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Synthesis of 2-Demethyl Vitamin K_2 and the Cytochrome System in *Haemophilus*

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ABSTRACT

WHITE, DAVID C. (University of Kentucky College of Medicine, Lexington). Synthesis of 2-demethyl vitamin K_2 and the cytochrome system in *Haemophilus*. J. Bacteriol. **89**:299–305. 1965.—The synthesis of the respiratory quinone, 2-demethyl vitamin K_2, is stimulated in *Haemophilus parainfluenzae* under conditions which provoke the synthesis of the cytochrome system. However, the various components of the electron-transport system can be formed in different proportions. The primary flavoprotein dehydrogenases are readily dissociated from the membrane without affecting the content of membrane-bound quinone, cytochrome b_1, or the cytochrome oxidases. These dehydrogenases must be membrane-bound to function, and each can be formed at a different rate. Molar ratios of various constituents of the electron-transport chain were calculated by use of reasonable extinction coefficients for the cytochromes. The molar ratio of quinone to cytochrome c_1 goes from 40 to 3 as the quinone content increases eightfold during the growth cycle. Similarly, the molar ratio of quinone to cytochrome oxidase a_2 varies from 27 to 17, and then increases to 31 as cytochrome oxidase a_1 assumes the oxidase function. The molar ratio of quinone to cytochrome b_1 remains 14 to 1 over a sixfold increase in both components measured in a mutant where cytochrome c_1 does not obscure cytochrome b_1. A similar consistency was noted between the quinone and cytochrome b_1 formation in the hemin-requiring *H. influenzae*.

Haemophilus parainfluenzae has the propensity to modify greatly its membrane-bound electron-transport system to compensate for various conditions of growth (White, 1962, 1963*b*). The functional electron-transport system is a part of a membrane complex (White and Smith, 1964) which contains the quinone, 2-demethyl vitamin K_2 (DMK_2) (Lester, White, and Smith, 1964). This quinone functions in the electron-transport system of *H. parainfluenzae* (White, J. Biol. Chem., *in press*). DMK_2 is reduced by each of the substrates capable of reducing the isolated electron-transport system and can be reoxidized by oxygen when DMK_2 is a part of the membrane complex. In the presence of inhibitors, DMK_2 is reduced sufficiently rapidly to account for the total rate of electron transport. If membrane preparations containing the respiratory chain are extracted with acetone, a neotetrazolium reductase activity, which is effective on addition of the substrates that can reduce the electron-transport system, is completely lost. The neotetrazolium reductase activity is completely recovered by adding DMK_2 to the extracted preparation in amounts equal to that removed with the acetone treatment. Both the original and restored activities are inhibited by the respiratory inhibitor 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide. No other quinones will substitute for DMK_2 in the restoration of the neotetrazolium reductase activity (White, *in press*). Consequently, it seemed important to examine the kinetics of DMK_2 formation in relation to the other members of the electron-transport system.

Studies with other microorganisms have shown a close relationship between cytochrome formation and the level of respiratory quinones. *Saccharomyces cerevisiae* when grown anaerobically contains no detectable cytochromes and very little coenzyme Q_6 (CoQ_6). When grown aerobically, the level of CoQ_6 can increase at least 350-fold (Lester and Crane, 1959). Anaerobically grown yeast, when aerated in phosphate buffer with glucose, can increase the CoQ_6 concentration 5- to 20-fold (Sugimura and Rudney, 1960; Sugimura, Okabe, and Rudney, 1964). Change of culture conditions of *Staphylococcus albus* from anaerobic to aerobic produces an increase in the Q_{O_2} with lactate of 100-fold, and also increases vitamin K_2-35 concentration at least 100-fold (Bishop, Pandya, and King, 1962). A twofold increase in both Q_{O_2} (lactate) and vitamin K_2-45 can be induced in *Corynebacterium diphtheria* by increasing the iron content of the medium (Bishop et al., 1962). Gram-negative *Escherichia*

coli, *Proteus vulgaris*, and *Pseudomonas aeruginosa* can increase the Q_{O_2} (lactate) 20- to 100-fold without much effect on the CoQ or vitamin K_2 content (Bishop et al., 1962). Kashket and Brodie (1960), however, found that, as the aeration of cultures of *E. coli* increased, the concentration of vitamin K_2 and one benzoquinone decreases, whereas the concentration of a second benzoquinone increased. *Rhodospirillum rubrum* decreases the content of CoQ_{10} when shifting from photosynthetic to oxidative metabolism (Sugimura and Rudney, 1962).

MATERIALS AND METHODS

Bacteria. The strain of *H. parainfluenzae*, media, and harvesting procedures were described previously (White and Smith, 1962). For the experiments illustrated in Fig. 2, the mutant strain of *H. parainfluenzae* which forms cytochrome b_1 as its predominant cytochrome was used (White and Smith, 1964). In these experiments, *H. parainfluenzae* was grown in 2.5-liter low-form Erlenmeyer flasks containing 1.5 liters of medium inoculated with 10^6 late stationary-phase bacteria. The flasks were incubated at 37 C without agitation, and periodic samples were withdrawn aseptically to measure the turbidity, cytochromes, and DMK_2. The strain of *H. influenzae* and medium used for the experiment illustrated in Fig. 3 were as described (White, 1963*a*). *H. influenzae* was grown in 1-liter flasks containing 200 ml of medium aerated as described (White, 1963*b*). The possibility of contamination with other organisms was checked in each experiment as described (White, 1962).

Oxygen utilization. Oxygen utilization was measured by use of the Clark electrode, as described previously (White, 1963*a*). The utilization of oxygen in the presence of a specific substrate as a measure of that primary flavoprotein dehydrogenase has been justified (White, 1964). In Table 1, the ferricyanide reductase activities were measured spectrophotometrically, as described previously (White, 1964).

Cytochromes. Cytochromes were assayed by difference spectroscopy (Chance, 1954), as described by White (1962). Briefly, the absorption spectrum of the respiratory pigments of a bacterial suspension, which has exhausted the oxygen in the cuvette in the presence of substrate, is compared with the spectrum of an identical suspension in which the respiratory pigments are oxidized. This comparison is possible because of the negligible endogenous respiration of these washed bacteria suspended in dilute phosphate buffer (White, 1963*b*). If, instead of a cuvette containing bacteria with oxidized respiratory pigments, ground glass with light-scattering characteristics similar to those of the bacterial suspension is used as a reference, the cytochromes can be assayed by use of half the quantity of bacteria. Four blanks of commercial Libby Owens Ford sand-blasted and acid-etched DS window glass 3 mm thick placed in the reference chamber of a Cary 14CM spectrophotometer balances the light-scattering produced by a suspension of *H. parainfluenzae* containing 30 to 40 mg of protein per ml. Use of the ground-glass reference balances the light scattering of the bacterial suspension so that it increases less than 0.1 absorbancy unit between 680 and 400 mμ. The ground glass does not introduce any specific absorbance in this wavelength range. This is an accurate and reliable procedure to assay for cytochromes b_1, c_1, and a_2, as shown by comparing difference spectra with the absolute spectra made versus ground glass. By use of the absolute spectra against ground glass, cytochrome c_1 is measured as the absorbancy increment between the maximum at 553 mμ and a line connecting 580 to 540 mμ; cytochrome b_1 is measured as the absorbancy increment between the maximum at 561 mμ and the same line; cytochrome oxidase a_2 is measured as the absorbancy increment between the maximum at 632 mμ and a line connecting 650 and 610 mμ. Cytochrome b_1 has no effect on the absorption of cytochrome c_1 in the experiments reported in Fig. 1. Cytochrome c_1 has less than 10% contribution to the absorbancy of cytochrome b_1 in the experiments reported in Fig. 2 calculated as described by White and Smith (1964). Complete reduction of the cytochromes occurs in 3 to 5 min in the presence of 10 mM formate and 5 mM reduced nicotinamide adenine dinucleotide ($NADH_2$) at 25 C as monitored by the oxygen electrode.

DMK_2 isolation. DMK_2 was isolated by extraction with acetone and assayed by its difference spectrum after KBH_4 reduction as described (White, *in press*) in the experiments reported in Fig. 1. The data in Fig. 2 and 3 were obtained by use of the isopropanol extraction method, which is equally effective (White, *in press*). These procedures recover all the DMK_2 that can be extracted by chloroform-methanol (2:1), which removes 95% of the esterified fatty acids from the membrane (Dittmer and White, *unpublished data*). In the original isolation and purification of the quinone (Lester et al., 1964), column chromatography revealed no other quinone in detectable amounts in the acetone extract of 120 g (dry weight) of bacteria. Further investigations have established that DMK_2 is the only neutral lipid present in significant amounts in the chloroform-methanol extracts of this organism (Dittmer and White, *unpublished data*). Calculations utilized extinction coefficients ($\epsilon_{264} = 15.5 \times 10^3$ in isooctane, $\epsilon_{246\text{-}265} = 26.7 \times 10^3$ in reduced versus oxidized difference spectra in ethanol; White, 1965).

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

Reagents. Reagents utilized in this study were as described previously (White and Smith, 1962, 1964; White, 1962; 1963*b*).

RESULTS

Relationship between the primary flavoprotein dehydrogenases and DMK_2. H. parainfluenzae

contains a membrane-bound electron-transport system that is reduced by $NADH_2$, reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), succinate, formate, D-lactate, and L-lactate (White, 1964). Each of these substrates reduces a distinct primary flavoprotein dehydrogenase which is the initiating reaction of the electron-transport system. Electrons are passed from the membrane-bound dehydrogenases, to DMK_2, to the cytochromes, to the oxidases, and thence to oxygen or nitrate. These flavoproteins on initial isolation are part of the same membrane complex which contains the phospholipids, cytochromes, DMK_2, and cytochrome oxidases (White, 1964; White and Smith, 1964). The primary flavoprotein dehydrogenases can be accurately assayed by use of their ferricyanide reductase capacity and can be dissociated from the membrane by various treatments (White, 1964; White and Smith, 1964).

The ferricyanide reductases which dissociate from the membrane can be quantitatively recovered in the supernatant portion after centrifugation. By exposure of the bacteria to sonic vibration, 29% of the succinic dehydrogenase activity, 36% of the $NADH_2$ dehydrogenase activity, and 92% of the L-lactate dehydrogenase activity can be separated from the membrane. During this same treatment, less than 8% of the DMK_2 is liberated into the supernatant portion (Table 1).

Formation of DMK_2 and the respiratory chain system. The formation of DMK_2 in *H. parainfluenzae* correlates well with the formation of cytochromes. Limited aeration was achieved by incubating the culture in flasks in which the liquid medium was 3 cm deep and was not agitated. Growth with limited aeration causes remarkable changes in the concentration of the primary dehydrogenases, cytochromes, and cytochrome oxidases (White, 1962, 1964). During the growth cycle in the presence of glucose, the primary flavoprotein dehydrogenases are synthesized at different rates (White, 1964).

Since these primary dehydrogenases are the rate-limiting components of the electron-transport system in the organized respiratory chain system, the utilization of oxygen in the presence of a substrate can be used to assay the specific dehydrogenase. A comparison of Fig. 1B and C shows little correlation between the time of maximal formation of succinate, $NADH_2$, D-lactate, or L-lactate dehydrogenases and the time of maximal DMK_2 synthesis. If the primary dehydrogenases are assayed by their ferricyanide reductase activity, essentially the same pattern results. DMK_2 seems to be formed independently of a given primary dehydrogenase.

TABLE 1. *Dissociation of DMK_2 from the flavoprotein dehydrogenases by sonic treatment of Haemophilus parainfluenzae membranes*

Sample	Dehydrogenase*			DMK_2† (*mμmoles/ml*)
	Succinic	$NADH_2$	L-Lactate	
Treated bacteria.....	25.2	250.0	2.4	37.0
Supernatant portion..	7.3	91.0	2.2	2.7
Resuspended pellet...	17.5	171.0	0.0	32.5

* Dehydrogenases were assayed by the ferricyanide method (White, 1964) on a suspension of bacteria containing 5.28 mg of protein per ml, treated with sonic vibration for 6 min at less than 10 C (treated bacteria), then centrifuged at 104,000 × *g* for 60 min and decanted (supernatant portion), and the pellet suspended to volume in 50 mM phosphate buffer, *p*H 7.6 (resuspended pellet). The supernatant portion and the resuspended pellet contained 2.5 mg of protein per ml. Results show the amount (in millimicromoles) of $Fe(CN)_6$ reduced per second per milliliter.

† DMK_2 isolated by the isopropanol method (White, *in press*).

During the growth cycle, under the conditions used for the experiment illustrated in Fig. 1, cytochrome c_1 increases 100-fold. A portion of the cytochrome c_1 is not enzymatically reduced. This cytochrome c_1 that is not reduced enzymatically has been shown to be in the bacterial cytoplasm and can be recovered quantitatively on cell rupture (Smith and White, 1962). The cytochrome c_1 that is functional remains membrane-bound (White and Smith, 1964). This functional membrane-bound cytochrome c_1 can be stripped from the membrane, and it appears to be identical to the cytochrome c_1 found in the cytoplasm during the stationary-growth phase. The retention time on Sephadex columns of partially purified cytochrome c_1 during its purification indicates that it has a molecular weight near 12,000 per hemin, and experiments indicate that the monomer contains one hemin with an extinction coefficient of 20.0×10^3. Consequently, it is not unreasonable to use the extinction coefficient for mammalian cytochrome *c* to compare the molar ratio of cytochrome c_1 to DMK_2. Early in the growth cycle, very little cytochrome c_1 is found, and the molar ratio of DMK_2 to cytochrome c_1 is 40:1. With the onset of late log phase, there is a rapid synthesis of cytochrome c_1 coupled with the appearance of cytochrome c_1 in the bacterial cytoplasm (White, 1962). During this period, there is an eightfold increase in DMK_2 per bacterium. As the stationary-growth phase continues, the molar ratio of DMK_2 to cyto-

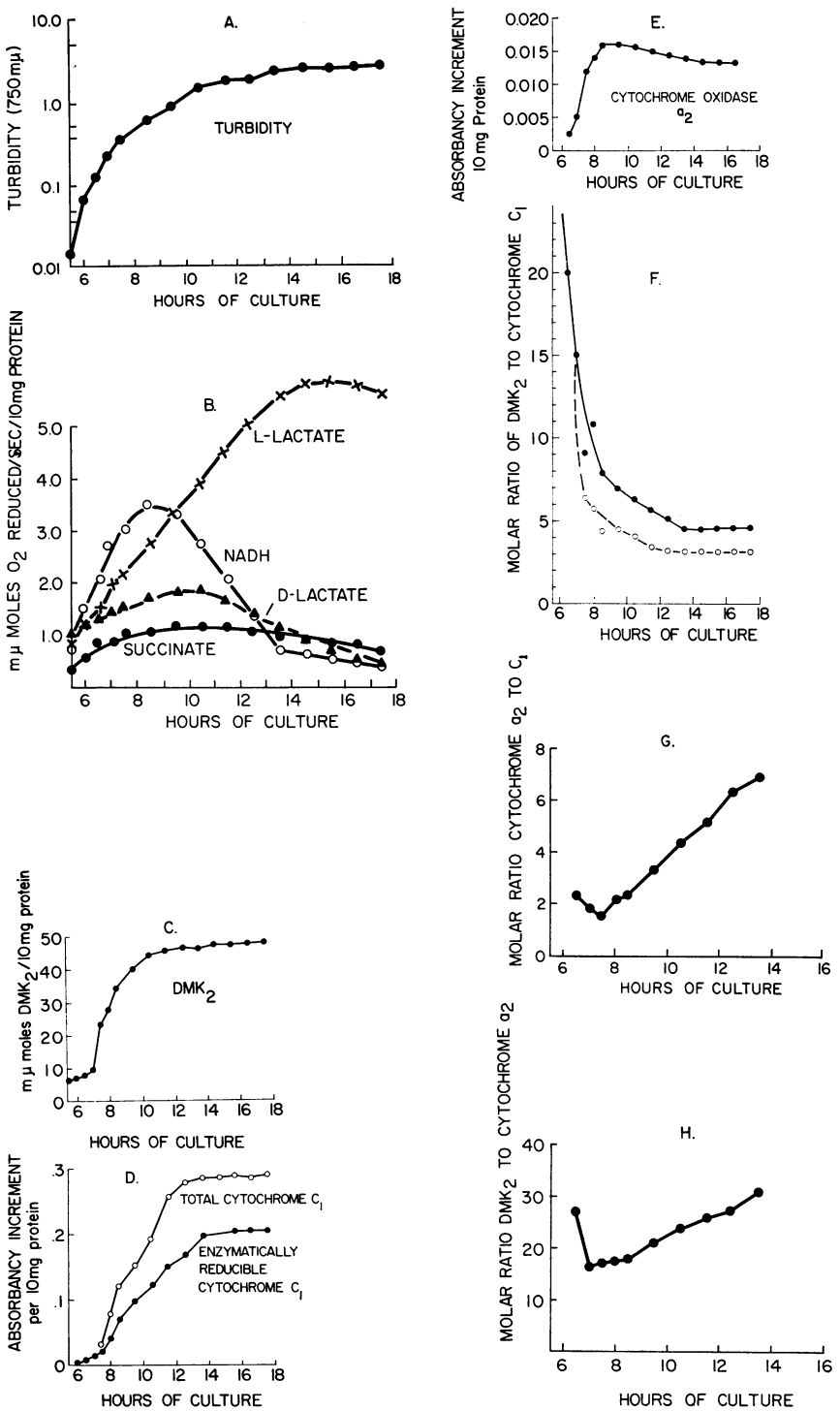

FIG. 1. *Changes in the composition of the electron-transport system during the growth cycle in Haemophilus parainfluenzae. (A) Changes in turbidity expressed as absorbance measured at 750 mμ with time of incubation; (B) changes in the activity of* D*-lactic,* L*-lactic, $NADH_2$, and succinic dehydrogenases per 10 mg of*

chrome c_1 remains at about 3:1 to 5:1 (Fig. 1C, D, F).

H. parainfluenzae can form three cytochrome oxidases. Cytochrome oxidase *o* is formed primarily under conditions of high aeration. Cytochrome oxidase a_2 concentration is maximal when oxygen tension in the medium is low. Cytochrome oxidase a_1 appears maximally during growth in the absence of oxygen (White, 1962). Cytochrome a_2 is formed maximally when the turbidity indicates a high cell density (Fig. 1A and E). The metabolic activity of the bacteria at high cell density (turbidity expressed as absorbance greater than 0.15) completely depletes media of oxygen (White, *unpublished data*). This is just prior to the period of most rapid DMK_2 formation. Assuming that cytochrome a_2 of *H. parainfluenzae* is like that purified from *P. aeruginosa* by Horio et al. (1961), a millimolar extinction coefficient of 8.5 can be estimated from the published spectra. By use of this information, the molar ratio of DMK_2 to cytochrome oxidase a_2 falls from an initial value of 27:1 to about 16:1 as the rapid synthesis of cytochrome oxidase a_2 eclipses the formation of DMK_2. Earlier experiments (White, 1962) indicated that cytochrome oxidase a_1 takes over the principal oxidase function as the growth cycle progresses. Consequently, the proportion of DMK_2 to cytochrome oxidase a_2 rises again to 31:1 (Fig. 1E and H). The molar ratio between cytochrome oxidase a_2 and cytochrome c_1 falls from 2:1 to 1.5:1, then climbs to 7:1 later in the growth cycle (Fig. 1G).

Coordinate formation of DMK_2 and cytochrome b_1. The strain of *H. parainfluenzae* used for the experiments in Fig. 1 forms large amounts of cytochrome c_1. The α maximum of the reduced form of this cytochrome obscures a second intermediate cytochrome, cytochrome b_1. In this strain, cytochrome b_1 can be detected only early in the growth cycle or after cytochrome c_1 has been removed (Smith and White, 1962; White, 1962). A mutant of this strain which forms very little cytochrome c_1, and then only in the late stationary phase, has been isolated. It is known to differ from the parental type only in this characteristic (White and Smith, 1964). With this mutant, the coordinate synthesis of cytochrome b_1 and DMK_2 can be demonstrated. This is of interest because experiments with respiratory inhibitors place the active locus of DMK_2 between the primary membrane-bound dehydrogenases and the cytochrome system (White, *in press*). Earlier experiments suggest that cytochrome b_1 is the first cytochrome to receive electrons during the oxidation of substrates (White and Smith, 1962). Consequently, the first known intermediate with which DMK_2 interacts is cytochrome b_1. In an experiment similar to that of Fig. 1, a culture of the mutant was allowed to go through the growth cycle, and the level of DMK_2 and cytochrome b_1 was measured. During the period of rapid depletion of oxygen in the medium, there is an increase in the components of the respiratory chain system. The absorbancy of cytochrome b_1 on reduction with formate and $NADH_2$ can be measured from an absolute spectrum between the maxima at 561 mμ and a line connecting 580 and 540 mμ. If with longer incubation the increase in absorbancy corresponding to cytochrome b_1 is plotted against the increase in DMK_2, the relationship illustrated in Fig. 2 is revealed. Assuming the extinction coefficient of *Haemophilus* cytochrome b_1 is $\epsilon_{mM} = 20.0$, which is reasonable from the studies with mitochondrial cytochrome *b* (Chance and Williams, 1956), DMK_2 and cytochrome b_1 stay at a fixed molar ratio of 14:1 during the growth period involving a sixfold increase in cytochrome b_1.

DMK_2 and cytochrome formation in H. influenzae. In the hemin-requiring *Haemophilus* species, the final growth yield, the level of the primary dehydrogenases, the level of cytochrome b_1, and the level of the cytochrome oxidases are proportional to the hemin content of the growth medium (White, 1963*b*). *H. influenzae* forms a quinone with the spectral characteristics of DMK_2. Over a hemin concentration range of three orders of magnitude, cytochrome b_1 and DMK_2 formation are reasonably coordinate (Fig. 3). In this experiment, the bacteria were incubated in the proteose peptone medium without yeast extract, in which maximal growth yield has been shown to be dependent on the hemin concentration added to the culture medium (White, 1963*a*). With the same extinction coefficient for *H. influenzae* cytochrome b_1 as used in Fig. 2, there are about 10 to 20 moles of DMK_2 per mole

protein with time of incubation; (C) changes in the content of DMK_2 per 10 mg of protein with time of incubation; (D) changes in cytochrome c_1 content reducible with formate and $NADH_2$ or $Na_2S_2O_4$ per 10 mg of protein with time of incubation; (E) changes in the content of cytochrome oxidase a_2 per 10 mg of protein with time of incubation; (F) ○ indicates the molar ratios between the quinone and total cytochrome c_1 (reduced with $Na_2S_2O_4$), ● indicates the molar ratios between the quinone and cytochrome c_1 reduced in the presence of formate and $NADH_2$; (G) changes in the molar ratios of cytochrome oxidase a_2 to cytochrome c_1 with time of incubation; (H) changes in the molar ratio of DMK_2 to cytochrome oxidase a_2 with time of incubation.

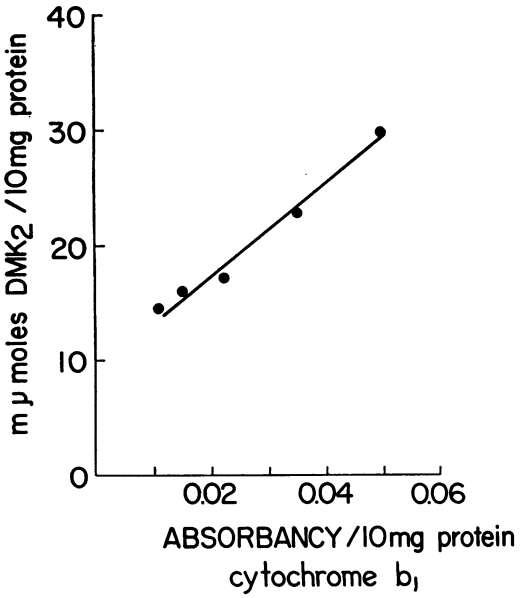

FIG. 2. *Comparison of the DMK_2 and cytochrome b_1 formed during the growth cycle of Haemophilus parainfluenzae. The mutant of H. parainfluenzae which forms cytochrome b_1 was used in an experiment similar to that illustrated in Fig. 1.*

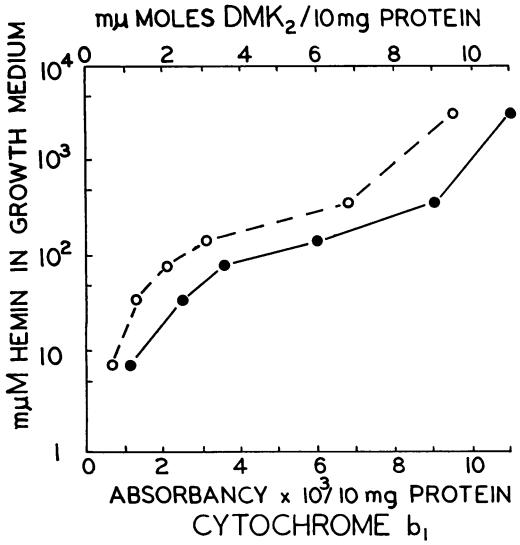

FIG. 3. *Comparison of cytochrome b_1 and DMK_2 formed by Haemophilus influenzae grown in medium containing various levels of hemin. The upper curve illustrates the amount of DMK_2 formed; the lower curve illustrates the amount of cytochrome b_1 formed.*

of cytochrome b_1. This molar ratio remains constant over the hemin concentrations shown.

DISCUSSION

DMK_2 remains part of the membrane system under conditions which allow the primary flavoprotein dehydrogenase to be dissociated from the membrane. The dissociated flavoprotein dehydrogenases are no longer active in the electron-transport system (White and Smith, 1964). Generally, growth conditions that cause increased cytochrome formation, also cause an increase in DMK_2 formation. This is in keeping with the studies of the correlation between increases in quinone and respiratory capacity. However, closer inspection of the synthesis of the various detectable members of the electron-transport system indicates there can be synthesis of most of the components of this membrane-bound system at different rates. The six membrane-bound flavoprotein dehydrogenases can be formed at different rates (White, 1964). Likewise, DMK_2 can be formed differentially with respect to succinic, D- and L-lactic, and $NADH_2$ dehydrogenases. Cytochrome c_1 is formed when the concentration of the terminal electron acceptor is low (White, 1962). This rapid synthesis of cytochrome c_1 allows the molar ratio of DMK_2 to cytochrome to fall from 40:1 to 3:1 during a period when the DMK_2 content increases eightfold. When the oxygen concentration falls off, cytochrome oxidase a_2 is formed, and the molar ratio of DMK_2 to oxidase falls from 27:1 to 17:1. As cytochrome oxidase a_1 assumes more and more of the oxidase function, the ratio rises again to 31:1. This rapid formation of cytochrome c_1 and the oxidases a_1 and a_2 serves the bacteria by substantially increasing the affinity for oxygen at low oxygen tensions (White, 1963*b*). When the cytochrome a_2 level is maximal, the molar ratio of cytochrome c_1 to cytochrome oxidase a_2 approaches 1:1. By use of a mutant in which cytochrome b_1 can be followed, or by interpolation between the early log phase and late stationary phase where cytochrome b_1 is detectable in the parental type, the molar ratio of DMK_2 to cytochrome b_1 remains a constant 14:1. This is also true of the hemin-requiring *H. influenzae*, in which growth and cytochrome b_1 formation are limited by the hemin supplied in the medium.

This study serves to emphasize that in *Haemophilus* the concept of the functional elementary unit of electron transport composed of multienzyme complexes of fixed composition is not compatible with the experimental data. Since this bacterium must have electron transport through the oxidases to grow (*unpublished data*), the widely varying proportions of the many com-

ponents of this system formed by the living bacterium must be functional.

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