RESPIRATORY SYSTEMS IN THE HEMIN-REQUIRING *HAEMOPHILUS* SPECIES

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ABSTRACT

WHITE, D. C. (Rockefeller Institute, New York, N.Y.). Respiratory systems in hemin-requiring *Haemophilus* species. J. Bacteriol. **85**:84–96. 1963.—If grown in Levinthal's medium or in proteose peptone medium with excess hemin, *Haemophilus influenzae*, *H. aegyptius*, and *H. canis* (*H. haemoglobinophilus*) form an electron-transport system consisting of six cytochromes and two respiratory flavoproteins. In proteose peptone, these species can greatly modify the composition of their electron-transport complex. With anaerobic incubation in the presence of nitrate, they produce increased amounts of cytochrome c_1 and the cytochrome oxidases a_1 and *o*. This anaerobic pattern is greatly exaggerated by growth under carbon monoxide, in which case large concentrations of cytochrome oxidase are produced. In the presence of the inhibitor secobarbital or of growth-limiting amounts of hemin, intermediate amounts of cytochromes and respiratory flavoproteins are formed. When only small amounts of hemin are present, these species grow but form no detectable cytochrome system. Catalase is the only hemoprotein found. Under these conditions, the addition of glucose induces the formation of a lactate oxidase flavoprotein if the system is incubated aerobically. This cytochromeless state also occurs when these species are grown in KCN or anaerobically without nitrate and with excess hemin. The ability of these species to modify the composition of the electron-transport system strongly suggests that this function unit is formed from individual components. Hemin-requiring *Haemophilus* species have a hemin-sparing compensatory mechanism that allows growth under conditions under which hemin-independent *Haemophilus* species will not grow.

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Growth of the hemin-independent *Haemophilus parainfluenzae* requires the formation of an electron-transport system involving six cytochromes and two respiratory flavoproteins (White and Smith, 1962). These pigments are part of a membrane system whose structural integrity must be preserved for function (Smith and White, 1962). From changes which occur in the concentrations of these respiratory pigments during various growth conditions, when the membrane itself is presumably not changing, it appears as if each component is formed individually and is subsequently added to the membrane (White, 1962). Several *Haemophilus* species require hemin which is used as prosthetic groups of the cytochrome system. With these species, the relationship between a prosthetic group and the whole membrane complex can be studied. During the stationary phase of growth, much greater changes in the concentrations of respiratory pigments can be demonstrated with the hemin-requiring species than with the hemin-independent species.

The studies of Lwoff and Lwoff (1937), Granick and Gilder (1946), and Gilder and Granick (1947) established that the hemin-requiring *H. influenzae* cannot grow in proteose peptone medium unless hemin and diphosphopyridine nucleotide (DPN) are present. Final growth is proportional to the hemin concentration in the medium. The hemin is used as part of the cytochrome system. The respiratory pigments of this bacterium, as determined with a hand spectroscope, are cytochromes *a*, *b*, and *c*. Cytochrome *b* is present in greater amounts than *c* (Fujita and Kodama, 1934). In view of these observations and of the finding that reduced diphosphopyridine nucleotide (DPNH), added to the intact bacterium, produces a rapid oxygen uptake characteristic of the cytochrome respiration, the interaction between hemin and the respiratory hemoproteins can conveniently be studied in this bacterium.

Changes in cytochrome concentrations in various bacteria were reviewed by Lascelles (1961). These primarily involve changes resulting from shifts between aerobic and anaerobic incubation. Richmond and Maaløe (1962) showed that *Salmonella typhimurium* can exist either with or without cytochromes as a function of the carbon source supplied in the medium. Shifts between these states involve a lag in growth lasting several generation times. The hemin-requiring mutant of *Staphylococcus aureus* forms a cytochrome system if grown in a peptone medium aerobically in the presence of hemin (Jensen and Thofern, 1954). This strain will grow as rapidly in medium supplemented with acetate or pyruvate without hemin (Lascelles, 1956). It is of particular interest that a strain of this bacterium resists 5-fluoro-2-deoxyuridine and has no respiratory activity on repeated subculture in the absence of this drug (Gause, Kochetkova, and Vladimirova, 1961). This compound specifically inhibits deoxyribonucleic acid (DNA) synthesis.

This study shows that three hemin-requiring species of *Haemophilus* can greatly modify the composition of their electron-transport system. With this information, it is hoped that further studies with these transformable *Haemophilus* species can lead to a clearer understanding of the genetic control of the formation of the electron-transport system.

Materials and Methods

Bacteria. The bacterial strains used in this study were *H. aegyptius* 15 and *H. influenzae*, rough type d (strain Garfinkel), both supplied by G. Leidy of Columbia University (Leidy, Hahn, and Alexander, 1956). These species required both hemin and DPN for growth in proteose peptone medium. *H. canis* 1659 (*H. haemoglobinophilus*), National Collection of Type Cultures, Mill Hill, England, required hemin but not DPN for growth. These species were maintained by daily transfer, or preserved in growth medium containing 15% (v/v) glycerin at −20 C.

Media. Levinthal's medium was prepared as described by Alexander (1958), except that the stock solution was autoclaved, and filtered DPN was added. The Levinthal stock was diluted by half with proteose peptone medium before use.

Proteose peptone medium consisted of proteose peptone no. 3 (Difco), 2%; NaCl, 0.6%; KNO_3, 0.1%; and tris(hydroxymethyl)aminomethane (Sigma), 0.02 M. The final pH was 7.8. The medium was boiled, filtered, and 0.01% $Na_2S_2O_4$ added before autoclaving. Just before use, filter-sterilized DPN (1 μg/ml) was added. Glucose (0.5%, w/v, autoclaved separately) was added where indicated. Hemin stock solution was prepared by adding 2.5 mg of hemin to 1 ml of 0.2 M KOH in 47.5% ethanol, and diluting to 5 ml with sterile water. This solution was stored in the dark at −20 C. By use of this method at the hemin concentrations employed in this study, colloidal mycelle formation by hemin apparently did not complicate the results.

For growth in cyanide, the culture apparatus was equipped with ground-glass joints and stopcocks. The medium was inoculated with 10 late stationary phase cells, and phosphate buffer (pH 7.8) added so the final concentration in the medium was 0.02 M. The flask was then deoxygenated with nitrogen, and the stopcocks closed. The ground-glass port was then opened and sufficient molar KCN added to achieve a final concentration of 5×10^{-3} M. This was done rapidly, and the apparatus sealed and incubated.

The apparatus used to grow the bacteria consisted of a water-jacketed Woulff bottle with ground-glass ports for aeration and sampling, as described previously (White, 1962). Cultures grown anaerobically were gassed with nitrogen or carbon monoxide while cooling after autoclaving, and throughout the growth period. The gases used in this study were deoxygenated by passing through a packed column of copper filings maintained at 500 C. Cultures were tested for contamination by incubating samples in proteose peptone not containing hemin. Cell counts and turbidity measurements were made as described previously (White, 1962).

Spectra. Bacterial suspensions were prepared and difference spectra were measured as described previously (White, 1962). Cytochromes were named as by White and Smith (1962).

Catalase activity. Catalase was measured under conditions where H_2O_2 does not damage the enzyme during the assay, as described previously (White, 1962). Catalase activity was expressed as Kat f (protein), which equals the apparent first-order rate constant per gram of bacterial protein.

Oxygen uptake. Oxygen uptake was measured by use of a Clark electrode (Yellow Springs Instrument Co., Antioch, Ohio). The electrode surface was covered by a polyethylene film and

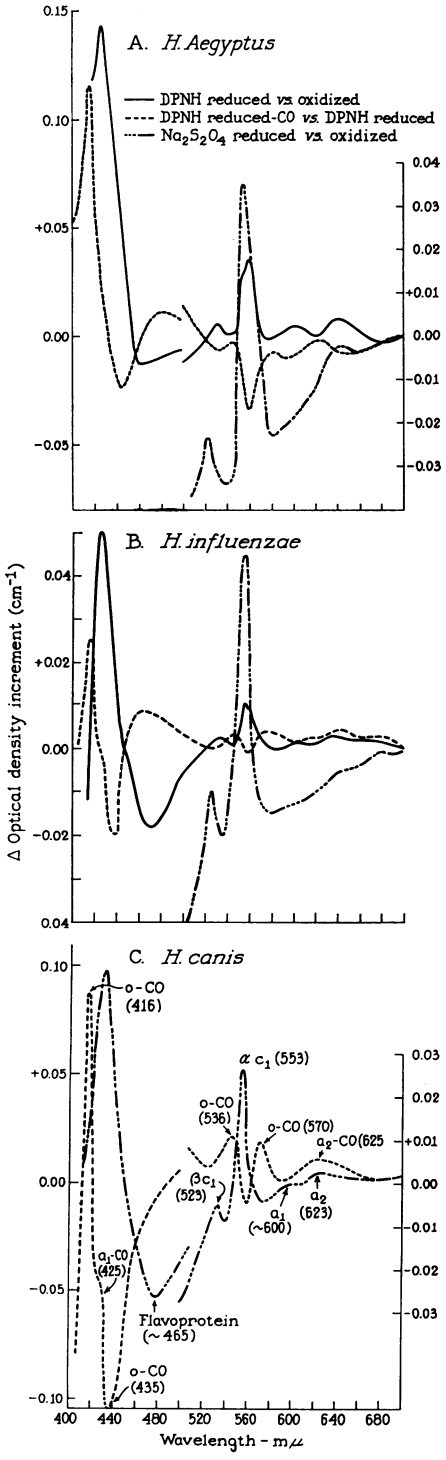


FIG. 1. *Cytochrome patterns formed by hemin-requiring Haemophilus species grown in Levinthal's medium. The physiologically reducible cytochromes can be seen in the DPNH-reduced vs. oxidized difference spectrum, and the cytochrome oxidases in the DPNH-reduced carbon monoxide vs. DPNH-reduced difference spectrum. Large amounts of non-physiologically reducible cytochrome c_1 can be detected after reduction with $Na_2S_2O_4$. Spectra represent 10 mg of bacterial protein per ml of suspension, measured in 0.05 M phosphate buffer (pH 7.0) and 20% (v/v) glycerin. The cytochromes and DPNH-oxidase flavoprotein are labeled in* C *at their characteristic maxima and minima.*

was polarized at 0.6 v. The electrode was inserted into 3 ml of test suspension of 0.05 M phosphate buffer (pH 7.0) maintained at 30 C by use of a water jacket. The suspension was stirred with a magnetic stirrer at a rate adjusted to give adequate stirring but not to change the oxygen concentration of partially deoxygenated buffer. The electrode was routinely standardized, using buffer saturated with air (220 μM O_2) and at zero oxygen after addition of excess $Na_2S_2O_4$. The bacteria (25 to 100 mg of bacterial protein in 0.3 ml) were then added to the air-saturated buffer, after which 0.03 ml of molar substrate (pH 7.0) was added and the oxygen uptake was recorded as illustrated in Fig. 6A, B, and C.

Protein determination. Protein was measured by the biuret reaction (Cornall, Bardawill, and David, 1949), using 0.2% sodium deoxycholate to aid cell lysis.

Reagents. The reagents were as described previously (White and Smith, 1962). Crystalline hemin was prepared by S. Granick (Granick and Gilder, 1946). The inhibitor, 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO), was the generous gift of J. W. Lightbown.

RESULTS

Levinthal's medium. The difference spectra of *H. influenzae*, *H. aegyptius*, and *H. canis* (Fig. 1) show cytochrome patterns typical of *H. parainfluenzae* (White and Smith, 1962). A DPNH-oxidase flavoprotein and cytochromes b_1, c_1, a_1, and a_2 are seen in the $DPNH_2$ reduced vs. oxidized spectra. The cytochrome oxidases are demonstrated in the DPNH-reduced carbon monoxide spectra to be cytochromes a_2, a_1, and *o*. As with other *Haemophilus* species (White and Smith, 1962), reduced cytochrome a_1 is more readily reoxidized by nitrate than the other oxidases (Fig. 7C and E).

It has been previously observed that large amounts of non-DPNH-reducible cytochrome c_1 appear in the cell sap as *H. parainfluenzae* ages, and this cytochrome can be recovered in solution on rupture of the cells with alumina (Smith and White, 1962). The isolated cytochrome c_1 from the hemin-requiring *Haemophilus* is spectroscopically identical to that of *H. parainfluenzae*. Other species of hemin-independent *Haemophilus* (*H. parahaemolyticus*, *H. "para" suis*, the animal parainfluenzae's *J-66* and *K-17* (Biberstein and Gills, 1961), and *H. aphrophilus*) show this same non-DPNH-reducible cytochrome c_1, which is recoverable on cell rupture (White, *unpublished data*). When the cytochrome patterns are followed during the growth cycle by examination of the difference spectra, during the transition between log and stationary phase the amount of non-DPNH-reducible cytochrome c_1 increases dramatically. There is also a marked increase in total cytochrome *o*, with a decrease in DPNH-reducible cytochrome *o* as stationary phase continues. Added DPNH produces a rapid oxygen uptake with intact bacteria of these species. When sufficient DPNH is added to the bacterial suspension, a maximal rate of oxygen uptake can be measured with the oxygen electrode (*see* Fig. 6D). Paralleling the loss of DPNH-reducible cytochromes *o* and c_1, there is a progressive decrease in oxygen uptake measured at saturating concentrations of DPNH (from 2.0 to 2.3 in the log phase to 0.5 to 0.2 in the late stationary phase, expressed in μM of O_2 per sec^{-1} per 10 mg of bacterial protein measured at 30 C). The catalase activity also falls progressively from Kat f (protein) of 6 to 0.2 in the late stationary phase. These findings are typical of those found in *H. parainfluenzae* (White, 1962).

In Levinthal's medium, *H. influenzae* may have a doubling time of 15 min with vigorous aeration and rapid stirring.

Proteose peptone medium. Proteose peptone medium made as described will support growth of *H. canis* when hemin is added, and growth of *H. influenzae* and *H. aegyptius* when both hemin and DPN are added. With this medium suitably supplemented with hemin and DPN, cell counts (Petroff-Hauser) and viable counts in pour plates indicate that in young cultures all cells can form colonies within the counting error. Viable counts are directly proportional to turbidimetric measurements. Growth measured turbidimetrically in

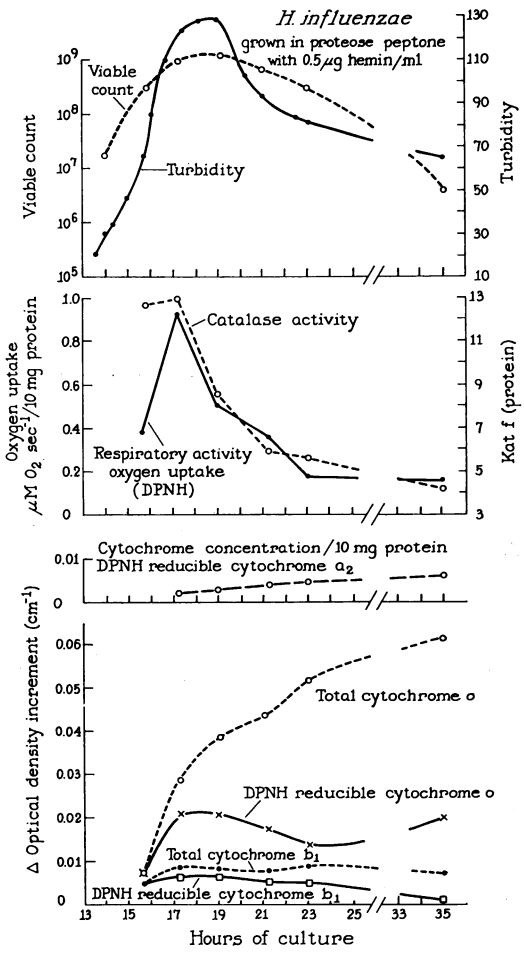


FIG. 2. *Changes in cytochrome patterns and respiratory and catalase activity during the growth cycle of Haemophilus influenzae incubated with excess hemin in proteose peptone medium at 37 C. The culture was aerated with 100 ml of air per min per liter of medium. Catalase activity is expressed at Kat f (protein) measured as described in Materials and Methods. Respiratory activity is measured at saturating DPNH concentration at 30 C. The cytochromes are measured in terms of optical density differences/10 mg of bacterial protein as: cytochrome b_1 between the maximum at 560 mµ and the minimum near 580 mµ; cytochrome a_2 between the maximum at 620 mµ and a line connecting 600 and 680 mµ; and cytochrome o between the maximum at 420 mµ and the minimum at 437 mµ. Difference spectra, respiratory, and catalase activity are measured in 0.05 M phosphate buffer (pH 7.0) containing 20% (v/v) glycerin.*

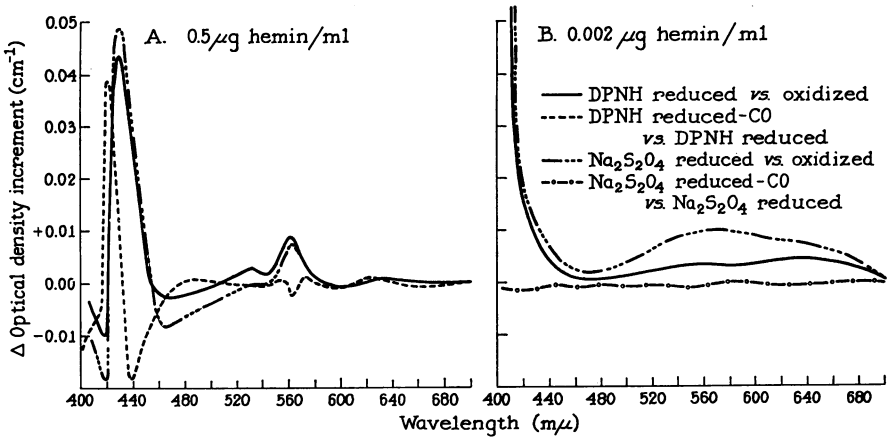


FIG. 3. *Difference spectra of Haemophilus influenzae grown in proteose peptone media with* (A) *0.5 μg of hemin/ml and* (B) *0.002 μg of hemin/ml. Spectra are plotted as described in Materials and Methods, and represent 10 mg of bacterial protein per ml. Bacteria were suspended in 0.05 M phosphate buffer (pH 7.0) containing 20% (v/v) glycerin.*

this medium is proportional to the hemin concentration between 0.001 and 0.1 μg of hemin per ml. At hemin concentration greater than 0.1 μg/ml, growth is not limited by the hemin concentration for any of the three species of hemin-requiring *Haemophilus*. If hemin is added to this medium at concentrations in which it is not growth limiting, these *Haemophilus* species form characteristic patterns of respiratory pigments, DPNH oxidase, flavoprotein, and cytochromes b_1, a_1, a_2, *o*; in *H. aegyptius* or *H. canis*, and *H. influenzae* grown anaerobically, cytochrome c_1 is detectable (*see* Fig. 3A, 6A, 7A). This pattern differs from that of the hemin-independent *Haemophilus* species, and these same hemin-requiring species grown in Levinthal's medium, in not having the large amounts of cytochrome c_1 which become nonreducible with DPNH during the stationary phase.

If the patterns of cytochrome formation are followed during the growth cycle, remarkable changes in oxidase cytochrome *o* occur. Figure 2 shows the changes in *H. influenzae* grown in proteose peptone with limiting aeration in the presence of excess hemin. There is no detectable cytochrome c_1 formed during the growth cycle. Cytochrome b_1 is formed rapidly during the log to stationary phase transition, after which very little more is formed. The amount of cytochrome b_1 that is DPNH-reducible falls during the stationary phase. There is a remarkable synthesis of cytochrome *o*, which increases even during the period of cell death. The DPNH-reducible cytochrome *o* decreases and then increases during the stationary phase. This is similar to the behavior of these bacteria grown in Levinthal's medium. Cytochrome a_2 progressively increases during the stationary phase, and all of it is DPNH reducible throughout the growth cycle.

When the maximal rate of oxygen uptake is followed throughout the growth cycle, there is an initial increase in respiratory activity which roughly parallels the increase in DPNH-reducible cytochrome *o* during the early stationary phase (Fig. 2). This maximal respiratory rate falls off with longer incubation, as it does during growth in Levinthal's medium.

TABLE 1. *Catalase activity of hemin-requiring Haemophilus species**

Growth conditions†	Cytochromes	Kat f (protein)		
		H. influenzae	*H. aegyptius*	*H. canis*
Aerobic, 0.5 μg of hemin/ml.....	+	16.1	10.6	11.9
Anaerobic, 0.5 μg of hemin/ml...	+	2.3	1.2	0.8
Aerobic, 0.002 μg of hemin/ml...	0	0.2	0.3	0.3

* Catalase was measured by iodometric titration on intact bacteria as described in Materials and Methods.

† In proteose peptone medium.

The catalase activity and cytochrome patterns are identical for *H. canis*, *H. aegyptius*, and *H. influenzae* grown in this manner. The hemin-independent *Haemophilus* species are able to grow anaerobically only in the presence of nitrate which reoxidizes the reduced electron-transport system; growth is inhibited by cyanide and carbon monoxide in the medium (White and Smith, 1962). This suggests that a functional electron-transport system including oxidase must be formed for them to grow. This necessitates the formation of several hemoproteins which could place hemin-requiring *Haemophilus* species at a selective disadvantage. Examination of these hemin-requiring *Haemophilus* grown in hemin concentration that limits the total growth shows they can partially compensate for their hemin-requirement by growth without forming a cytochrome system. There is still an absolute requirement for hemin under these conditions, and the only detectable hemoprotein formed is catalase.

Figure 3B shows the difference spectra of *H. influenzae* grown in proteose peptone medium containing a growth-limiting hemin concentration. Detectable cytochromes are not formed. In the same medium under the same conditions of growth, addition of 0.5 μg of hemin/ml allows

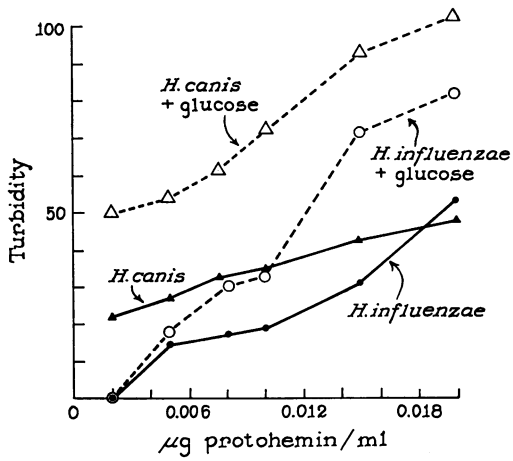


FIG. 4. *Potentiation of growth-limiting hemin by glucose (0.5%). Turbidity was measured with a Klett colorimeter in a 13-mm tube, after 36 hr of growth in 20-mm test tubes, containing 5 ml of media, incubated in air. Under these conditions, a turbidity of 50 corresponds to 5 × 10⁸ cells/ml. Inoculum consists of 100 late stationary phase cells previously incubated in media for 3 hr without hemin.*

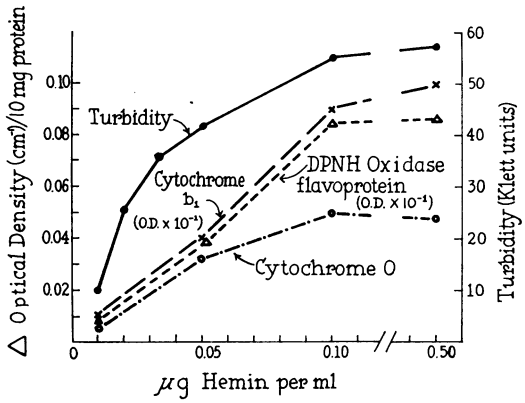


FIG. 5. *Formation of DPNH-reducible flavoprotein and cytochromes by Haemophilus influenzae at various hemin concentrations. Turbidity and concentrations of cytochromes b_1 and o measured as in Fig. 2. DPNH-oxidase flavoprotein measured between minima at 465 and 500 mμ. Cytochrome b_1 and DPNH-oxidase flavoprotein are plotted at ten times the measured value. The cytochromes represent those reducible with DPNH measured in the early stationary phase cells. (Incubation at 37 C for 18 hr.)*

the formation of a typical cytochrome pattern (Fig. 3A). The cells used for the difference spectra of Fig. 3A have an oxygen uptake at saturating DPNH concentration of 1.32 μM of O_2 per sec^{-1} per 10 mg of bacterial protein at 30 C, whereas the cells used for the difference spectra of Fig. 3B have no detectable oxygen uptake in 30 min in the presence of 60 mg of bacterial protein and 0.005 M DPNH. Neither the difference spectra nor the functional activity (oxygen uptake) reveal any cytochromes in *H. influenzae* grown aerobically in medium containing limiting hemin. This also can be shown for *H. cannis* (Fig. 6B) or *H. aegyptius* (Fig. 7B).

The only hemoprotein detected in the cytochromeless state with the hemin-requiring *Haemophilus* is catalase (Table 1). The activity during the early stationary phase of growth is low, but consistently present in the cytochromeless state. At hemin concentrations between these extremes, the cytochromes formed per cell are proportional to the hemin added (Fig. 4). This is true of the DPNH-oxidase flavoprotein as well. The total growth, cytochrome, or flavoprotein formed is independent of hemin concentration above 0.1 μg per ml of hemin. The oxygen uptake measured with saturating DPNH is likewise proportional

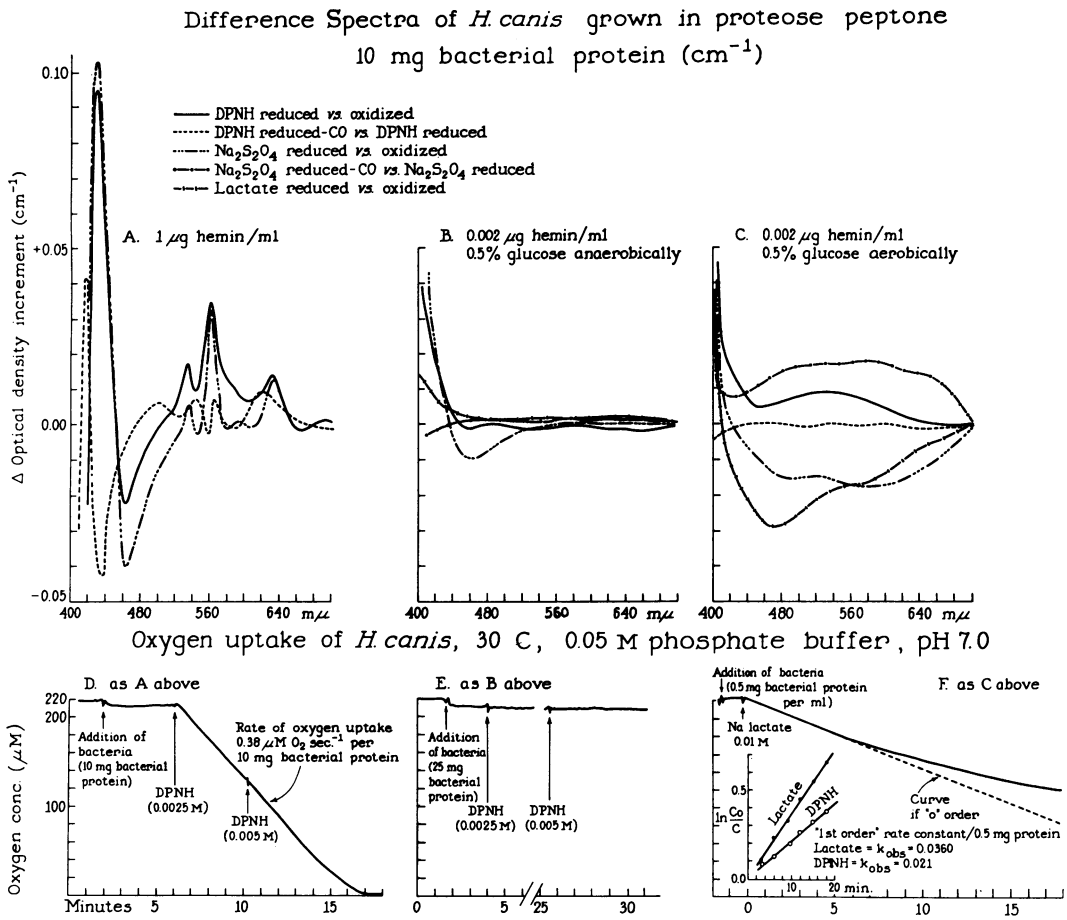


FIG. 6. *Difference spectra of Haemophilus canis grown in proteose peptone media with* (A) *0.5 μg of hemin/ml;* (B) *0.02 μg of hemin/ml and 0.5% glucose, anaerobically; and* (C) *0.002 μg of hemin/ml and 0.5% glucose, aerobically. Oxygen uptake curves* (D, E, F) *for each of the samples* A, B, *and* C *are plotted, showing the effect of adding the bacterial suspension, DPNH, or sodium lactate, and following the oxygen uptake with time. Bacteria for spectra and oxygen-uptake measurements were suspended in 0.05 M phosphate buffer (pH 7.0) and 20% (v/v) glycerin. In* E *inset, the oxygen uptake is replotted to show the dependence of rate on the oxygen concentration.*

to the hemin added to the medium in the range 0.005 μg to 0.1 mg/ml.

Figure 5 illustrates the effect of glucose (0.5% w/v) which potentiates the effect of hemin at growth-limiting concentrations. *H. aegyptius* behaves nearly identically to *H. influenzae* and is omitted from Fig. 5 for clarity.

The electron-transport system formed in the presence of glucose, which is similar in all three species, is illustrated in Fig. 6 with *H. canis*. Grown aerobically in excess hemin, *H. canis* has a typical difference spectrum (Fig. 6A). The oxygen uptake of cells grown in this manner is shown in Fig. 6D, which illustrates the rapid oxygen uptake initiated by adding DPNH to the bacterial suspension. The rapid oxygen uptake that continues at a uniform rate until very low oxygen concentrations are achieved is typical of cells respiring via cytochrome oxidase where the oxidase concentration is high (Chance, 1957). Figure 6B illustrates the difference spectra of *H. canis* grown anaerobically in limiting hemin in the presence of glucose. The oxygen uptake of these cells is illustrated in Fig. 6E, where no detectable oxygen uptake occurs upon adding DPNH.

When this medium containing limiting hemin

and glucose is incubated aerobically, a flavoprotein can be demonstrated which reacts with sodium lactate and DPNH (Fig. 6F). No cytochromes are formed, however. The oxygen uptake (Fig. 6F) by cells grown aerobically with limiting hemin and glucose is typical of that of a flavoprotein (Laser, 1952). The rate of oxygen uptake is proportional to the oxygen concentration (Fig. 6F inset) and is fastest with sodium lactate. Perhaps the formation of a lactate-DPNH oxidase flavoprotein(s) explains the potentiation by glucose of growth at limiting hemin concentrations. Flavoprotein reacts with oxygen to form H_2O_2. The catalase activities of these bacteria grown aerobically with glucose and limiting hemin are, however, similar to those of bacteria grown aerobically with limiting hemin without glucose.

The flavoprotein type of oxygen uptake can be examined by use of the inhibitor HOQNO. *H. aegyptius* grown aerobically with excess hemin (0.5 μg per ml) has a difference spectrum that shows that all of the cytochrome b_1 and DPNH-oxidase flavoprotein are reduced in the presence of 6 μg/ml of HOQNO and 0.005 M DPNH. To form the typical carbon monoxide complex, the cytochrome oxidase must be reduced. Using the formation of the CO complex as a measure of oxidase reduction, only 15% of the cytochrome *o* reducible with DPNH is reduced in the presence of 6 μg/ml of HOQNO. These measurements were made after the cells were incubated in HOQNO and DPNH ten times longer than is necessary for complete reduction with DPNH alone. This concentration reduces the oxidation rate by 90% of that observed at saturating DPNH concentration. HOQNO is an effective inhibitor of respiration, and blocks electron transport between cytochrome b_1 and the oxidases in hemin-requiring *Haemophilus*. This is the case with *H. parainfluenzae* (White and Smith, 1962) and with heart muscle sarcosomes and other bacteria (Lightbown and Jackson, 1956; Jackson and Lightbown, 1958).

HOQNO does not interfere with respiration by DPNH-oxidase flavoproteins (Lightbown and Jackson, 1956; Lightbown and Kogut, 1959). In *H. aegyptius*, *H. canis*, and *H. influenzae*, the flavoprotein type of respiration, which is pseudo-first order with oxygen concentration, is insensitive to 12 μg/ml of HOQNO. This adds further evidence that cytochrome oxidases are not functional in bacteria grown with limiting hemin. This flavoprotein respiration is apparently not active in cells grown with adequate hemin or with hemin-independent *Haemophilus*.

The electron-transport systems formed by the hemin-requiring *Haemophilus* species grown in various inhibitors in the presence of 0.5 μg of hemin/ml are illustrated in Fig. 7 with *H. aegyptius*. Cells taken from early stationary phase incubated aerobically form the typical *Haemophilus* cytochrome pattern (Fig. 7A). If these bacteria are grown in 0.005 M KCN in a sealed grower, the typical pattern of cytochromeless growth can be demonstrated (Fig. 7B). *H. aegyptius* grown anaerobically with nitrate in the media forms an electron-transport system where cytochrome c_1, rather than cytochrome b_1, forms the predominant intermediate cytochrome, and larger amounts of cytochrome a_1 (nitrate reductase), nearly twice the cytochrome *o*, and no detectable cytochrome a_2 are present (Fig. 7C). This pattern is typical of *H. parainfluenzae* grown anaerobically (White, 1962).

Barbiturates inhibit mitochondrial DPNH-oxidase flavoprotein, but not succinic dehydrogenase (Ernster et al., 1955). Sodium secobarbital [sodium 5-allyl-5(1-methylbutyl) barbituric acid] inhibits the respiration of aerobically grown *H. aegyptius* at a concentration of 0.001 M. The oxygen uptake at saturating DPNH concentration is reduced 60% and the oxygen uptake with saturating succinate concentration is reduced 80% by this concentration of sodium secobarbital. *H. aegyptius* grown in this inhibitor forms less than one-tenth of the cytochrome b_1 and DPNH-oxidase flavoprotein, and one-third of the cytochrome *o* formed when grown without the inhibitor, in excess hemin (Fig. 7D).

Carbon monoxide is an inhibitor of cytochrome oxidase. If the bacteria are grown in a medium saturated with carbon monoxide, four times the cytochrome *o* present in aerobically grown cells appears (Fig. 7E). Cytochrome c_1 appears as the principal intermediate cytochrome, as in anaerobically grown cells, and nitrate reductase activity is present. The oxygen uptake of the bacteria grown in carbon monoxide is typical of flavoprotein respiration (*see* Fig. 6E). The CO complex with cytochrome oxidase is readily dissociable with nitrogen, and, in the cells prepared for the difference spectra, it does not until carbon monoxide is bubbled through the sus-

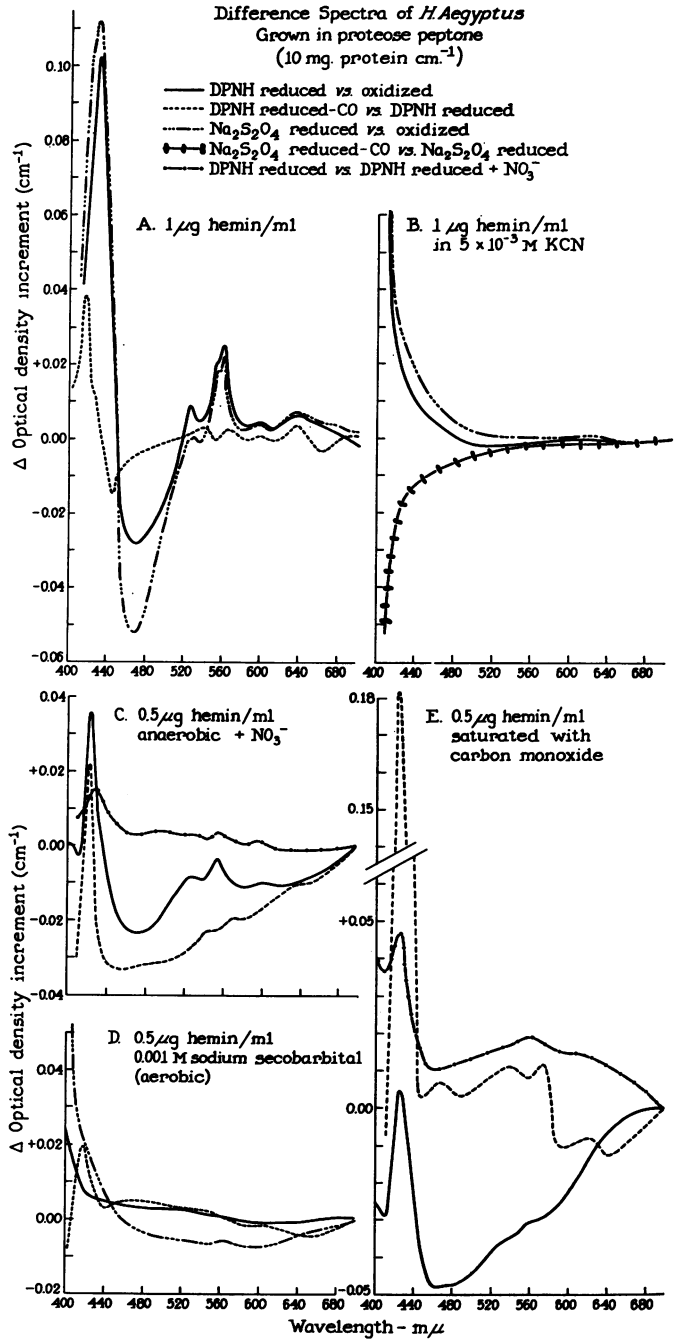


FIG. 7. *Difference spectra of Haemophilus aegyptius grown* (A) *aerobically,* (B) *with 0.005 M KCN,* (C) *anaerobically,* (D) *with 0.001 M sodium secobarbital aerobically, and* (E) *with saturating carbon monoxide in proteose peptone media containing 0.5 μg of hemin/ml. Suspensions were made in 0.05 M phosphate buffer (pH 7.0) and 20% glycerin (v/v) at a density of 10 mg of bacterial protein per 1 ml.*

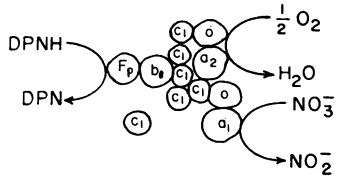
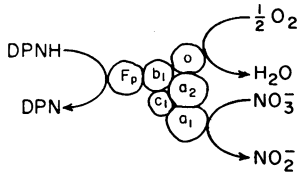
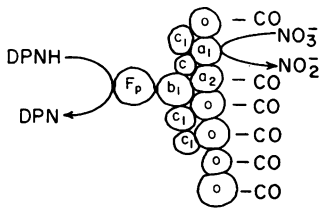
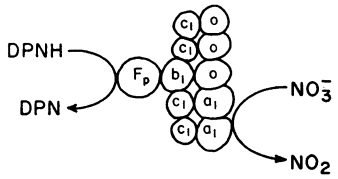
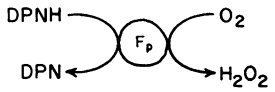
Electron Transport System	Growth Condition	Doubling Time (minutes)		
		H. influenzae	*H. aegyptus*	*H. canis*
	Levinthal's medium (aerobic)	25	45	60
	Proteose Peptone 0.5 μg hemin/ml (aerobic)	40	70	50
	Proteose Peptone 0.5 μg hemin/ml (carbon monoxide)	220	280	280
	Proteose Peptone 0.5 μg hemin/ml (anaerobic)	200	180	220
	Proteose Peptone 0.002 μg hemin/ml 0.5% glucose (aerobic)	60	75	135
No detectable respiratory pigments (catalase)	Proteose Peptone a) 0.002 μg hemin/ml (aerobic) b) 0.002 μg hemin/ml 0.5% glucose (anaerobic) c) 0.5 μg hemin/ml 0.005 M KCN (anaerobic) d) 0.5 μg hemin/ml without nitrate (anaerobic)	~250	~320	~360

FIG. 8. *Representation of the electron-transport system formed by the hemin-requiring Haemophilus species in various growth conditions. Cytochromes are labeled* b_1, c_1, a_1, a_2, *and o and DPNH-oxidase flavoprotein as* F_p. *Doubling time is the time (min) for the turbidity to double, measured during early log phase. For these experiments, bacteria were grown in stationary 250-ml Erlenmeyer flasks, with 13-mm side arms, containing 15 ml of media for aerobic growth, and in graduated cylinders with glass joints and side arm that could be sealed after gassing for anaerobic and carbon monoxide growth. Inoculum was between 10 and 100 early stationary-phase cells and incubation was at 37 C.*

pension. The catalase activity of these bacteria is similar to that formed during anaerobic growth.

DISCUSSION

Figure 8 illustrates diagrammatically the electron-transport systems and the doubling times of these three hemin-requiring *Haemophilus* species under various conditions of growth. The fastest growth rate is seen in Levinthal's medium, with the formation of an electron-transport system which increasingly branches during the growth cycle. Multiple oxidases interact with a DPNH-flavoprotein oxidase-cytochrome b_1 complex through a network of cytochrome c_1 molecules. With prolonged incubation, the cytochrome c_1 leaves the cell membrane, becomes nonreducible with DPNH, and permits only some of the oxidase to be reduced by DPNH. The non-DPNH-reducible cytochrome c_1 can be recovered, on cell rupture, as the soluble hemoprotein. During this period when DPNH-reducible cytochrome c_1 is decreasing, there is increased formation of the oxidases *o* and a_2 and of cytochrome c_1. The patterns are typical of those described for the hemin-independent *H. parainfluenzae* (White, 1962).

By use of proteose peptone medium, in which growth is dependent on added hemin, the electron-transport system can be greatly modified during growth. These three species grown aerobically with a hemin concentration that does not limit growth form a cytochrome system containing very little cytochrome c_1. These cells grow at rates nearly as rapid as those in Levinthal's medium. Grown under these conditions, the principal intermediate cytochrome is cytochrome b_1. In this medium, during the stationary phase, there is a loss of DPNH reducibility by the intermediate cytochrome, and at the same time there is increased synthesis of cytochrome oxidase a_2 and *o*. In the transition from log to stationary phase, there is first an increase, then a decrease to a fairly constant level, of the maximal rate of oxygen uptake with DPNH.

Cells grown anaerobically with excess hemin form more cytochrome *o* and a_1 in an electron-transport system involving cytochrome c_1 as the principal intermediate cytochrome. In the three hemin-requiring species, growth with excess hemin in medium saturated with carbon monoxide greatly exaggerates the effect of anaerobiosis in stimulating oxidase production. The principal oxidase is cytochrome *o*, which reaches values four times that found in cells grown aerobically. The oxidases formed in carbon monoxide function as nitrate reductase, but neither give the characteristic constant rate of oxygen uptake to very low oxygen tensions with added DPNH nor allow as rapid growth as under anaerobic conditions without carbon monoxide in the presence of nitrate. It appears as if carbon monoxide produces an imbalance in cytochrome-oxidase production, such that a partially defective electron-transport system results.

The addition of glucose to this medium, and aerobic incubation with limiting hemin, allows greater total growth at a faster rate than growth under the same conditions without glucose (Fig. 8). In the presence of oxygen, flavoprotein DPNH and lactate oxidase(s) function by reaction with oxygen. No detectable cytochromes are formed, and the inhibitor HOQNO has no effect on the oxygen uptake. Addition of glucose to medium containing excess hemin has no effect on the doubling time.

H. influenzae, *H. aegyptius*, and *H. canis* grown with limiting hemin form no detectable cytochromes or flavoprotein systems that react with nitrate or oxygen. This cytochromeless condition also appears when these bacteria are grown anaerobically in glucose with limiting hemin, with excess hemin in 0.005 M cyanide, or anaerobically with excess hemin without nitrate. Growth under these conditions is slow (Fig. 8). Hemin is still required, and catalase is the only hemoprotein demonstrable. Growth occurs by unknown processes which involve a change in pH only if glucose is present. In this condition, there are a maximum of 10^3 to 10^4 hemin molecules per bacterium. At the concentration where hemin is no longer growth limiting, at least 10^6 hemin molecules are present in each bacterium.

Growth of the hemin-requiring *Haemophilus* species at intermediate levels of hemin or in the presence of the respiratory inhibitor sodium secobarbital forms intermediate amounts of respiratory pigments. Addition of increasing amounts of hemin to cells grown in limiting hemin (Lwoff and Lwoff, 1937) or the growth of cells in increasing concentrations of hemin (Biberstein and Spencer, 1962) results in progressively greater rates of oxygen uptake. The amount of cytochromes formed (Fig. 5) and the flavoprotein oxidase activity of cells grown in glucose are

directly proportional to the hemin concentration between 0.05 mg/ml and 0.1 mg/ml for the three hemin-requiring species. No sharp discontinuity between the cytochromeless state and the full electron-transport system as a function of hemin concentration has been found. Cyanide, which reacts with the cytochrome oxidase, completely represses the formation of the electron-transport system in the presence of excess hemin. Sodium secobarbital, which inhibits both DPNH and succinic oxidase flavoprotein, depresses the formation of the electron-transport system in the presence of excess hemin. Carbon monoxide greatly increases the formation of oxidase.

Hemin-requiring strains of *Haemophilus* can change the composition of the electron-transport system in response to the growth condition. They form: no respiratory flavoprotein or cytochromes; various intermediate levels in secobarbital or intermediate hemin concentration; the full complement of pigments in a complex branching array with excess hemin; or the greatly increased oxidase in carbon monoxide. The great variation in patterns of the individual components suggests that the pattern in a given growth state is similar in all the members of the population rather than some with none and the rest with some fixed amount. This great variability of response in the hemin-requiring *Haemophilus* species reinforces the contention (White, 1962) that the respiratory pigments in *Haemophilus* are formed individually under controls capable of regulating the concentration of each member. Once formed, they are then placed in the membrane in the functional unit. The ability to grow without forming an electron-transport system has not been detected in hemin-independent *Haemophilus* species. Hemin-independent species will not grow in cyanide, carbon monoxide, or anerobically without nitrate. The ability to form cytochrome c_1 can be depressed by the growth of *H. parainfluenzae* in 0.1 M sodium malonate. The other cytochromes, catalase, and respiratory flavoproteins are formed in normal amounts. Under other conditions of culture, the full complement of cytochromes are uniformly found. It appears, then, that this wide variability in the composition and concentration of respiratory pigments with various growth conditions is a compensatory mechanism characteristic of hemin-requiring strains of *Haemophilus*. By growth without cytochromes, these bacteria conserve at least 10^3 molecules of hemin per cell, under conditions where hemin is limiting, or respiratory inhibitors are present.

Biberstein and Gills (1961) found the catalase activity of these *Haemophilus* species to be proportional to the hemin concentration in the medium during growth. The hemin-requiring *Haemophilus* species lose catalase activity during the stationary phase if incubated aerobically. This is true of other bacteria as well (McCarthy and Hinshelwood, 1959; White, 1962). Aerobic incubation in excess hemin induces greater catalase activity than anaerobic incubation early in the growth cycle in *Haemophilus*. The function of this enzyme is obscure, but its presence under conditions of limited hemin where the electron-transport system is not formed suggests some unique function.

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