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Effect of Glucose on the Formation of the Membrane-Bound Electron Transport System in *Haemophilus parainfluenzae*

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ABSTRACT

The catabolism of glucose by *Haemophilus parainfluenzae* affected the formation of the primary dehydrogenases of the membrane-bound electron transport system. The formation of other components of the respiratory system, 2-demethyl vitamin K_2, cytochrome b_1, cytochrome c_1, and the cytochrome oxidases a_1, a_2, and o, is not affected by the catabolism of glucose. The formation of all components of the electron transport system is controlled by the identity and concentration of the terminal electron acceptors present in the growth medium.

In yeast, the formation of the mitochondrial electron transport system is repressed in the presence of glucose (5). If a concentration of 10^{-4} M glucose is maintained, it represses the synthesis of the respiratory pigments of the yeast mitochondria (R. L. Lester, *personal communication*). In *Staphylococcus aureus* (2, 23) or the enteric bacteria (7, 10, 19), the presence of glucose in the growth media reduces the specific activity of the respiratory pigments.

Haemophilus parainfluenzae has been shown to form a membrane-bound electron transport system (32) that can be modified during its growth cycle (24, 27, 29). In *Haemophilus*, the activity of this membrane-bound respiratory system is an obligatory requirement for glucose catabolism, reduced pyridine nucleotide oxidation, and growth (32). This study presents evidence that what is affected by glucose is the formation of the primary membrane-bound dehydrogenases and that the concentrations of 2-demethyl vitamin K_2 (DMK_2), cytochromes, and cytochrome oxidases are determined by the nature and concentration of the terminal electron acceptors of the electron transport system.

MATERIALS AND METHODS

Turbidity. Bacterial growth was followed by measuring the turbidity (bacterial density) in terms of absorbance at 750 mμ in 13-mm test tubes with the Spectronic-20 colorimeter. The relationship between the turbidity and dry weight of these bacteria has already been given (30).

Oxygen utilization measurements. The Clark oxygen electrode was used to measure the oxygen tension in the growth medium. The electrode was standardized in 50 mM phosphate buffer (*p*H 7.6) at 38 C (25), and washed with 70% (v/v) ethyl alcohol from a washing bottle; the tip of the electrode was placed 5 to 10 mm below the surface of the medium. Recalibration of the instrument at the end of the experiment with air-saturated phosphate buffer and phosphate buffer deoxygenated with excess $Na_2S_2O_4$ established that the instrument remained stable throughout the experiment.

Cytochromes. Cytochromes b_1 and c_1 were measured by comparing the absorbance in suspensions of whole cells or membrane preparations in which the pigments were reduced to sand-blasted glass blanks in the Cary 14 CM recording spectrophotometer (29). Cytochrome b_1 was measured as the absorbance increment between the maximum at 561 mμ and a base line connecting points at 540 and 580 mμ in an absolute spectrum of the reduced cytochrome; cytochrome c_1 was measured as the absorbance increment between the maximum at 553 mμ and the same base line. Cytochrome oxidase a_2 was measured as the absorbance increment between the maximum at 635 mμ and a line connecting points at 660 and 610 mμ.

The cytochrome oxidases a_1 and o were measured from difference spectra between a suspension of bacteria in which the respiratory pigments were reduced and a similar suspension with reduced pigments that was saturated with carbon monoxide (24, 32). Cytochrome oxidase a_1 was measured as the absorbancy increment between the maximum at 435 mμ and a line extending from the side of the cytochrome o maximum at 416 mμ in the CO spectra as previously described (24). Cytochrome oxidase o was measured as the abbance increment between the maximum at 540 mμ and the minimum between 500 and 520 mμ. Cytochrome and cytochrome oxidase absorbancies were calculated per 10 mg of bacterial protein. Reduction of the res-

piratory pigments in the presence of 10 mM formate and 5 mM reduced nicotinamide adenine dinucleotide ($NADH_2$) has been established to be complete (32).

Measurements of the reoxidation of cytochromes and cytochrome oxidases were performed by comparing the difference spectra of bacterial suspensions with pigments reduced in the presence of 10 μM formate to those of similar suspensions with the reduced pigments reoxidized by shaking in air or after the anaerobic addition (31) of deoxygenated nitrate or fumarate solutions to final concentrations of 20 mM. Correction for the effects of the absorbancy of cytochromes b_1 and c_1 on each other was calculated with the following expressions: absorbance at 553 mμ = $x + 0.45\ y$ and absorbance at 561 mμ = $0.61\ x + y$, where x = absorbance of cytochrome c_1 at 553 mμ and y = absorbance of cytochrome b_1 at 561 mμ. The factors used in these equations were derived from the data presented previously (32).

Primary membrane-bound dehydrogenases. The spectrophotometric assay of ferricyanide reduction on addition of substrate has been established to be an adequate assay for the membrane-bound primary dehydrogenases of the electron transport system in these bacteria (27).

DMK_2. The quinone was extracted with isopropanol and assayed spectrophotometrically (28).

Glucose. Glucose was measured colorimetrically (17) after deproteinization of the medium (22).

Nitrite. Nitrite in the growth medium was measured colorimetrically (9).

Protein. Protein was measured by a modified biuret procedure (24).

Growth of bacteria. Two strains of *H. parainfluenzae*, one a mutant of the other, were used in this study. The parental type forms large amounts of cytochrome c_1 when grown anaerobically (24). The mutant does not form significant amounts of cytochrome c_1 (32). The strains, medium, growth conditions, culture preservation, and harvesting procedures have already been described (24).

Reagents. Reagents were as described in previous publications (30, 32).

RESULTS

Factors affecting the formation of the respiratory pigments. Haemophilus requires both a functional electron transport system and the presence of terminal electron acceptors for glucose catabolism, reduced pyridine nucleotide oxidation, and growth (30). Reduced pyridine nucleotides [$NADH_2$ and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$)], oxygen, nitrate, fumarate, and pyruvate have been shown to serve as suitable terminal electron transport acceptors (30). The concentration and nature of the terminal electron acceptors have a pronounced effect on the composition of the electron transport system (Table 1). If oxygen is the terminal electron acceptor for bacterial growth, cytochrome c_1 was not detectable. Cytochrome oxidase *o* was the only terminal oxidase found in bacteria grown with vigorous aeration. At low oxygen tensions, the bacteria contained double the concentration of cytochrome b_1 and at least 100-times the concentration of cytochrome oxidase a_2 present in the cells grown with vigorous aeration. Anaerobic growth with nitrate as the terminal electron acceptor resulted in high cytochrome c_1 and cytochrome oxidase a_1 con-

TABLE 1. *Cytochromes and cytochrome oxidases formed by Haemophilus parainfluenzae during growth with oxygen, nitrate, or fumarate as the terminal electron acceptors*[a]

Terminal electron acceptor	Doubling time	Turbidity at harvest[b]	Cytochrome formation[c]		Cytochrome oxidase formation[c]		
			b_1	c_1	a_1	a_2	*o*
	min						
Oxygen							
Vigorous aeration	35	0.70	0.021	<0.005	<0.005	<0.0002	0.002
Poor aeration	50	0.70	0.040	<0.010	<0.005	0.0200	0.009
Nitrate	65	0.90	<0.020	0.100	0.070	<0.0002	0.012
Fumarate	60	0.80	<0.010	0.048	0.040	0.007	0.060

[a] Parental-type *H. parainfluenzae* was grown for 14 to 18 hr at 37 C in these experiments in the proteose-peptone medium (24) under the following conditions: with oxygen and vigorous aeration (26); with oxygen, poor aeration, and 1.5 liters of medium added to 2.5-liter low-form Erlenmeyer flasks rotated at 20 cycles per min on a rotary shaker; grown in the all-glass anaerobic vessel and gassed with deoxygenated nitrogen as described (30) in the presence of 20 mM nitrate; grown as with nitrate in the presence of 20 mM fumarate. Cultures were harvested in the early stationary phase of growth. Cytochromes measured from absolute and difference spectra as described in Materials and Methods. Cytochrome concentrations are expressed as absorbancy difference per 10 mg of protein and were measured at bacterial densities of 25 to 50 mg of protein per ml. Cytcohrome b_1 and c_1 corrected for cytochrome c_1 and b_1, respectively.

[b] Turbidity is expressed as absorbance at 750 mμ.

[c] Measured in terms of absorbance per 10 mg of protein.

centrations. Anaerobic growth with fumarate as the terminal electron acceptor resulted in large amounts of cytochrome oxidase *o*.

Addition of the terminal electron acceptors to suspensions of bacteria with reduced respiratory pigments resulted in the reoxidation of these pigments (Table 2). If the spectra were measured within 4 min after vigorous shaking in air at 4 C, there was no significant reduction of the oxidized respiratory pigments. The addition of oxygen caused the reoxidation of all the pigments in bacteria grown with oxygen as the terminal electron acceptor. Nitrate added anaerobically produced partial oxidation of cytochrome c_1 and complete oxidation of cytochrome oxidase a_1 in experiments with bacteria grown with nitrate as the terminal electron acceptor. Fumarate added anaerobically to bacteria with their reduced respiratory pigments resulted in the reoxidation of half the cytochrome c_1 and of little of the cytochrome oxidases a_1 or a_2 in experiments with bacteria grown with fumarate as the terminal electron acceptor. The reoxidation of the cytochrome c_1 by the anaerobic addition of fumarate may involve cytochrome oxidase *o* and is suggested by the following reasons: the reoxidation by fumarate is inhibited by KCN (32); KCN inhibits oxygen utilization in bacteria in which cytochrome oxidase *o* is the only oxidase present (32); the inhibition by KCN appears in difference spectra to be at the level of this cytochrome oxidase (28). Since neither cytochrome oxidase a_1 nor a_2 is oxidized by the anaerobic addition of fumarate to reduced bacteria, the cyanide-sensitive oxidation is believed to involve cytochrome oxidase *o*.

TABLE 2. *Per cent of cytochrome oxidases reoxidized on addition of air, nitrate, or fumarate to suspensions of reduced Haemophilus parainfluenzae*[a]

Terminal electron acceptor		Per cent of cytochrome reoxidized			
Used for growth	Used for oxidation	b_1	c_1	a_1	a_2
Oxygen, vigorous aeration.....	Oxygen	100	ND[b]	ND	ND
Oxygen, poor aeration.....	Oxygen	100	100	ND	100
Nitrate.....	Nitrate	ND	24	100	ND
Fumarate.....	Fumarate	ND	50	7	0

[a] Suspensions of *H. parainfluenzae*, grown as described in Table 1, were reduced in the presence of 10 μM formate. Oxygen was added to bacteria grown with oxygen as the terminal electron acceptor by shaking the suspension of bacteria vigorously in air, and the reoxidation of the respiratory pigments was compared with a similar suspension not shaken in air in the spectrophotometer (25). Cytochromes and cytochrome oxidases were measured as in Table 1.

[b] ND indicates cytochrome was not detectable.

In experiments not shown in Table 2, oxygen completely reoxidized the respiratory pigments formed during growth with either nitrate or fumarate as the terminal electron acceptors. Anaerobic addition of fumarate to bacteria grown with nitrate as the terminal electron acceptor caused the partial reoxidation of cytochrome c_1. This was also true with the anaerobic addition of nitrate to bacteria grown with fumarate as the terminal electron acceptor.

Effect of glucose on bacterial growth. The effect of glucose utilization on the formation of the electron transport system must be measured under conditions in which the changes in the composition of the electron transport system formed with different electron acceptors are known. If bacteria are grown with vigorous aeration to a bacterial density of 10^7 cells per milliliter and the aeration is suddenly stopped, conditions which produce the maximal change in the composition of the electron transport system occur (24, 29). With the shift from aerobic to anaerobic conditions, there were changes in the concentrations of primary dehydrogenases, increases in DMK_2, cytochrome b_1, and cytochrome oxidase *o*, and the appearance of cytochrome oxidases a_1 and a_2. Much higher concentrations of cytochrome oxidase a_1 appeared if nitrate was present in the medium. Changes in the concentrations of the terminal electron acceptors in an aerobic to anaerobic shift are illustrated by the curves in Fig. 1. After the shift, the oxygen concentration dropped rapidly, and the rate of nitrite formation from nitrate increased. If glucose was present in the medium, its concentration dropped rapidly after the aeration was stopped. The rate of bacterial growth during such an aerobic to anaerobic shift was unaffected by the presence of glucose in the growth medium (Fig. 2).

Numerous experiments have established that the concentrations of the dehydrogenases, cytochromes, and cytochrome oxidases remain essentially constant during log-phase growth in a highly aerated environment. There is no reduction in the oxygen tension in the medium nor are there changes in the respiratory-pigment concentrations between cell densities of 10^6 and 5×10^8 cells per milliliter (turbidity, 0.05 to 0.30) so long as vigorous aeration is maintained. In the experiments that follow, the bacteria were grown in medium with or without glucose to bacterial densities near 10^7 cells per milliliter with vigorous aeration. At this point, a sample was removed for assay of the steady-state aerobic respiratory-pigment concentrations. The aeration was then

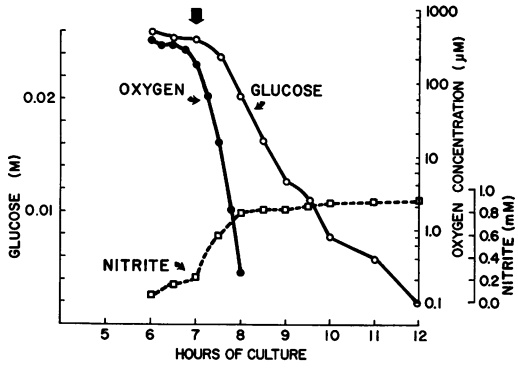

FIG. 1. *Relationship among glucose utilization, oxygen concentration, and nitrite production during an aerobic to anaerobic growth cycle. The mutant type of Haemophilus parainfluenzae was grown in a low-form Erlenmeyer flask containing 1.5 liters of proteose-peptone medium with 20 mM nitrate at 38 C with shaking at a rate of 100 cycles per min (26). The medium was 3 cm deep. At the time indicated by the arrow, the shaking was stopped. Symbols: ●, oxygen concentration measured 5 mm below the surface of the medium with the Clark oxygen electrode as described under Materials and Methods; ○, glucose concentration in the medium; □, nitrite concentration in the medium.*

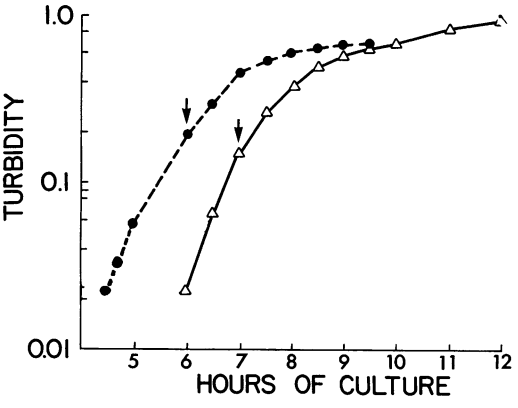

FIG. 2. *Growth of Haemophilus parainfluenzae during the shifts from aerobic to anaerobic growth in the presence and absence of glucose. At the times indicated by the arrows, the aeration was stopped by stopping the agitation. Symbols: △, experiment conducted in proteose-peptone medium with 40 mM glucose; ●, experiment conducted with this medium without glucose. Bacterial density (turbidity) was measured as the absorbance at 750 mμ. The doubling time during aeration in both experiments was about 20 min.*

stopped at the points indicated by arrows in Fig. 1 to 5, and the changes in respiratory pigment concentration were assayed as the bacterial metabolism changed the concentrations of terminal electron acceptors. In this manner, the effect of glucose catabolism on the changes in the respiratory pigments could be examined. The glucose concentration was 40 mM at the start of the experiments. By the time of the last determination, the glucose concentration was 2 to 5 mM.

Effect of glucose on the formation of the primary membrane-bound dehydrogenases. The presence of glucose during the shift from aerobic to anaerobic growth resulted in an 11-fold increase in the activity of formic dehydrogenase. In bacteria growing under the same conditions but without glucose, there was little net change in the formic dehydrogenase activity. Succinic dehydrogenase activity was relatively unaffected during the shift from aerobic to anaerobic growth in bacteria incubated in the presence of glucose. In the absence of glucose, the bacteria produced a fivefold increase in succinic dehydrogenase activity (Fig. 3). The activities of the dehydrogenases for $NADPH_2$, $NADH_2$, D-lactate,

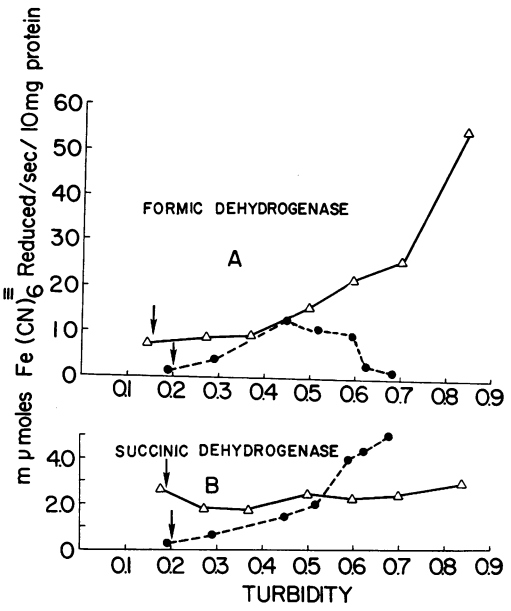

FIG. 3. *Effect of growth in the presence and absence of glucose on the formation of formic and succinic dehydrogenases in Haemophilus parainfluenzae. Formic dehydrogenase activity (upper curve) and succinic dehydrogenase activity (lower curve) were measured spectroscopically by ferricyanide reduction (27). Symbols: △, samples taken from bacteria grown in the presence of 40 mM glucose [samples were taken at the turbidities (bacterial density at 750 mμ) indicated on the abcissa from the experiment in Fig. 1 and 2]; ●, samples taken from bacteria grown without glucose; arrows indicate the points at which aeration was stopped.*

and L-lactate were similar during the shift from aerobic to anaerobic growth in bacteria incubated both with and without glucose in the medium. All these enzymes are part of the membrane-bound electron transport system in *Haemophilus* (27, 32).

Effect of glucose on the formation of DMK_2 and cytochrome b_1. If *Haemophilus* is subjected to the shift from aerobic to anaerobic growth (Fig. 1 and 2) both in the presence and absence of glucose, the effect of the presence of glucose on DMK_2 and cytochrome b_1 concentrations can be examined. The data in Fig. 4 illustrate that glucose catabolism had no effect on the formation of cytochrome b_1 and DMK_2. The formation of DMK_2 and cytochrome b_1 have been shown to be coordinate (29).

Effect of glucose on the formation of cytochrome c_1 and the cytochrome oxidases. The parental type of *H. parainfluenzae* differed from the strain used in Fig. 1 to 4 in having the ability to form large amounts of cytochrome c_1 if grown anaerobically. In all other respects, the parental and mutant types appear to behave similarly (32). The synthesis of cytochromes as followed throughout the shift from aerobic to anaerobic growth is illustrated in Fig. 5. The syntheses of cytochrome c_1 and cytochrome oxidase a_2 were not affected by the presence of glucose. The formation of the cytochrome oxidases a_1 and o is similarly not affected by the catabolism of glucose.

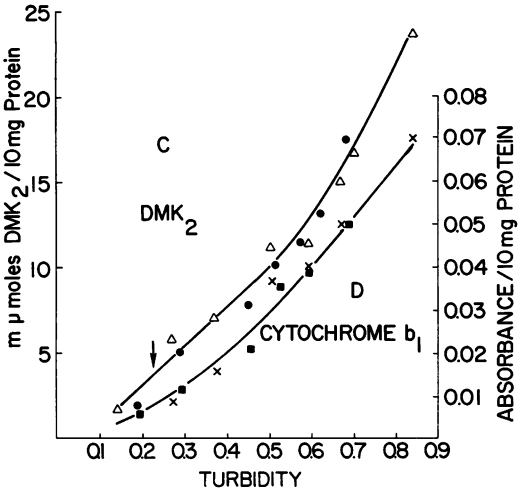

FIG. 4. *Effect of growth in the presence and absence of glucose on the formation of DMK_2 and cytochrome b_1. Curve C (upper curve) illustrates the formation of DMK_2 during the shift from aerobic to anaerobic growth of Fig. 1 and 2. Symbols: ●, samples of bacteria grown without glucose; △, samples of bacteria grown in the presence of 40 mM glucose [curve D (lower curve) illustrates the formation of cytochrome b_1 under the same growth conditions]; ■, samples taken from bacteria grown without glucose in the medium; ×, samples taken from bacteria grown in the presence of 40 mM glucose. The turbidity was measured as in Fig. 3. The absorbance increment for cytochrome b_1 was measured between the maximum at 561 mμ and a line connecting 540 and 580 mμ as described in Materials and Methods. Arrows indicate the points at which aeration was stopped.*

DISCUSSION

The effect of culture conditions on the formation of the cytochrome system apparently divides bacteria into two classes. In one group, oxygen acts as an inducer for the formation of the electron transport system. This group includes yeast (5), *Bacillus cereus* (21), *Salmonella typhimurium* (19), and *Pasteurella pestis* (3, 4). In the other class, oxygen acts as a repressor of cytochrome formation as in *Pseudomonas* species (11, 20), *Bacillus subtilis* (1), or *H. parainfluenzae*

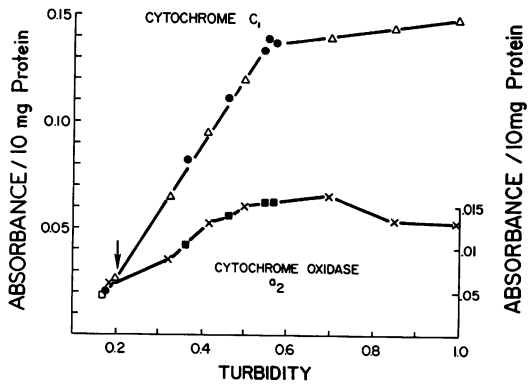

FIG. 5. *Effect of growth in the presence and absence of glucose on the formation of cytochrome c_1 and cytochrome oxidase a_2 in Haemophilus parainfluenzae. The upper curve represents experiments with the parental type of H. parainfluenzae during the shift from aerobic to anaerobic growth similar to that illustrated in Fig. 1 and 2. Symbols: △, cytochrome c_1 concentration in bacteria grown in the presence of 40 mM glucose; ●, cytochrome c_1 concentration in bacteria grown in the absence of glucose. The lower curve represents the experiments like those of the upper curve in which the formation of cytochrome oxidase a_2 was measured in bacteria grown with 40 mM glucose (×), and grown in the absence of glucose (■). The right-hand ordinate indicates the absorbancy increment for cytochrome a_2 and the left-hand ordinate indicates the absorbancy increment for cytochrome c_1. Cytochrome oxidase a_2 was measured between the maximum at 635 mμ and a line connecting 660 and 610 mμ, and the cytochrome c_1 was measured between its maximum at 553 mμ and a line connecting 540 and 580 mμ as described in Materials and Methods. Arrows indicate the points at which aeration was stopped.*

(24). This division of bacteria into two classes is also reflected in the response of the cytochrome-forming system to the utilization of glucose from the growth medium. In the group of organisms in which oxygen induces cytochrome formation, glucose exerts a marked repressive effect on the formation of cytochromes. Examples of organisms in which glucose repression is observed include the enteric bacteria (6, 7, 10, 19), staphylococci (2, 23), and yeast (5). If *H. parainfluenzae* is typical of the class in which oxygen represses cytochrome formation, then glucose catabolism would have little effect on the formation of the cytochrome system.

In some respects, the responses of the cytochrome system to changes in environment in the different classes of bacteria are similar. Low oxygen tension in the growth medium induces the formation of cytochrome oxidase a_2 in both classes of bacteria. In *Escherichia coli* and *Aerobacter aerogenes* (14, 15) as well as in *H. parainfluenzae*, cytochrome oxidase a_2 is induced during growth with low oxygen tension. Nitrate that is the only terminal acceptor of electrons in the growth medium causes the induction of cytochromes and cytochrome oxidases in many bacteria in which oxygen induces cytochrome formation (16), as well as in bacteria in which oxygen represses cytochrome formation [*Pseudomonas* (8) and *H. parainfluenzae*]. Oxygen counteracts the induction of cytochrome formation by nitrate in both bacterial classes [e.g., *A. aerogenes* (18) and *H. parainfluenzae*].

Glucose has been shown to affect the formation of the cytoplasmic catabolic enzymes in many bacteria (7, 12, 13). Glucose metabolism induces the formation of the enzymes of the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways and depresses the formation of some Krebs cycle enzymes in *H. parainfluenzae* (30). Unlike with many bacteria in which the utilization of glucose during bacterial growth inhibits the formation of the respiratory system, with *H. parainfluenzae* glucose utilization has no measurable effect on the formation of the respiratory quinone, the cytochromes, or the cytochrome oxidases. The formation of the respiratory quinone, the primary membrane-bound dehydrogenases, the cytochromes, and cytochrome oxidases by *H. parainfluenzae* seems to be dependent primarily on the type and concentration of the terminal acceptor of electron transport in the growth medium (Table 1). During the shift from aerobic to anaerobic growth, the presence of glucose in the medium seems to cause a marked increase in formate dehydrogenase activity when compared to growth in the absence of glucose. In contrast, in the absence of glucose the shift results in the bacterial synthesis of more succinic dehydrogenase than is found in bacteria grown in the presence of glucose. The possible rationale for this effect of glucose on the formation of the primary membrane-bound dehydrogenases remains unclear. The experiments reported in this paper show that, in addition to being responsive to the type and concentration of the terminal electron acceptors, the control mechanisms governing the synthesis of the membrane-bound electron transport system also are affected by glucose catabolism. This effect of glucose or its catabolites (13) on the control mechanism for membrane-bound electron transport system formation acts on the primary dehydrogenases and not on the other elements of the system.

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