.0		0.053
$Na_2S_2O_4$			0.034			0.102
B. Added sequentially						
Succinate + DPNH	2.95	3.25	0.033	5.10	5.07	0.062
Succinate + DPNH + L-lactate	6.75	7.85	0.031	8.90	8.75	0.063
Succinate + DPNH + formate	18.9	46.75	0.030	44.0	51.07	0.054
D- + L-lactate	3.85	6.83	0.031	1.35	4.35	0.054

manner described previously (9). Rates of oxygen uptake are expressed as millimicromoles of oxygen per second per 10 mg of bacterial protein or as millimicromoles of oxygen per second × 0.05/the difference in absorbance at 561 and 580 mμ in the anaerobic minus aerobic difference spectrum.

Ferricyanide reduction was followed spectrophotometrically at 425 mμ (4, 5). Under these conditions of assay, the rate of reduction is zero order (5).

Protein was measured by the biuret method (10) in the presence of 0.06% sodium deoxycholate.

Reagents—Rotenone (K and K Laboratories), thenoyltrifluoroacetone (Fisher Scientific Company), 2,4-dinitrophenol (Amend Drug Company), and carbonyl cyanide *m*-chlorophenylhydrazone (CalBioChem) were added as solutions in 95% ethanol. The inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, the generous gift of Dr. J. W. Lightbown, was dissolved in 0.001 M KOH, and Dicumarol (Mann Research Laboratories) in 0.02 M KOH. The addition of equivalent volumes of ethanol or of KOH to the buffered reaction mixtures has no measurable effect on the rate of oxygen uptake. Quinacrine (3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine hydrochloride), from Nutritional Biochemicals Corporation, and sodium secobarbital (sodium 5-allyl-5-(1-methylbutyl)barbiturate), from Eli Lilly and Company, were prepared in aqueous solutions. Crystalline perfluorosuccinate was synthesized by oxidation of cyclic $C_4F_4Cl_2$ (synthesized by Dr. T. R. Walton, Ohio State University) with permanganate (11).

RESULTS

H. parainfluenzae is characterized by an unusual permeability to pyridine nucleotides and substrates. One washing with weak buffer usually removes endogenous respiration, and an additional washing with weak buffer uniformly removes endogenous respiration. Addition of DPNH to the intact bacteria results in an immediate rapid oxygen uptake with no measurable lag (3). Somewhat lower rates of respiration are observed in the presence of substrates involved with pyridine nucleotide-linked enzymes (8). Since these bacteria have no apparent mechanism capable of oxidizing reduced pyridine nucleotide at a rate sufficient to promote growth at a detectable rate that does not involve electron transport to oxygen or nitrate (8), the addition of various substrates to bacteria suspended in phosphate buffer results in the accumulation of reduced pyridine nucleotide once the oxygen in the suspension is utilized. Consequently, the addition of substrates to bacteria suspended in phosphate buffer is followed by an increase in absorbance at 340 mμ, after the oxygen in the suspension is exhausted. This absorbance change is not a light scattering phenomenon as indicated by the sharp maximum at 340 mμ. These results are illustrated in Fig. 1. Various substrates produce reduced pyridine nucleotide at different rates, but the total amount produced is always the same. Adding other metabolites, TPN, or DPN after reduced pyridine nucleotide production is complete (*right-hand arrow* in Fig. 1*A*) does not result in further increases in reduced pyridine nucleotide. Adding two substrates simultaneously produces a greater rate of reduced pyridine nucleotide production than either added singly but does not effect the total amount produced. Nitrate can cause the reoxidation of reduced cytochromes in this bacterium (3), and the addition of nitrate can cause the disappearance of reduced pyridine nucleotide absorbance. The capacity of the electron transport system to reduce pyridine nucleotide is much greater than the rate at which it is generated. The amount of reduced pyridine nucleotide produced in the presence of substrates is between 100 and 200 mμmoles per g, dry weight.

If stationary phase bacteria are held at 0° for several hours in phosphate buffer, the rates of oxygen utilization with these substrates can be increased by adding DPN. Such an experiment with the use of citrate is illustrated in Fig. 2. Anaerobically these bacteria can produce DPNH from the DPN added to intact cells in the presence of a suitable substrate. The DPNH produced can reach levels 100 times that detected with freshly harvested bacteria in the presence of substrate. The

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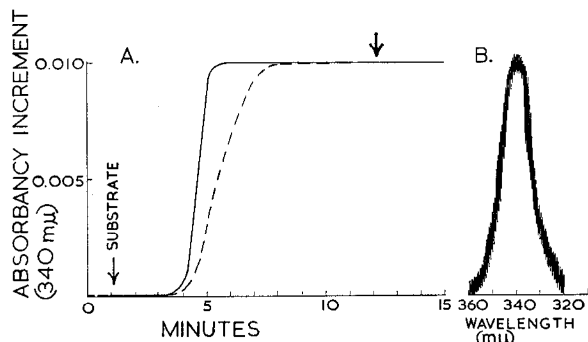

FIG. 1. *A*, change in absorbance at 340 mμ with time in the presence of 10 mM α-ketoglutarate (——) or 10 mM citrate (- - -) to suspensions of intact parental type *H. parainfluenzae*. The sample cuvette was allowed to go anaerobic in the presence of substrate, which was added when indicated by the *left-hand arrow*. Experiments with an identical suspension with the oxygen electrode established that pyridine nucleotide reduction began after anaerobiosis and that there was no endogenous respiratory activity. Addition of other substrates or 1 mM DPN or TPN at the time indicated by the *right-hand arrow* had no effect on the amount of pyridine nucleotide reduced. Data were obtained with the Cary model 14CM spectrophotometer with the scattered transmission accessory and intense visible light source between identical bacterial suspensions containing 14.5 mg of protein per cm of light path in 50 mM phosphate buffer, pH 7.6, at 25°. Under these conditions the dispersion of the spectrophotometer at 340 mμ was ±9 A. *B*, the difference spectrum of the reduced pyridine nucleotide produced 18 minutes after the addition of α-ketoglutarate, plotted to give an indication of the noise level. Similar difference spectra are produced with glucose, malate, citrate, and pyruvate.

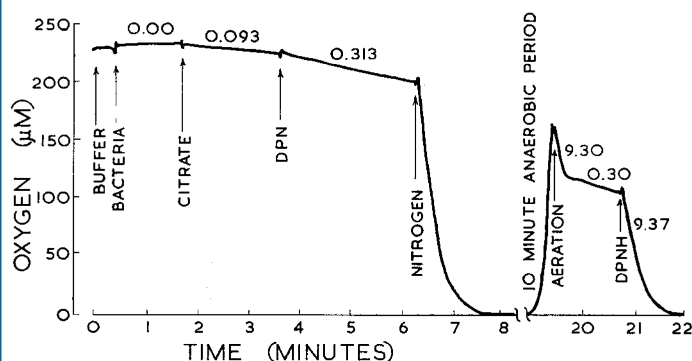

FIG. 2. Tracing of the respiration of intact parental type *H. parainfluenzae* (34.0 mg of protein) grown with 20 mM glucose, added to 3 ml of 50 mM phosphate buffer, pH 7.6, at 30° in the oxygen electrode as described (7, 9). Irregularities result from adding bacteria, substrates, or bubbling nitrogen or air (aeration). The bacteria have no endogenous respiration, and utilize oxygen in the presence of 20 mM citrate and 6 mM DPN. The suspension is then deoxygenated with nitrogen and incubated with bubbling nitrogen for 10 minutes in the presence of 0.05 ml of Dow-Corning Antifoam B. After 10 minutes, the suspension is aerated with air, and the initial rate observed after aeration is equivalent to that of added DPNH (3 mM). Respiratory rates are written above the tracing, expressed as millimicromoles of O_2 per second.

parental type reacts more readily with added DPN. The ability to reduce added DPN can be used to show that DPNH formed anaerobically can be oxidized in the presence of oxygen at the same rate as DPNH added to the bacterial suspension. This result is illustrated in Fig. 2.

Although intact cells can oxidize a large variety of substrates (8), the insoluble particles which bear the electron transport chain can only respire in the presence of formate, DPNH, D- and L-lactate, and succinate. These are the substances most rapidly oxidized by the intact cells (8). A comparison of the rates of respiration of intact cells with respiratory particles is shown in Table II. The electron transport system is concentrated in the membrane fragments as indicated by the increase in specific activity of the substrate-provoked respiration (Table II) and the demonstration that all the substrate-reducible cytochromes are found in this fraction (4). The membrane-linked primary dehydrogenases of these bacteria can be assayed readily by use of ferricyanide reduction in the presence of cyanide (5). The ferricyanide reduction capacity of the particles with DPNH, lactate, or succinate corresponds to the oxygen uptake per electron transferred. Formate ferricyanide reductase has about one-fourth of the activity predicted from its respiratory activity (5). Very little ferricyanide reductase activity is found in the supernatant, indicating that these primary dehydrogenases are all membrane-bound.

The respiratory capacity of the particles can be reduced differentially by prolonged incubation in phosphate buffer at 0° as seen in Table III. There is considerable decrease in specific activity with D- or L-lactate. Even so, the rates of respiration expressed in terms of the extent of reduction of cytochrome b_1 by a given substrate are quite similar for the intact bacteria and aged particles. When fresh membrane preparations are exposed to ultrasonic energy, there ensues a loss of both ferricyanide and oxygen utilization capacity in the membrane fraction, which is exactly balanced by ferricyanide reductase in the supernatant (5). The concentration of substrate giving half-maximal reaction rates for the different substrates are not very different with whole cells and with the small particles, as seen in Table IV.

TABLE II

Comparison of rates of oxygen utilization and ferricyanide reduction of bacteria, respiratory particles, and supernatant fractions of H. parainfluenzae

Bacteria were harvested after 12 hours of incubation, centrifuged, washed with 50 mM phosphate buffer, pH 7.6, ruptured with alumina, and resuspended in phosphate buffer. Centrifugation at 6,000 × *g* for 10 minutes removed alumina and whole cells; then the particles were collected at 12,000 × *g* for 10 minutes. The supernatant was centrifuged at 105,000 × *g* for 45 minutes, and the small membrane fragments were combined with those in the 12,000 × *g* pellet in phosphate buffer. Oxygen uptake measurements were made as in Table I. Ferricyanide reduction was followed spectrophotometrically at 425 mμ after addition of substrates to cuvettes containing about 5 mg of protein, 0.5 mM ferricyanide, and 5 mM KCN in 50 mM phosphate buffer, pH 7.6, at 30° as described (5). DPN (10 mM) was added with glucose and malate.

Substrate	Oxygen uptake		Ferricyanide reduced	
	Bacteria	Particles	Particles	Supernatant
	mμmoles/sec/10 mg protein			
Formate	52.00	58.00	63.0	<0.02
DPNH	6.40	14.00	61.5	6.6
D-Lactate	1.88	2.54	11.2	<0.02
L-Lactate	3.40	6.24	25.0	<0.02
Succinate	1.27	3.00	13.6	<0.02
Malate (+DPN)	0.68	<0.01		
Glucose (+DPN)	0.62	<0.01		

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The relative concentrations of substrate-reducible cytochrome c_1 can be varied over a wide range and still provide the organism with the efficient electron transport system that it needs to survive. The same variability is also possible in the relative concentration of cytochrome b_1. Some of the data on which this conclusion is based are illustrated in Fig. 3. Since the α maximum of reduced cytochrome c_1 is 553 mμ (3) and that of reduced cytochrome b_1 is 561 mμ, the presence of one cytochrome could obscure the presence of the other. Certain information is necessary to evaluate the relative concentration of cytochrome b_1 in the presence of membrane-bound cytochrome c_1. Membrane preparations can be depleted of cytochrome c_1 (4). The membrane-bound and the cytochrome c_1 rendered soluble have essentially identical absorption spectra (4), indicating that functionally active, membrane-bound cytochrome c_1 and soluble cytochrome c_1 have very similar if not identical extinction coefficients. Membrane preparations depleted of cytochrome c_1 have an absorbance at 561 mμ that is proportional to the protein content of the membrane. Intact bacteria of the mutant strain harvested in the late log phase of an appropriate growth condi-

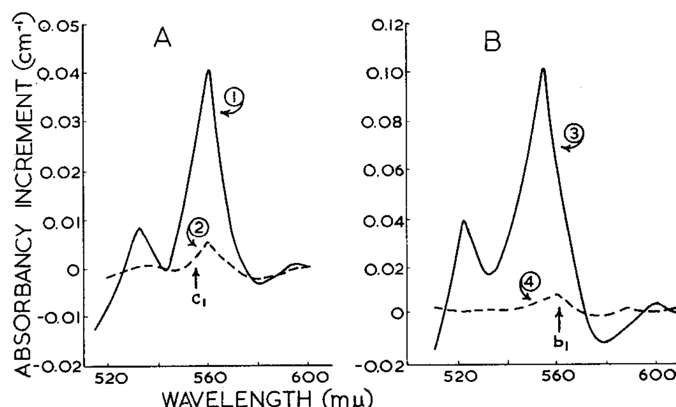

Fig. 3. The difference spectra between membrane fragments of *H. parainfluenzae* allowed to go anaerobic in the presence of DPNH (10 mM) compared to an identical aerobic suspension. Spectra were measured at a protein concentration of 10 mg per ml with membranes prepared by grinding with alumina (4) in 50 mM phosphate buffer, pH 7.6, at 25°. *A*, washed membrane fragments of the mutant which does not form large amounts of cytochrome c_1: *Curve 1* (——), measured with bacteria grown anaerobically with nitrate harvested in the late log phase; *Curve 2* (- - -), measured with bacteria grown with vigorous aeration (7). *B*, *Curve 3* (——), resuspended alumina-ruptured cells of the parental type grown in a deep, unshaken culture flask in medium without glucose were harvested at the initiation of the stationary phase; *Curve 4* (- - -), difference spectrum of these particles washed four times with phosphate buffer, frozen, and thawed twice as described (4). Rates of oxygen uptake were measured at DPNH concentrations that were not rate-limiting and are expressed as millimicromoles of oxygen per second per 10 mg of protein at 30°: *Curve 1*, 3.48; *Curve 2*, 1.00; *Curve 3*, 3.43; *Curve 4*, 0.005. The *arrow* in *Part A* indicates 553 mμ; the *arrow* in *Part B* indicates 561 mμ.

tion contain no detectable cytochrome c_1 on reduction with $Na_2S_2O_4$. On rupture of the bacteria by grinding with alumina, sonic vibration, or the Hughes press, no detectable cytochrome b_1 is found in the supernatant suspension, and all the cytochrome b_1 found in the intact bacteria can be accounted for by that found in the membrane fraction. Subjecting the membranes to the washing and freeze-thaw procedure (4), which removes cytochrome c_1, does not alter the content of cytochrome b_1. If the washings are lyophilized and treated with acid-acetone, and the resultant protohemin chromatographed and detected by its pyridine-hemochromogen or by the ultrasensitive benzidine spray according to classical methods (13), essentially no protohemin can be detected. Bacteria harvested under these growth conditions contain an insignificant catalase activity (6). Treatment of the membrane by this procedure uniformly yields protohemin with a content that roughly parallels the cytochrome b_1 content (14). Consequently the absorbance at 561 mμ on reduction is a real measure of the relative cytochrome b_1 concentration if the influence of cytochrome c_1 on this spectrum can be established. We have established that soluble cytochrome c_1 has a c-type pyridine-hemochromogen (3) and that hematohemin is liberated only after reduction of the thioester bonds (14). If membranes similar to those used for the experiments illustrated in Fig. 3*A*, which show no spectral evidence for cytochrome c_1, are treated with acid-acetone, then reduction with Ag_2SO_4 followed by isolation of hemin or porphyrin, no significant evidence for amounts of the hemato derivative can be found.

Table III

Comparison of respiratory rate of bacteria and aged respiratory particles of H. parainfluenzae

Bacteria were harvested after 24 hours of incubation, centrifuged, washed with 50 mM phosphate buffer, pH 7.6, ruptured with alumina, and resuspended in phosphate buffer. After centrifugation at 6,000 × g for 10 minutes to separate intact bacteria and alumina, particles were collected by centrifugation at 12,000 × g and aged at 0° for 6 hours before use. Oxygen utilization was measured as in Table I and expressed as rate per 10 mg of protein or as rate per 0.05 absorbance unit of cytochrome b_1 reduced after anaerobiosis produced with that substrate.

Substrate	Oxygen utilization per 10 mg of protein		Oxygen utilization per 0.05 absorbance of cytochrome b_1	
	Bacteria	Particles	Bacteria	Particles
	mμmoles/sec		mμmoles/sec	
Formate	26.0	16.0	35.0	34.0
DPNH	4.16	3.12	7.9	7.9
D-Lactate	6.31	0.13	6.5	6.1
L-Lactate	7.40	0.25	7.1	7.0
Succinate	4.66	3.48	6.7	6.7

Table IV

K_m values for formate, DPNH, D-lactate, L-lactate, and succinate measured with intact bacteria and respiratory particles

K_m values were obtained from a Lineweaver-Burk (12) plot of reciprocal respiratory rate *versus* reciprocal substrate concentration with bacteria and respiratory particles similar to those used for Table II.

Substrate	K_m with intact bacteria	K_m with respiratory particles
	mM	mM
Formate	0.04	0.16
DPNH	0.20	0.06
D-Lactate	1.7	0.37
L-Lactate	2.0	0.31
Succinate	0.29	0.10

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The absorbance of soluble reduced cytochrome c_1 at 553 mμ is proportional to the protein concentration. Examination of the spectra of reduced, soluble, partially purified cytochrome c_1 indicates that at 561 mμ it has 65% of the absorbance that it shows at 553 mμ. The influence of cytochrome b_1 on the absorbance of cytochrome c_1 is a bit more difficult to access. We can set an upper limit on the possible effect of cytochrome b_1 on membrane-bound cytochrome c_1 by use of a preparation of the mutant strain incubated in the stationary growth phase until it develops a respiratory system that has the spectrum illustrated in Fig. 1*B* of Reference 7. Bacterial membranes can be depleted of all detectable cytochrome c_1 by repeating the washing and freeze-thaw technique (4) 10 times. This treatment reduces the respiratory activity 1000-fold, and no further cytochrome c_1 can be removed. The soluble cytochrome c_1 removed and the absorption of the remaining cytochrome b_1 roughly correspond to the facts that at 561 mμ cytochrome c_1 accounts for 61% of the absorbance, and that at 553 mμ cytochrome b_1 accounts for 45% of the absorption at 553 mμ. In the case of the wild-type strain illustrated in Fig. 3*B*, the complication of the overlap of reduced cytochrome b_1 and c_1 is readily corrected. Removing all detectable cytochrome c_1 as described (4), a procedure which lowers the rate of oxygen uptake with DPNH 400-fold, indicates that cytochrome b_1 can account for less than 3% of the absorbance at 553 mμ. We have established that this procedure does not remove cytochrome b_1.

With this information in hand, the data of Fig. 3 can be examined. The maximal rates of oxygen utilization measured

TABLE V

Inhibition of respiration of intact H. parainfluenzae in presence of various substrates

Data were calculated from the initial rate of oxygen utilization with substrate alone and the slowest rate achieved within 30 seconds after addition of inhibitor. Inhibitors were added when oxygen tension was greater than 100 μM. Oxygen utilization was measured as in Table I.

Inhibitor	Concentration	Inhibition in the presence of:				
		Formate	DPNH	D-Lactate	L-Lactate	Succinate
	μmoles/3 ml	%	%	%	%	%
Quinacrine	0.12	34	59	97	56	84
Secobarbital	12.0	86	89	98	92	97
Thenoyltrifluoroacetone	30	50	71	96	89	92
Oxalate	60	14	53	95	92	99
Rotenone	0.05	0	0	0	0	25
Malonate	60	0	0	0	0	87
Monoethyloxalacetate	30	0	0	0	0	100
Perfluorosuccinate	60	0	0	0	0	61
Oxamate	60	0	0	0	0	0
2,4-Dinitrophenol	0.1	25	30	50	33	40
CCP	0.01	40	96	60	60	95
Dicumarol	0.09	60	56	96	66	85
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide	0.006	74	73	94	87	98
Azide	60	73	97	97	50	88
Cyanide	5	97	100	100	100	100
Carbon monoxide	40% (v/v)	27	25	20	27	26

in the presence of DPNH concentrations that are not rate-limiting are given in Fig. 3. By comparing the mutant bacteria grown with high aeration (*Curve 2*) and the parental type (*Curve 3*), a respiratory system capable of rapid electron transport can be fashioned by the living bacteria, involving at a *minimum* a 32-fold variability in functional cytochrome c_1 concentration. This assumes that all the absorbance at 553 mμ in *Curve 2* is due exclusively to cytochrome c_1. With correction for the increment that cytochrome b_1 adds to the absorbance at 553 mμ, the *maximal* level of cytochrome c_1 that could be present in this bacterium is 72-fold less than that constructed by the bacterium grown under conditions producing maximal enzymatically reducible cytochrome c_1. This is also an underestimate of the variability of functional cytochrome c_1, since the absence of hematohemin isolated from the membranes similar to those reduced in *Curve 2* indicates that actually little if any cytochrome c_1 is present. *Curve 4* (Fig. 3) clearly indicates that essentially all the absorbance at 553 mμ is due to cytochrome c_1.

In Fig. 3*A*, variation in the growth condition can lead to a 9-fold change in the content of enzymatically reducible cytochrome b_1 and yet provide for an active electron transport system. This assumes that cytochrome c_1 makes no contribution to the absorbance at 553 mμ. On the assumption that there is no cytochrome c_1 in the bacteria grown with high aeration and that the maximal amount is present in the anaerobically grown bacteria, the range of variability would still be 6-fold.

It has been established that the rate-limiting component in the electron transport system of this bacterium is near the flavoprotein dehydrogenases (5). It has also been established that the greater the concentration of a given membrane-bound primary dehydrogenase, the greater the proportion of cytochromes c_1 or b_1 or both reduced, and the more rapid the rate of oxygen utilization produced in the presence of the particular substrate (Table VII of Reference 5). In the experiments illustrated in Fig. 3, conditions of growth were chosen such that DPNH reduced all the membrane-bound cytochrome reducible by $Na_2S_2O_4$. If growth conditions are chosen in such a way that all five dehydrogenases are present in roughly similar amounts, combinations of substrates can be tested effectively for their effects on the level of cytochrome reduction and the rate of electron transport in both mutant and parental types. Such data are illustrated in Table I, Part B, and show that the respiratory rate in the presence of combinations of substrates is additive with the parental strain except for the mixture of D- and L-lactate and a combination containing formate. The order of addition has no effect on the final rate of respiration. With the mutant, the rates observed with mixtures of substrates were always less than additive, and the discrepancy was particularly marked with mixtures containing formate. The cytochrome b_1 of the mutant could be completely reduced in the presence of formate and L-lactate and was incompletely reduced by the other substrates. In the parental type, the greatest amount of cytochrome c_1 was reduced by DPNH and succinate, and somewhat less was reduced with the other substrates. As reported previously (3, 4, 6), some of the cytochrome c_1 could only be reduced with $Na_2S_2O_4$, and this is the cytochrome c_1 which is not membrane-bound (4). Addition of mixtures of substrates does not produce increases in the proportions of cytochromes b_1 and c_1 reduced with either strain.

The effect of the addition of a number of known respiratory chain inhibitors is shown in Table V. Both inhibited and unin-

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hibited rates were measured when the respiratory chain system was saturated with both substrate and oxygen. This was done by making the measurements at relatively high oxygen concentrations, since inhibition of electron transport can result in an apparent increased K_m value for oxygen (7). In most cases maximal inhibition was observed within 30 seconds after the addition of inhibitor to the cells or particles.

Azide, cyanide, and carbon monoxide, typical inhibitors of cytochrome oxidases, showed the expected inhibitory effect on the respiration of whole cells and insoluble particles. Representative data with whole cells are shown in Table V. The inhibition by carbon monoxide was observed to be partially relieved by illumination with strong white light.

2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide, which inhibits the oxidation of cytochrome *b* in some bacteria (15) and inhibits transfer of electrons from cytochrome *b* to cytochrome c_1 in the mammalian cytochrome system (16), depressed respiration between 70 and 100% with all substrates tested when present in a concentration of 18 μM.

Respiration in the presence of succinate is strongly inhibited by malonate and by monoethyloxaloacetate, typical inhibitors of succinic dehydrogenase (17, 18), and by perfluorosuccinate. The enzyme of *H. parainfluenzae* is unusually sensitive to inhibition by malonate. However, no inhibition was observed on addition of 0.05 M oxaloacetate.

The respiration of the cells is inhibited to varying extents with the five substrates if tested by a number of substances which inhibit flavoprotein enzymes: quinacrine (19), secobarbital (20), thenoyltrifluoroacetone (21), and oxalate (Table V). The respiration with D-lactate is unusual in that it varies depending upon whether the cells are grown in glucose or in gluconate (or glucuronate). When glucose is the carbon source during growth, the respiration of the cells in the presence of D-lactate can be blocked by all inhibitors of electron transport down the cytochrome chain, and the cytochromes are reduced in the presence of D-lactate under anaerobic conditions. If the bacteria are grown in media supplemented with gluconic or glucuronic acid, the addition of D-lactate produces respiration insensitive to inhibition by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or inhibitors of cytochrome oxidases, and the cytochromes are not reduced when the cells are anaerobic in the presence of D-lactate. The respiration of cells grown in gluconate is inhibited by secobarbital, thenoyltrifluoroacetone, oxalate, and, to a small degree, by quinacrine. The respiration is dependent upon the concentration of oxygen in solution up to 100%; this resembles the respiration of the hemin-requiring *Hemophilus* species grown with aeration in the presence of limiting concentrations of hemin; under these conditions an autoxidizable flavoprotein mediates the respiration (9).

The data of Table V also show that rotenone and oxamate, which inhibit DPNH dehydrogenase and lactate dehydrogenases, respectively, in mammalian cells (22, 23), were without effect on the DPNH- and lactate-stimulated respiration of intact cells. The uncoupling agents of mammalian systems, 2,4-dinitrophenol, CCP,[1] and Dicumarol, showed inhibitory effects on respiration. The inhibition with CCP always occurs after a lag period in agreement with observations of Avi-Dor (24) on *E. coli*.

[1] The abbreviation used is: CCP, carbonyl cyanide *m*-chlorophenylhydrazone.

The respiration of the insoluble particles was also inhibited by the above substances.

DISCUSSION

From the data presented in this paper plus our previous observations (3–7), the following picture of hydrogen and electron transfer in *H. parainfluenzae* emerges.

1. The cytochrome system and at least five dehydrogenases can be bound to insoluble cellular membranes, presumably the cytoplasmic membrane. The five dehydrogenases appear to be flavoproteins (see also White (5)). The five flavoproteins, like the cytochromes, are synthesized at different rates, and these vary with the growth conditions and the growth phase. They appear to be arranged in varying numbers around the different cytochrome chains in a three-dimensional array, as previously suggested for two of the dehydrogenases (4, 6), with some overlapping of the flavoproteins and the different cytochrome assemblies. This arrangement would explain the observations in Table I, Part B, and in Table VII of White (5) that (*a*) respiration with a combination of substrates of these dehydrogenases may or may not be additive, and (*b*) the extent of reduction of the predominant cytochrome under anaerobic conditions is different with the different substrates and is usually the same as the amount reduced with one substrate when a combination of substrates is added.

The linkages of some of the dehydrogenases (*e.g.* D- or L-lactate) with the membrane system are more easily broken than others (formate or succinate or DPNH); they are dissociated at different rates as a suspension of the particles ages, even at 0°. The formate dehydrogenase is particularly tightly bound and appears to be less accessible for reaction with added oxidation-reduction substances (ferricyanide) or inhibitors.

2. The numerous other cellular dehydrogenases are not membrane-bound, or are so loosely associated with the membrane that they are easily detached on rupture of the cells (8). Various substrates can reduce what appears to be a single pool of pyridine nucleotide. The pool of pyridine nucleotide that is capable of enzymatic reduction is not in rapid equilibrium with DPN added to intact bacteria unless the cells have been damaged by incubation at 0°. The reduced pyridine nucleotide formed enzymatically can be oxidized by the membrane-bound electron transport system as rapidly as DPNH added to the bacterial suspension. There is no evidence for compartmentation of DPN or DPNH within this bacterium.

3. The rate of oxidation of substrates of pyridine nucleotide-linked enzymes is low compared to the oxidation of DPNH by the DPNH dehydrogenase and the cytochrome chain. The capacity for electron transport in the cytochrome chain is still larger than the rate of oxidation of DPNH by the DPNH dehydrogenases, as shown by the rate of oxidation of formate. Apparently, various metabolic reactions in this bacterium produce DPNH, succinate, formate, D-lactate, and L-lactate, which diffuse to the membrane-bound electron transport system where they are rapidly oxidized.

4. The ease of removal of substrates and pyridine nucleotides from the electron transport system of *H. parainfluenzae* and the rapid penetration of these substances into intact cells make these bacteria suitable for comparison of the reactions of this system in intact bacteria with that on the derived membrane fragments collected on rupture of the cells. Immediately upon rupture, the specific activity of the isolated respiratory chain

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particles with the different substrates is increased on a protein basis, and the K_m values for the different substrates are rather similar with intact cells and particles. As the dehydrogenases dissociate from the membranes on standing, the respiratory rates with the appropriate substrates decrease; then the respiratory rates are related to the amount of cytochrome that can be reduced under anaerobic conditions. Experiments reported elsewhere (5) show that the dehydrogenases lost from the membrane are found in the supernatant fluid. Thus only the dehydrogenases that remain associated with the membrane system are active in electron transport. Similar observations have been reported (4) for the cytochrome c_1 of the parental type.

5. The respiratory chain system of *H. parainfluenzae* is sensitive to inhibition by a number of substances which inhibit other respiratory chain systems. For example, the oxidases of the mutant used in these experiments are inhibited by cyanide, azide, and carbon monoxide. 2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide inhibits the bacterial electron transport chain in the region of cytochrome b_1, as in mammalian cells, and the flavoprotein dehydrogenases are inhibited by typical inhibitors of other flavoprotein enzymes. The succinate dehydrogenase of *H. parainfluenzae* is unusually sensitive to inhibition by malonate, as compared with the mammalian enzyme (25), but is insensitive to oxaloacetate. Rotenone, which is strongly inhibitory to the mammalian DPNH dehydrogenase (22), has no effect on the dehydrogenase of *H. parainfluenzae*. The most startling difference in the effect of inhibitors on the bacterial respiration is seen in the strong inhibition by dinitrophenol, Dicumarol, and CCP, which are uncouplers of mammalian oxidative phosphorylation but do not inhibit respiration in such low concentrations.

The data presented above give a picture of the localization of the enzymes for hydrogen and electron transport in this bacterial cell. They also show that in most aspects the bacterial system resembles that in mammalian mitochondria. On rupture of the cells, electron transport proceeds rapidly to the extent that the pigments remain associated with the membrane in the proper orientation. There is one really important difference between the bacterial and mammalian respiratory chain systems. This difference is the great variability of the relative proportions of the different pigments (both cytochromes and flavoproteins) in bacteria grown under different conditions or harvested at different times during the growth cycle. The bacteria have the ability to modify greatly the composition of the electron transport complex. Also, the differences in the relative rates of synthesis of the various cytochromes by the mutant as compared to the parental type make possible the harvesting of bacteria with widely different proportions of the cytochromes. We have established that a membrane-bound respiratory system capable of rapid electron transport can be fashioned by the living bacteria with a 3-fold range in DPNH dehydrogenase, a 27-fold range in formate dehydrogenase, a 500-fold range of D-lactate dehydrogenase, a 1400-fold range of L-lactate dehydrogenase, and an 8-fold range of succinate dehydrogenase activities (5). The cytochrome b_1 concentration can be modified over *at least* a 6- to 10-fold range, and the cytochrome c_1 concentration over *at least* a 32- to 72-fold range that is compatible with the rapid electron transport system which we have shown to be absolutely necessary for growth in this bacteria. The cytochrome oxidase *o* can vary over a 4-fold range, the cytochrome oxidase a_1 can vary over *at least* a 70-fold range, and the cytochrome oxidase a_2 can vary over *at least* a 10-fold range (3). Three, four, or five dehydrogenases or one, two, or three oxidases may be present in the functionally active electron transport system. In spite of the great variabilities in the quantities of the different cytochromes making up the respiratory chain system, the rates of respiration do not differ greatly (Table I and Fig. 3) (2, 5) unless the content of the flavoprotein dehydrogenases is changed. The rate-limiting step never appears to be in the cytochrome chain. It has been suggested that these observations with these bacteria mean that the pigments are synthesized separately, then incorporated into the membrane-bound system (4, 6, 7). In addition, the data also give additional insight into the structure of the respiratory chain system. There is no evidence that this system in *Hemophilus* is composed of several multienzyme packets, each with a fixed proportion of respiratory pigments. In fact, the type of elementary repeating unit of electron transport formed by the stoichiometric accretion of four multienzyme complexes, each of fixed composition, used as a model for beef mitochondria (26), obviously does not apply to this bacterium. In spite of the lack of consistency of the proportion of the different pigments, the bacterial respiratory chain system is always capable of rapid electron transport.

The two lactate dehydrogenases of *H. parainfluenzae* are not DPN-linked enzymes; in this respect they resemble the enzymes of yeast (27, 28). The L-lactate dehydrogenase of *H. parainfluenzae* can pass reducing equivalents to the cytochrome system, although it is not clear that the enzyme of yeast can do this (29). It has recently been reported that the D-lactate dehydrogenase of yeast does react with the respiratory chain system (30, 31).

The D-lactate dehydrogenase activity of *H. parainfluenzae* is different in cells grown in different media. In cells grown with glucose, the dehydrogenase appears to be a membrane-bound enzyme which can react directly with the cytochrome system. Like the other dehydrogenases, it does not react with oxygen at a detectable rate when dissociated from the membrane (5). In cells grown with gluconate, the enzyme is not linked to the respiratory chain system and reacts with oxygen as a typical flavoprotein oxidase. This observation is reminiscent of the work of Somlo (32), who showed that the activity of yeast L-lactate dehydrogenase is different depending upon whether or not it is membrane-bound.

SUMMARY

Studies of respiration and difference spectra in the presence of a number of substrates and inhibitors show the respiratory chain system of *Hemophilus parainfluenzae* to have many properties similar to that in mammalian tissues. The main differences are the marked inhibition of respiration produced by uncouplers of oxidative phosphorylation and the variability in the proportions of the respiratory pigments that can be induced environmentally. Two forms of *H. parainfluenzae*, one a mutant of the other, show similar respiratory activity even when they contain widely differing proportions of cytochromes. These data, plus previous observations with these bacteria, show that the bacterial respiratory chain system cannot be composed of "elementary particles" with a fixed composition. The data can only be explained in terms of a variable three-dimensional array of pigments.

The membrane-bound respiratory chain system contains the cytochromes and five flavoprotein dehydrogenases, while the pyridine nucleotide-linked dehydrogenases are in the cytoplasm.

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The reduced diphosphopyridine nucleotide generated diffuses rapidly to the membrane-bound reduced diphosphopyridine nucleotide dehydrogenase. The over-all rate of respiration is always limited by the activity of the dehydrogenases. Insoluble respiratory particles collected after rupture of the cells contain the respiratory chain system with increased specific activity. On aging, even at 0°, the pigments become dissociated at different rates. Only the pigments which remain membrane-bound participate in rapid electron transport.

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