The obligatory involvement of the electron transport system in the catabolic metabolism of *Haemophilus parainfluenzae*

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The catabolic metabolism of *Haemophilus parainfluenzae* involves the activity of enzymes of the Embden-Meyerhof-Parnas pathway, the hexose monophosphate pathway, and the tricarboxylic acid cycles. The enzymes of these pathways are very likely localized in the cytoplasm of the bacteria and generate the catabolites which have an obligatory reaction with the membrane-bound electron transport system. The catabolites formed interact to reduce or in some cases to reoxidize six distinct primary membrane-bound dehydrogenases of the electron transport system. The obligatory interaction of the catabolic enzyme systems and the membrane-bound electron transport system involves the generation of DPN as beef heart lactic dehydrogenase can substitute for the membrane-bound electron transport system.

The utilization of glucose, the reoxidation of reduced pyridine nucleotide, and the growth of *H. parainfluenzae* all depend on: 1. a functional electron transport system; and 2. suitable externally supplied electron acceptors. Studies with respiratory inhibitors indicate that the primary dehydrogenases, the quinone, the cytochromes and possibly the cytochrome oxidases are involved. An electron acceptor, either DPN, TPN, fumarate, pyruvate, nitrate or oxygen, must be present. Nitrate and oxygen reoxidize the reduced electron transport system by reoxidizing the cytochrome oxidases. There is preliminary evidence that fumarate, possibly TPN, and pyruvate cause pyridine nucleotide reoxidation by oxidizing the proper primary dehydrogenase.

INTRODUCTION

Haemophilus parainfluenzae forms a complex membrane-bound electron transport system consisting of primary dehydrogenases, a respiratory quinone, cytochromes and cytochrome oxidases under all tested growth conditions (White and Smith, 1964; Lester, White and Smith, 1964). The proportions of the various components of the electron transport system differ widely during these various growth conditions (White, 1962; 1965*a*). These compensatory

changes increase the apparent affinity of the entire electron transport system for the terminal electron acceptor when the organism is grown under conditions where the terminal electron acceptor is limiting (White, 1963*b*). This elaborate compensatory mechanism suggests that the electron transport system was indispensable in the metabolism of *H. parainfluenzae.*

METHODS AND MATERIALS

Turbidity and dry weight. Turbidity was measured as the absorbance at 750 mμ in 13 mm round cells with the Bausch and Lomb Spectronic 20 spectrophotometer. Dry weight was measured by drying bacteria suspended in 50 mM phosphate buffer. Water was removed under vacuum at 40 C until the weight was constant. The relationship between dry weight and turbidity is given in Fig. 1.

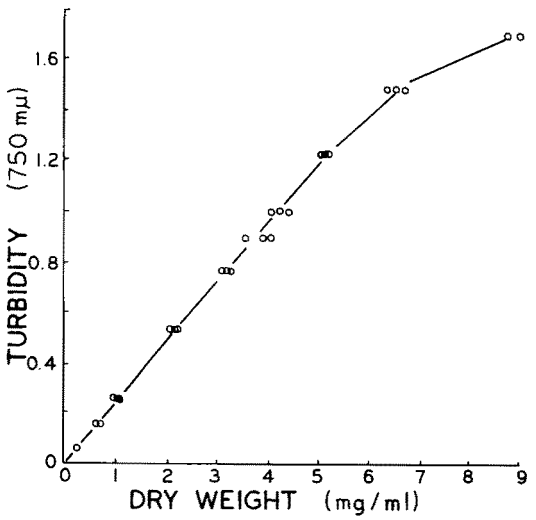

Fig. 1. The relationship between the turbidity measured at 750 mμ and the dry weight of *H. parainfluenzae.*

Oxygen utilization. Oxygen utilization was measured with the Clark oxygen electrode as described (White, 1963*a*). Measurements were made at substrate concentrations sufficiently high so that the reaction rates were independent of the substrate concentrations. Where indicated sequential substrate additions were made as previously described (White and Smith, 1964). Inhibitors were added at oxygen concentrations higher then 100 μM as inhibitors affect the affinity of the cytochrome system for oxygen (White, 1963*b*).

Pyridine nucleotide reduction measurements. The formation of reduced pyridine nucleotide in the intact bacteria was assayed at 340 mμ using the Cary 14CM spectrophotometer with the DuMont 7664 end window photomultiplier

as detector (White and Smith, 1964). For these measurements the bacteria were suspended in 50 mM phosphate buffer at pH 7.6 at a bacterial concentration of 40 to 50 mg bacterial protein per ml.

Experiments involving the formation or reoxidation of pyridine nucleotide with washed membrane preparations were measured with the Cary spectrophotometer at 340 mμ. In these experiments membrane suspensions and substrate solutions were deoxygenated by continuous bubbling with deoxygenated nitrogen. Additions to glass-stoppered cuvettes were made under a stream of nitrogen with the technique previously described (White, Bryant and Caldwell, 1962). Additions of deoxygenated phosphate buffer had no effect on the level of pyridine nucleotide reduction with these conditions.

DPN and TPN extraction and assay. A sample of intact bacteria was mixed aerobically to completely oxidize the respiratory system (White, 1965*b*) and an equal volume of ice-cold 0.7 M perchloric acid was added. The resulting suspension was centrifuged and the pellet washed twice with ice-cold 0.05 M TRIS buffer pH 7.6. The supernatants were combined and neutralized to pH 8.0 with ice-cold KOH. After a quick centrifugation, the cold supernatant was made to pH 3.4 with HCl and assayed after neutralization for DPN by use of the highly purified aldolase dehydrogenase and for TPN with highly purified galactose dehydrogenase supplied by Dr. A. S. L. Hu. These enzymes were isolated from *Pseudomonas saccharophila* (Hu and Cline, 1964).

Ferricyanide reductase. Ferricyanide reductase activity with D-lactate, L-lactate, succinate, formate, DPNH and TPNH was measured spectrophotometrically as previously described (White, 1964).

Cytochromes and cytochrome oxidases. Cytochromes and cytochrome oxidases were measured from difference spectra (White and Smith, 1964). The detection of reoxidation of reduced cytochromes and cytochrome oxidases was accomplished by shaking or by anaerobically adding deoxygenated solutions of fumarate, pyruvate, DPN, TPN or nitrate (White and Smith, 1962). Addition of similar volumes of deoxygenated phosphate buffer had no effect on the level of cytochrome reduction.

2-*Demethyl vitamin* K_2. The respiratory quinone was isolated by the isopropanol method and assayed spectrophotometrically as described previously (White, 1965*b*).

Rupture of the bacteria. The membrane-free preparations were made by suspending the bacteria in 50 mM phosphate buffer pH 7.6 and subjecting them to sonic vibration under the conditions previously described (White, 1964). Membranes were recovered and washed with phosphate buffer by three successive centrifugations of 25,000 $\times$ *g* for 20 minutes. This treatment effectively removes substrate-reducible cytochrome components from the supernatant

portion (White and Smith, 1964). In some experiments bacteria were ruptured with the Ribi-Servall French pressure cell at 20,000 psi, in a Hughes press, or by grinding with alumina (White, 1962).

Enzyme assays. The following enzymes were assayed as described: aldolase (Sibley and Lehninger, 1949), phosphohexose isomerase (Bodansky, 1954), glyceraldehyde-3-phosphate dehydrogenase (Velick, 1955), isocitrate lyase (Daron and Gunsalus, 1962), fumarase (Massey, 1955), phosphoenol pyruvate kinase (Bücher and Pfleiderer, 1955), and malic dehydrogenase (Ochoa, 1955). Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase were measured by the increase in absorbance at 340 mμ at 25 C after addition of substrate to membrane-free supernatant solutions containing 1 to 2 mg of protein in the presence of 3.2 mM TPN. Substrate was added to give a final concentration of 30 mM. No absorbance due to formation of TPNH developed unless substrates were added. Hexokinase and gluconate kinase were assayed by following the absorbance at 340 mμ after adding glucose or gluconate to membrane-free supernatant solution. Measurements were carried out in the presence of $MgCl_2$ (30 mM), TPN (3.2 mM), ATP (10 mM) and substrate (30 mM) at 25 C.

Glucose utilization. Glucose utilization was measured with the supernatant portions of high speed centrifugations of sonically disrupted bacteria with 100 mM phosphate buffer pH 7.6, 2 mM ATP, 1.5 μM DPN, 2 mM $MgCl_2$, 1 mM KNO_3, 1 mM fumarate, and 15 mM glucose.

Substrate assays. Pyruvate was assayed with lactic dehydrogenase (Segal, Blair and Wyngaarden, 1956). D- and L-Lactate were assayed colorimetrically (Barker and Summerson, 1941) using lithium lactate as standard. Glucose was assayed (Nelson, 1944) after deproteinization (Somogyi, 1945).

Protein. Protein was measured by the modified biuret reaction (Gornall, Bardawill and David, 1949; White and Smith, 1962).

Bacteria. The strain of *H. parainfluenzae* and the yeast extract, proteose peptone medium used in this study have been described (White and Smith, 1962). Glucose was autoclaved separately and was added to a final concentration of 20 mM. The incubation, inoculum and harvesting procedures have been described (White and Smith, 1962). Before harvesting, the culture was checked for contamination with other bacteria (White, 1962). The growth experiments illustrated in Table 1 were performed with media made without glucose, nitrate, or yeast extract. Aerobic growth was measured with 15 ml of this medium in 300 ml Erlenmeyer flasks fitted with side arms 13 mm in diameter. These flasks were incubated without agitation. Anaerobic growth experiments were performed in 13 mm test tubes containing 15 ml of medium that was deoxygenated with nitrogen for 90 min after inoculation and then sealed with

rubber stoppers. The nitrogen used for deoxygenation was itself deoxygenated by passage through copper filings heated to 600 C. The completeness of the deoxygenation of the nitrogen was monitored by passing the gas through a 0.01% (w/v) solution of resazurin at pH 7.6. In the experiments illustrated in Table 1, sterile reagents were added to the media before inoculation. The cultures were incubated for 36 hours at 37 C.

The bacteria used in the experiment illustrated in Table 6, were grown in 1.5 liters of proteose peptone media made without yeast extract, or nitrate to which sterile glucose (40 mM), lactic dehydrogenase (7 mg), and DPN 2.0 μM were added after autoclaving. The lactic dehydrogenase and the DPN were filter-sterilized. The vessel used for growth consisted of a 2 liter Florence flask fitted with a standard taper joint at the top. The top was fitted with a glass sparger and outlet port both of which contained glass valves. The medium was gassed with deoxygenated nitrogen through the sintered glass sparger from the time it was removed from the autoclave as it cooled. Deoxygenation continued while the supplements and inoculum were added and for at least an hour after inoculation. The medium was stirred during the gassing with a teflon-covered magnetic stirring bar.

Reagents. Sodium glyoxylate (Sigma Chemical Co.) was made to pH 7.6 and used immediately after preparation. Sodium glyceraldehyde-3-phosphate (Sigma Chemical Co.) was prepared from the Barium salt with Dowex 50 in the acid form. Hexafluoroglutarate (Calbiochem Corp.) was recrystallized before use. Phosphoenolpyruvate, glucose-6-phosphate, D- and L-lactate (all from Calbiochem Corp.), 6-phosphogluconate, 2-deoxyglucose (General Biochemical Corp.), gluconate (Cowels Chemical Corp.), glucuronolactone (Fisher Chemical Corp.) were prepared in solution at pH 7.6 as sodium salts and used directly. Glucose and gluconate were shown to be chromatographically pure by Dr. A. S. L. Hu. All other reagents were as described in earlier publications (White and Smith, 1964; White, 1962; 1963*b*).

RESULTS

Requirements for growth. Growth of *H. parainfluenzae* in the rich proteose peptone medium takes place aerobically and is inhibited by the respiratory inhibitors KCN or NOQNO [1]. Fermentative growth in the presence of glucose or gluconate is detectable only in the presence of the terminal electron acceptors oxygen, nitrate, pyruvate or fumarate. If 10^4 times the optimal DPN or TPN concentration is supplied some anaerobic growth is possible in the absence of nitrate, pyruvate or fumarate (Table 1). There is no anaerobic growth

[1] The abbreviation is: NOQNO, 2-n-nonyl-4-hydroxyquinoline-N oxide.

TABLE 1

Effect of respiratory inhibitors and terminal electron acceptors on the growth of *H. parainfluenzae*.

The growth of bacteria was measured as the absorbance at 750 mμ. In the experiments illustrated in Column A, the bacteria were incubated in air. In Column B the medium was deoxygenated as described in the text and sealed with a rubber stopper. The highest turbidity achieved after incubation at 37 C for 18 to 36 hr is illustrated in the table.

Supplements added to proteose peptone media	Turbidity	
	A Aerobically	B Anaerobically
None	0.23	0.00
Glucose (20 mM)	1.50	0.00
Glucose + KCN (5 mM)	—	0.00
Glucose + NOQNO (17 μM)	0.09	0.02
Glucose + DPN (150 μM)	1.50	0.45
Glucose + TPN (150 μM)	1.50	0.32
Glucose + nitrate (20 mM)	1.58	0.92
Glucose + fumarate (20 mM)	1.85	0.96
Glucose + pyruvate (20 mM)	1.50	0.32
Gluconate (20 mM)	0.89	0.00

detectable with the addition of sulfate, sulfite or nitrite to this medium. Similar dependence on nitrate, fumarate, pyruvate, TPN, DPN or oxygen can be shown with the very rich Levinthal's (Alexander, 1958) medium. It seems unlikely that other common electron acceptors besides oxygen, nitrate, pyruvate, DPN, TPN or fumarate are effective in allowing growth of *H. parainfluenzae*.

Requirements for glucose utilization. Washed intact *H. parainfluenzae* suspended in phosphate buffer are able to use glucose only in the presence of the terminal electron acceptors nitrate, fumarate, pyruvate, oxygen or large amounts of DPN (Fig. 2). In their absence there is no glucose utilization. Inhibition of the electron transport system with NOQNO or KCN results in the inhibition of glucose utilization even in the presence of the terminal electron acceptors. A membrane-free preparation of the bacteria prepared by exposure of the bacterial suspension to sonic vibration was centrifuged at 104,000 × *g*. The supernatant portion was tested for glucose utilization. The data of Fig. 3 indicate that there is no glucose utilization even in the presence of the terminal electron acceptors. This supernatant does not contain membrane fragments. The lack of oxygen utilization in the presence of any substrates, and the absence of respiratory quinone, cytochrome b_1 or cytochrome oxidases indicate that no membrane fragments are present in the supernatant. The phospholipids contain the major portion of the fatty acids of *H. parainfluenzae* and these lipids are found exclusively in the membrane[1]. No fatty acids can be detected

[1] J. C. Dittmer and D. C. White, unpublished data.

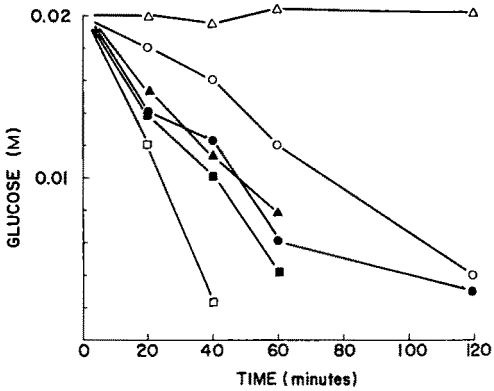

Fig. 2. Factors necessary for the utilization of glucose by intact *H. parainfluenzae*. Anaerobic glucose utilization at 37 C in the presence of the following acceptors is indicated as: Δ—Δ control without acceptor; O—O fumarate (20 mM); ■—■ DPN (5 mM); ▲—▲ pyruvate (20 mM); and □—□ KNO_3 (20 mM). ●—● indicates glucose utilization in a suspension gassed with air at a rate of 8 ml/min.

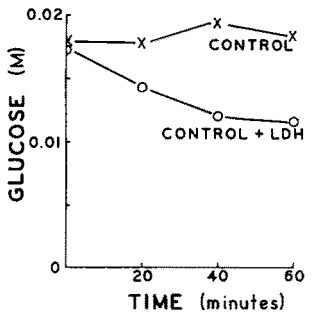

Fig. 3. Aerobic utilization of glucose by membrane-free preparations of *H. parainfluenzae*. Crystalline beef heart lactic dehydrogenase was added to the experiment illustrated by the data in the lower curve.

in the supernatant. Consequently, both an intact functional electron transport system and a sufficient concentration of terminal electron acceptor are necessary for glucose catabolism.

The obligatory involvement of the electron transport system in glucose catabolism involves the oxidation of DPNH (Figs. 3 and 4).

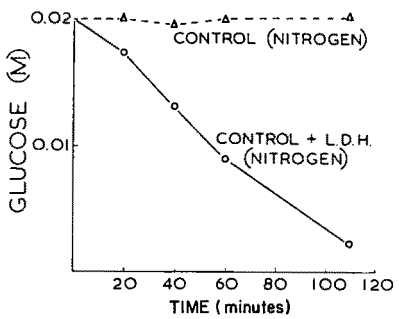

Fig. 4. Anaerobic utilization of glucose by cell-free homogenates of *H. parainfluenzae* in the presence of lactic dehydrogenase.

The addition of 5 mg of crystalline beef heart lactic dehydrogenase (generously provided by Dr. A. D. Winer) allows glucose utilization either in the absence

of oxygen or in the absence of a functional electron transport system. The fact that in the presence of lactic dehydrogenase the membrane-free supernatant portion of *H. parainfluenzae* can utilize glucose, indicates that the enzymes necessary for glucose catabolism are present and active in the membrane-free supernatant portion of a broken-cell preparation.

The generation of reduced pyridine nucleotide in the absence of a terminal electron acceptor. Incubation of suspensions of intact washed *H. parainfluenzae* in the presence of various substrates under conditions where the terminal electron acceptor is completely utilized results in an absorbance change suggesting the reduction of pyridine nucleotide. Measurements with the Cary 14 spectrophotometer have indicated that the maximum of the absorbance of the postulated reduced pyridine nucleotide is near 340 mμ (White and Smith, 1964). If a Clark electrode is inserted into the cuvette near the optical pathway, one can observe that the reduction of pyridine nucleotide occurs after the largest part of the oxygen is utilized by bacterial metabolism. At the time of maximal pyridine nucleotide reduction, the cytochromes and cytochrome oxidases are reduced. The reduced pyridine nucleotide can be reoxidized after the addition of fumarate (Fig. 5), nitrate, pyruvate, or by shaking in air. The

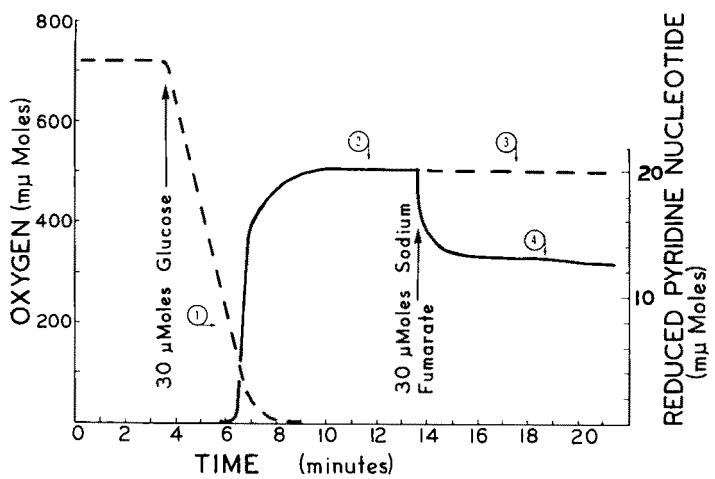

Fig. 5. Oxygen utilization and reduced pyridine nucleotide formation in *H. parainfluenzae*. Oxygen uptake and pyridine nucleotide reduction were replotted for a bacterial suspension containing 10 mg protein/ml. The oxygen utilization (curve 1), and the absorbance change (curve 2) were followed after addition of glucose (5 mM). Addition of an anaerobic solution of fumarate (10 mM) causes a decrease in the proportion of reduced pyridine nucleotide (curve 4). Repeating the experiment in the presence of 5 mM KCN (curve 3) shows there is no effect on the formation of reduced pyridine nucleotide but that the reoxidation of reduced pyridine nucleotide is inhibited.

oxidation of reduced pyridine nucleotide by these electron acceptors is inhibited in the presence of 5 mM KCN. This concentration of KCN inhibits electron transport and cytochrome oxidation (White and Smith, 1964). It does not inhibit the actual reduction of pyridine nucleotide.

The amount of reduced pyridine nucleotide generated by electron transport in the absence of a terminal electron acceptor is less than the total content of acid-extractable oxidized pyridine nucleotide. Repeated measurements have shown that in the absence of a terminal electron acceptor 4 to 20 mμmoles per 10 mg of bacterial protein of reduced pyridine nucleotide can be generated. Perchloric acid extraction of bacteria that have been vigorously aerated and assayed by use of the highly purified specific dehydrogenases of *Pseudomonas saccharophila* (Hu and Cline, 1964) indicated that about 100 to 120 mμmoles of TPN and 70 to 80 mμmoles of DPN per 10 mg of protein are present. These conditions of vigorous aeration completely oxidize the cytochromes and pyridine nucleotides (White, 1965*b*).

Catabolic enzyme systems. The activity of the enzymes by which glucose may be catabolized can be demonstrated in the supernatant portion of a broken-cell suspension (Table 2). The activities of these enzymes suggest the

TABLE 2

Enzymatic activities in the supernatant portion of sonically disrupted *H. parainfluenzae* grown with supplemental glucose, gluconate, glutamate, or without supplement

Enzyme:	Growth supplement			
	None	Glucose	Gluconate	Glutamate
	mμmoles/sec per 30 mg protein			
Glucose-6-phosphate dehydrogenase[1)]	103.7	445.1	136.7	255.0
6-Phosphogluconate dehydrogenase[1)]	108.8	159.0	222.5	68.5
Hexokinase[2)]	<0.009	3.72	18.9	—
Gluconate kinase[2)]	<0.009	20.3	10.9	—
Aldolase[3)]	174.0	609.0	520.0	53.8
Phosphohexose isomerase[4)]	104.0	248.0	218.0	36.1
Glyceraldehyde-3-phosphate dehydrogenase[5)]	248.0	681.0	167.1	70.0
Phosphoenolpyruvate kinase[6)]	247.5	2139.0	133.5	238.0
Malic dehydrogenase[6)]	2488.0	1775.0	890.0	2310.0
Fumarase[7)]	144.0	26.0	20.8	44.9

The supernatant portion of bacteria ruptured by sonic vibration was used to assay the enzyme activities as follows: [1)] TPNH appearance at 25 C; [2)] TPNH appearance at 30 C; [3)] fructose-6-phosphate disappearance at 37 C; [4)] fructose-6-phosphate appearance at 37 C; [5)] DPNH appearance at 25 C; [6)] DPNH disappearance at 25 C; and [7)] 0.01 absorbance (300 mμ) decrease at 25 C. The data are expressed as the rate in mμmoles per second per 30 mg protein.

presence of an Embden-Meyerhof-Parnas pathway, the hexosemonophosphate shunt, and the tricarboxylic acid cycle systems. The activities of these enzymes can differ markedly as a function of the major catabolites added to the growth media. These changes in enzyme activities suggest that these enzymes are vitally involved in the catabolic processes of the growing bacteria.

The obligatory involvement of the electron transport system in catabolism coupled with the remarkable permeability of *H. parainfluenzae* to both substrates and pyridine nucleotides (White and Smith, 1964) allows oxygen utilization in the presence of various substrates to be used for a measure of the function of the enzymes demonstrated in Table 2. Washed cells of *H. parainfluenzae* have no detectable endogenous respiration (White, 1964). Consequently, respiration produced on the addition of a substrate, especially if oxygen utilization can be inhibited by relatively specific inhibitors, suggests the involvement of various metabolic pathways in the intact bacteria.

Typical glycolysis can result if beef heart lactic dehydrogenase is added to the membrane-free supernatant portion of ruptured *H. parainfluenzae*. This indicates that the enzymes of the Embden-Meyerhof-Parnas pathway detected in the experiment illustrated in Table 2 are functional.

Respiration produced on the addition of glucose can be inhibited by 2-deoxyglucose and by inhibition of the tricarboxylic acid cycle (Table 3). The Embden-Meyerhof-Parnas pathway enzyme, phosphohexose isomerase is inhibited by 2-deoxyglucose (Wick et al., 1957). This inhibitor reduces the utilization of glucose, suggesting that the hexose monophosphate shunt is active in *H. parainfluenzae*. Respiration of gluconate or ribose which presumably involves the shunt is not inhibited by 2-deoxyglucose. Monofluoroacetate, perfluorosuccinate, and malonate inhibit the tricarboxylic acid cycle as indicated by the inhibition of respiration produced by aspartate or succinate. Again the respiration stimulated by gluconate or ribose is not inhibited by these agents which significantly depress the activity of the tricarboxylic acid cycle. In the assay of the enzymes of the hexosemonophosphate shunt, TPN is about 70 times more effective than DPN. The levels of enzyme activities of the first two enzymes of the hexosemonophosphate shunt are dependent on the addition of metabolites to the growth media.

Data in Tables 2 and 4 show evidence for the presence of several enzymes of the tricarboxylic acid cycle. Specific inhibitors of this cycle, monofluoroacetate which inhibits aconitase (Morrison and Peters, 1954) perfluorosuccinate and malonate which inhibit succinic dehydrogenase, inhibit the respiration stimulated on the addition of substrates of the tricarboxylic acid cycle. Hexafluoroglutarate was observed to inhibit the respiration of α-ketoglutarate. None of these inhibitors stimulate oxygen utilization when added in the absence of substrate.

TABLE 3

Effect of 2-deoxyglucose and of blocking the tricarboxylic acid cycle on respiration produced by carbohydrates in *H. parainfluenzae*.

Washed suspensions of intact bacteria were used in the Clark oxygen electrode to measure the respiratory activity. Substrate and 2-deoxyglucose were added sequentially where indicated by the + sign (Column A). Similar experiments measured in phosphate buffer containing 30 mM monofluoroacetate, malonate, and hexafluoroglutarate are illustrated in Column B. With a given bacterial suspension and a given substrate the rates of respiration are reproducible to ± 5%.

Substrate (60 μmoles/3 ml)	Rate of oxygen utilization mμmoles oxygen/sec per 10 mg protein	
	A	B
Endogenous	<0.001	<0.001
2-Deoxyglucose	<0.001	<0.001
Glucose	3.48	1.27
+ 2-deoxyglucose	1.62	0.58
Gluconate	2.32	2.32
+ 2-deoxyglucose	2.32	2.32
D-Ribose	0.69	0.68
+ 2-deoxyglucose	0.67	0.68
L-Lactate	13.90	2.54
D-Lactate	3.48	1.35
DL-Aspartate	1.50	0.16
Succinate	2.80	0.21

TABLE 4

Cyclic reactions of terminal oxidation in *H. parainfluenzae*

The measurement of the respiratory activity of washed suspensions of intact bacteria were performed as in Table 3. Sequential addition of substrates or inhibitors to a suspension of bacteria are indicated by the + sign; * indicates a second bacterial preparation was used.

Substrate	Concentration (μmoles/3 ml)	Oxygen uptake in mμmoles O_2/sec per 10 mg protein
	Tricarboxylic acid cycle	
Endogenous	—	<0.001
Pyruvate	30	1.24
+ $NaAsO_2$	60	0.20
Pyruvate	30	1.26
+ monofluoroacetate	60	0.00
+ citrate	90	0.20
Pyruvate	30	1.26
+ malonate	60	0.81
Pyruvate	30	1.32
+ perfluorosuccinate	60	0.81

TABLE 4 *(continued)*

Substrate	Concentration (μmoles/3 ml)	Oxygen uptake in mμmoles O_2/sec per 10 mg protein
	Tricarboxylic acid cycle	
Acetate	30	0.18
Citrate	30	0.15
Isocitrate	30	0.21
α-Ketoglutarate	30	0.20
Fumarate	30	0.24
Malate	30	0.30
Oxalacetate	30	0.20
	Glyoxylate cycle	
Isocitrate	30	0.21
+ perfluorosuccinate	60	0.18
+ malonate	60	0.15
Glyoxylate	30	0.46
	Aspartate-glutamate cycle	
Aspartate	30	0.54
+ malonate	60	0.54
+ perfluorosuccinate	60	0.54
* Aspartate	30	0.17
+ hexafluoroglutarate	30	0.17
+ monofluoroacetate	30	0.09
	Glutamate-aspartate cycle	
Glutamate	30	0.57
+ malonate	60	0.21
* Glutamate	30	0.60
+ perfluorosuccinate	60	0.21
* Glutamate	30	0.42
+ monofluoroacetate	30	0.42
+ hexafluoroglutarate	30	0.09
* α-Ketoglutarate	30	0.19
+ hexafluoroglutarate	60	0.06

The rapid utilization of glyoxylate and the relative insensitivity of isocitrate respiration to inhibitors of succinic dehydrogenase suggest the presence of enzymes of the glyoxylate by-pass (Wong and Ajl, 1955; Kornberg and Madsen, 1958). Isocitrate lyase activity can be demonstrated in the supernatant from a cell-free extract prepared from gluconate-grown bacteria: 0.204 μmoles of glyoxylate were formed from DL-isocitrate in 10 min at 30 C by a sample of extract containing 9.3 mg protein.

The utilization of aspartate in the presence of inhibitors of succinic dehydrogenase (malonate and perfluorosuccinate) or α-ketoglutarate dehydrogenase (hexafluoroglutarate) and the inhibition of aspartate respiration by monofluoroacetate suggest the presence of the aspartate-glutamate and glutamate-aspartate cycles (Terner, Eggleston and Krebs, 1950; Krebs, 1950) in *H. parainfluenzae*. Glutamate respiration is inhibited by hexafluoroglutarate, malonate and perfluorosuccinate but not by monofluoroacetate.

Consequently, the cyclic terminal catabolic reaction sequences include the tricarboxylic acid cycle and parts of that cycle involving the glyoxylate, aspartate-glutamate, and glutamate-aspartate bypasses.

Cellular localization of the primary dehydrogenases. Most of the Embden-Meyerhof-Parnas, hexose monophosphate, and cyclic terminal pathways appear to be either very loosely membrane-bound or to be in the cytoplasm of the bacterial cell. Subjecting *H. parainfluenzae* to sonic vibration for 1 min followed by centrifugation, separates a membrane system containing the respiratory quinone, the cytochromes and the cytochrome oxidases (White and Smith, 1964) from the supernatant portion containing the enzyme activities listed in Table 2. The membrane system contains primary dehydrogenases which react with D-lactate, L-lactate, succinate, formate and DPNH. These five substrates cause oxygen utilization in washed membrane preparations of the electron transport system (White, 1964). The membrane fragments contain the entire respiratory system whether prepared by rupture of penicillin spheroplasts, grinding with alumina, exposure to sonic vibration, rupture with the Hughes press or French pressure cell. The addition of 15 μM DPN to washed membrane preparations does not increase the number of substrates capable of stimulating oxygen utilization.

TPNH dehydrogenase. An enzyme system capable of coupling the reoxidation of TPNH to the electron transport system was detected. TPNH added to a washed membrane preparation of *H. parainfluenzae* provokes oxygen utilization. The oxygen utilization caused by TPNH is depressed in the same manner as with other substrates by respiratory inhibitors. The respiration produced in the presence of TPNH does not appear to result from pyridine nucleotide transhydrogenase activity as the addition of either DPN or TPN has no effect on the rate of respiration of TPNH.

TPNH reacts with a primary dehydrogenase distinct from DPNH dehydrogenase (Table 5). The respiration and ferricyanide reductase activity produced by both DPNH and TPNH is additive. Ferricyanide reduction has been established as an adequate assay for the other five dehydrogenases (White, 1964). There is differential sensitivity to inactivation in phosphate buffer at 49 C between DPNH and TPNH ferricyanide reductase activities. Half the TPNH ferricyanide

TABLE 5

TPNH and DPNH dehydrogenase activities in *H. parainfluenzae*.

Oxygen utilization and ferricyanide reductase activities were measured with washed suspensions of intact bacteria. TPNH and DPNH were added to a final concentration of 0.5 mM. Further additions of either substrate did not result in increases in the rates of oxygen uptake or ferricyanide reduction.

Substrate	Ferricyanide reductase	Oxygen utilization
	mμmoles/sec per 10 mg protein	
TPNH	16.6	4.15
DPNH	68.0	16.7
DPNH+TPNH	82.0	22.1
TPNH+DPNH	83.0	21.8
Sum of 1 + 2	84.6	20.85

reductase activity is destroyed at 49 C in 27 min compared with 12 min for DPNH ferricyanide reductase activity.

Reactions of the primary membrane-bound dehydrogenases. Only D-lactate, L-lactate, succinate, formate, DPNH and TPNH are capable of causing oxygen utilization with the washed membrane fractions of all the substrates used in this paper. The oxidation of DPNH or TPNH results in the disappearance of absorbance with its maximum near 340 mμ with membrane fragments, suggesting that DPN and TPN are formed during the reoxidation of DPNH and TPNH. Formate oxidation measured manometrically is coupled with the uptake of oxygen and release of carbon dioxide. In the presence of 1 mM arsenite, pyruvate accumulation and either D-lactate or L-lactate disappearance increase with the time of incubation. Succinate oxidation is coupled with the appearance of a compound which very strongly absorbs light below 300 mμ. Malonate is a competitive inhibitor of succinate oxidation in these bacteria (White and Smith, 1964) suggesting that fumarate and succinate are substrates for succinic dehydrogenase.

Anaerobic growth in the presence of lactic dehydrogenase. H. parainfluenzae is permeable to both DPN and DPNH (White and Smith, 1964). *H. parainfluenzae* is capable of forming DPN from TPN or TPN from DPN as either is effective for the nicotinamide riboside requirement for growth (Gingrich and Schlenk, 1944). If the essential function of electron transport were the reoxidation of metabolically reduced pyridine nucleotide, then growth could

be possible either in the presence of excessive oxidized pyridine nucleotide or in the presence of an oxidized pyridine nucleotide generating system. Excessive DPN or TPN in anaerobic media allows some growth (Table 1). If bacteria are incubated anaerobically in media not containing nitrate or fumarate but in the presence of glucose, DPN and beef heart lactic dehydrogenase, growth occurs. No growth occurs in media prepared identically except for the omission of lactic dehydrogenase. The final turbidity reaches 0.30 after 48 hours. Under these growth conditions bacterial growth is dependent on the presence of lactic dehydrogenase. The doubling time is about 1.5 hours. The bacteria grown in this manner appear microscopically as long thin thread-like forms. This pleomorphic morphology is characteristic of *Haemophilus* grown with limiting DPN (Henriksen, 1948). If these bacteria are harvested, washed in phosphate buffer with centrifugation, they have an oxygen utilization stimulated in the presence of formate of 0.99 mμmoles oxygen utilized per second and per 10 mg protein. This respiratory activity is inhibited in the presence of 5 mM KCN and 3 μM NOQNO. Examination of the difference spectra of washed intact bacterial suspension indicates cytochrome c_1 and cytochrome oxidase o are formed (Table 6). The levels of cytochromes and the respiratory quinone

TABLE 6

Respiratory chain components formed during anaerobic growth in the presence of lactic dehydrogenase with *H. parainfluenzae.*

Respiratory pigments were determined from difference spectra of *H. parainfluenzae* grown anaerobically without an electron acceptor but in the presence of lactic dehydrogenase.

Component	mμmoles per 10 mg protein
2-Demethyl vitamin K_2	6.10
	Absorbance per 10 mg protein
Cytochrome c_1	0.019
Cytochrome oxidase o	0.004
Cytochrome oxidase a_1	<0.002
Cytochrome oxidase a_2	<0.0005

are comparable to those formed in this bacterium when grown with aeration (White, 1962; 1965*a*). Consequently, the addition of a mechanism to reoxidize pyridine nucleotide does not relieve the formation of a functional membrane-bound electron transport system.

DISCUSSION

The catabolic metabolism of *H. parainfluenzae* involves enzymes which provide the substrates that reduce and reoxidize the membrane-bound electron transport system. An outline of the catabolic network involved in the formation of these substrates is provided in Fig. 6. Evidence in support of the activity of the Embden-Meyerhof-Parnas, hexose monophosphate, and tricarboxylic acid cycle pathways in the catabolism of *H. parainfluenzae* consists of 1) the

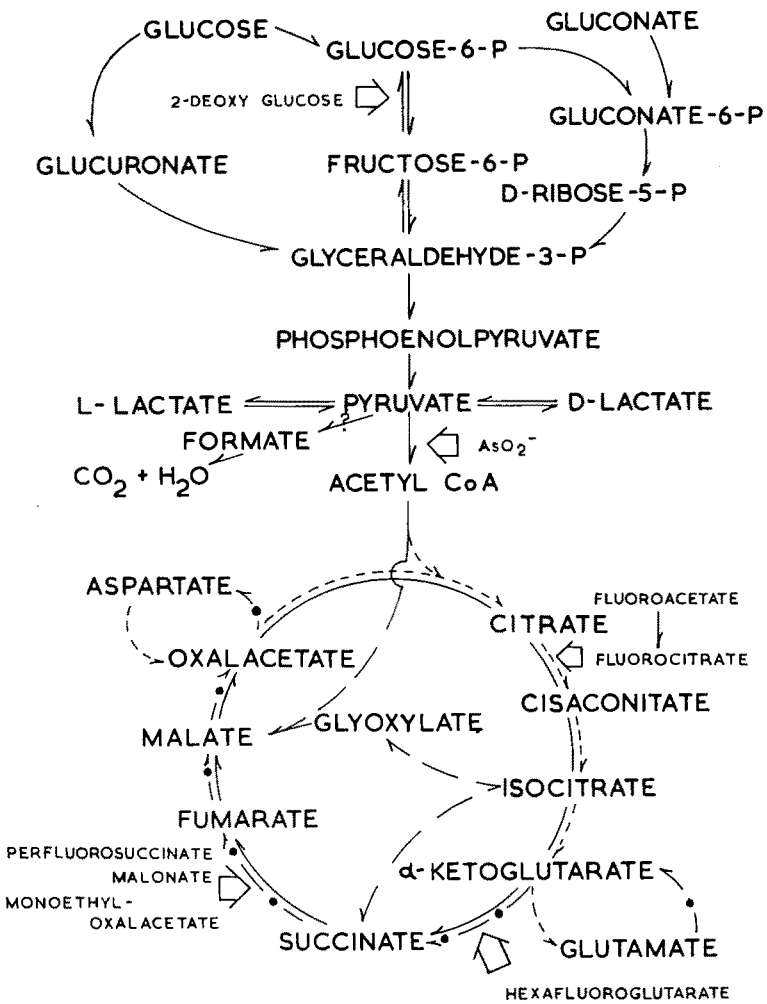

Fig. 6. Summary of the proposed catabolic systems in *H. parainfluenzae*. ▷ indicates the sites of inhibition by the inhibitors. The aspartate-glutamate cycle – – – →, the glyoxylate bypass —— ——→, and the glutamate-aspartate cycle —• —• → are indicated.

change in the activites of enzymes of these pathways during growth with various substrates; and 2) the specific inhibition of substrate-provoked respiration in a pattern that is consistent with the operation of these pathways. The enzymes of these pathways are found in the supernatant portion of bacteria that have been disrupted by sonic vibration and centrifuged at high speed. These cytoplasmic enzymes provide the substrates which presumably interact with six distinct membrane-bound dehydrogenases. Succinate, D-lactate, L-lactate, DPNH, TPNH or formate reduce all components of the isolated electron transport system and can cause either oxygen utilization or nitrate reduction. These six substrates each react with a specific dehydrogenase that must be a part of the same membrane which contains the respiratory quinone, the cytochromes, and the cytochrome oxidases (White and Smith, 1964).

Previous work with these bacteria had demonstrated the presence of enzymes of the tricarboxylic acid cycle (Klein, 1940).

The utilization of oxygen or the reduction of nitrate by the electron transport system in the presence of these substrates is inhibited by respiratory inhibitors. These inhibitors act at various levels in the electron transport system (White, 1965*b*). Both oxygen and nitrate act at the level of the cytochrome oxidases. The anaerobic oxidation of the reduced electron transport system by fumarate or pyruvate seems to be a bit more complicated. Preliminary evidence indicates that in the absence of oxygen, the cytochrome c_1 in isolated membrane fragments that are reduced in the presence of formate can be oxidized by the anaerobic addition of pyruvate or fumarate. Formate added to anaerobic washed membrane fragments of *H. parainfluenzae* can cause both TPN and DPN reduction. In each case the reductions or oxidations are inhibited by thenoyltrifluoracetone, NOQNO and KCN. Since the flavo protein dehydrogenases are the sites of the succinate $\rightleftarrows$ fumarate, D- or L-lactate $\rightleftarrows$ puryvate, DPNH $\rightleftarrows$ DPN and TPNH $\rightleftarrows$ TPN reactions, and the overall reactions are inhibited by the three respiratory inhibitors, the necessity for the function of the entire electron transport system is indicated. The fact that malonate specifically inhibits cytochrome and reduced pyridine nucleotide reoxidation in the presence of fumarate, indicates that at least in the case of fumarate, the terminal reaction takes place at the specific dehydrogenase.

This study indicates that *H. parainfluenzae* needs a functioning electron transport system for catabolic metabolism of glucose. Growth of this bacterium depends on the presence of electron acceptors in the media. Oxygen, nitrate, pyruvate, fumarate, DPN and TPN can serve as electron acceptors. These are the only reagents thus far found that can react with the isolated electron transport system. No growth occurs in the presence of the respiratory inhibitors KCN or NOQNO.

The reoxidation of DPNH seems to be an essential reaction which depends on the functional electron transport system. There are no soluble dehydrogenases similar to alcohol or lactic dehydrogenases to allow glycolytic growth in the absence of the functional electron transport system. If sterile beef heart lactic dehydrogenase as an artificial DPN regenerating system is present in the medium, glycolytic growth is possible in the absence of any electron transport system or a proper electron acceptor.

The hypothesis that the membrane-bound respiratory system cannot function without DPN being regenerated is further supported by the increased absorption at 340 mμ in a bacterial suspension allowed to go anaerobic in the presence of a substrate but in the absence of an electron acceptor (Fig. 5). The amount of reduced pyridine nucleotide generated when substrate is added but an electron acceptor is lacking, is not increased on addition of more substrate, different substrates capable of reducing the electron transport system, DPN or TPN to freshly prepared bacterial suspensions (White and Smith, 1964). Reagents capable of reoxidizing the elements of the electron transport system cause decrease in absorbancy at 340 mμ. This presumably involves the reoxidation of enzyme-bound pyridine nucleotide. This reoxidation in inhibited by respiratory inhibitors. The amount of bound reduced pyridine nucleotide represents 2 to 20% of the total pyridine nucleotide in the bacteria. Perhaps the requirement for DPN in this bacterium somehow necessitates that a part of the pyridine nucleotide not be involved in oxidation reduction reactions.

An obligatory requirement of oxygen for glucose catabolism via the Embden-Meyerhof-Parnas pathway has been demonstrated for *Pasteurella tularensis*, *Agrobacterium tumefaciens* (Hill and Mills, 1954), and for *Streptomyces coelicolor* (Cochrane, 1955). These organisms like *H. parainfluenzae* require an additional mechanism to generate DPN in the absence of oxygen.

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