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The Function of 2-Demethyl Vitamin K_2 in the Electron Transport System of *Hemophilus parainfluenzae**

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There is a rapidly growing body of evidence for the implication of quinones in the electron transport systems of mitochondria (1, 2) and various bacteria (3–5). *Hemophilus parainfluenzae* contains a vitamin K_2 analogue which differs from vitamin K_2 in having the methyl substituent at the 2-position of the naphthoquinone replaced by hydrogen (6). A similar quinone has been isolated from *Streptococcus fecalis* (7). With the discovery of large amounts of this new class of naphthoquinones in *H. parainfluenzae*, it is of interest to examine the possible function of this quinone in relation to the respiratory system. Fortunately, accurate extraction and assay procedures were developed that allowed the demonstration of enzymatic reduction of the quinone by substrates reacting with the electron transport system. With these techniques, advantage is taken of some of the remarkable properties of the electron transport system in *H. parainfluenzae* (8–12) in order to perform experiments reported here which suggest a respiratory function for this quinone.

EXPERIMENTAL PROCEDURE

Extraction of Demethyl-K_2[1]—Intact bacteria suspended in 2 ml of phosphate buffer (about 10 to 20 mg of protein) were put in a 300-ml stainless steel centrifuge bottle, and 38 ml of redistilled acetone were added. The suspension was stirred for 5 minutes with a Teflon-covered magnetic stirring bar at room temperature, centrifuged at 14,000 × *g* for 10 minutes and the acetone decanted. The acetone was transferred to a 125-ml separatory funnel, 7.5 ml of spectral grade isooctane were added, the contents mixed, and 7.5 ml of water were added. The isooctane layer was removed and taken to dryness under a vacuum on a rotary evaporator at a temperature of 40°. The residue was dissolved in 10 ml of redistilled ethanol containing 1% (v/v) 1.0 M ammonium acetate, pH 5.0. The ethanol solution was added to 2-ml cuvettes of 10-mm light path and 4 μmoles of freshly prepared aqueous KBH_4 added in 0.04 ml and the contents shaken until hydrogen evolution ceased. The difference spectra between the reduced and oxidized ethanol solutions were recorded. The difference spectrum of 1.98 mμmoles per ml of highly purified demethyl-K_2 is illustrated in Fig. 1. The sample used for Fig. 1 represents a 1,250-fold purification of the demethyl-K_2 present in the initial acetone extract (6). From the data illustrated in Fig. 1, an extinction coefficient of 26.7 × 10^3 can be calculated between the minimum at 265 mμ and the maximum at 246 mμ. Calculations with the absolute spectra of this solution before and after reduction indicate that in this procedure the quinone is completely oxidized in the ethanol solution before the addition of KBH_4. The completeness of reduction can be tested by adding an additional 1 μmole of KBH_4. The experiments are performed in a darkened laboratory. This method is essentially that used previously (6).

If care is taken to protect the demethyl-K_2 from light and alkali, this procedure can uniformly extract demethyl-K_2 to an accuracy of ± 2%. Less than 3% of the demethyl-K_2 is left in the acetone after one extraction with isooctane or in the residue to be extracted by a second acetone treatment of the bacteria. These results are illustrated in Table I, Extraction Procedure A. Chloroform-methanol, 2:1, which extracts 99% of the esterified fatty acids of *H. parainfluenzae*[2] does not remove more demethyl-K_2 from the bacteria than acetone. Rupture of the bacteria by exposure to sonic vibration does not significantly increase demethyl-K_2 recovery (Table II).

Attempts to show enzymatic reduction of demethyl-K_2 necessitated the development of a technique involving fewer manipulations and not involving acetone or other solvents with great ultraviolet absorption. To this end a system involving isopropyl alcohol was developed that extracts all the demethyl-K_2 within 3% of the acetone method. This is illustrated in Table I, Extraction Procedure B. It was found that if the bacteria were carefully added to an equal volume of deoxygenated 3.5 M perchloric acid and the extraction carried out with solvents and vessels deoxygenated with a gas mixture of 20% CO_2 and 80% N_2 freed from any oxygen by passage through hot copper, the quinone can be recovered in its reduced form. The method involves adding 1 ml of bacteria (10 to 20 mg of protein) to 1 ml of 3.5 M perchloric acid in a deoxygenated screw cap test tube. The mixture is shaken slowly and washed into a deoxygenated 50-ml mixing cylinder with glass stopper containing 4 ml of deoxygenated isooctane with 20 ml of deoxygenated spectral quality isopropyl alcohol under a stream of CO_2-N_2. The cylinder is then inverted 10 times, 16 ml of deoxygenated water added and the cylinder inverted an additional 10 times. After 5 minutes the clear isooctane layer is removed. If necessary the isooctane layer is centrifuged at 1500 × *g* for 5 minutes in

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[1] The abbreviations used in this paper are: demethyl-K_2, 2-demethyl-1,4-naphthoquinones with a 5-unit isoprenoid side chain at the 3-position (see Reference 6); CoQ_{10}, 2,3-dimethoxy-5-methylbenzoquinone with a 10-unit isoprenoid side chain at the 6-position; vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone; NHQO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; and neotetrazolium, 2,2′,5,5′-tetraphenyl-3,3′-(4,4′-biphenylene) ditetrazolium chloride.

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

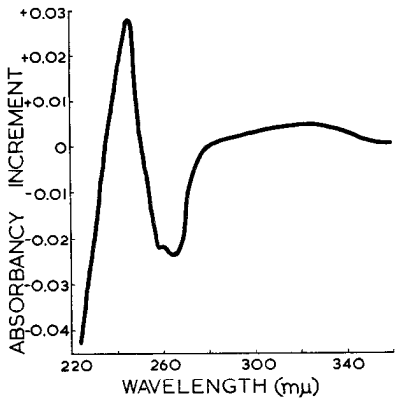

FIG. 1. Difference spectra of 1.98 mμmoles of highly purified demethyl-K_2. Demethyl-K_2 reduced with KBH_4 in ethanol containing 1% 1 M ammonium acetate buffer, pH 5.0, compared to a similar ethanolic solution containing the oxidized quinone. Reduction procedure is that described previously (6) with the Cary model 15 recording spectrophotometer. Additional KBH_4 established the completeness of reduction in the sample cuvette.

deoxygenated screw cap centrifuge tubes. The absolute spectrum is then determined against a solvent blank prepared with the same procedure without added bacteria. There are no pigments which significantly affect the spectra that are extracted by this procedure. Calculating the expected absorbance at 254, 248, and 244 mμ from the spectra of highly purified demethyl-K_2 on the basis of $\epsilon^{264} = 15.5 \times 10^3$, no significant deviation between purified and isopropyl alcohol extracted demethyl-K_2 can be detected. This result is illustrated in Table III. The enzymatically reduced demethyl-K_2 must be carefully protected from reoxidation during the manipulations prior to extraction. Careful addition to deoxygenated perchloric acid stops the reoxidation and the acid protects the quinone from alkali destruction. If purified demethyl-K_2 is completely reduced in ethanol containing 1% ammonium acetate, mixed with various proportions of an identical solution of oxidized demethyl-K_2 and extracted by the isopropyl alcohol-isooctane-water method, reduced quinone can be recovered quantitatively. This is illustrated by Fig. 2. Consequently the proportion of reduced demethyl-K_2 can be assayed by measuring the absolute spectra of demethyl-K_2 isolated from bacteria with oxidized respiratory pigments, measuring the difference in absorbance between the maxima at 264 mμ and the isosbestic point at 275 mμ normalized to 1.57 mμmoles per ml as seen in Fig. 2. The accuracy of this method is illustrated by the recovery of completely reduced demethyl-K_2 within 1% from $Na_2S_2O_4$ reduced bacteria in Table V. Reduced demethyl-K_2 can be shaken in isooctane in air for 3 minutes without significant reoxidation.

Cytochromes and Respiratory Capacity—The cytochromes were measured by using their absolute spectra measured against ground glass with the Cary model 14 CM spectrophotometer with the scattered transmission accessory and intense light source as described previously (12). The rate of respiration was measured with the Clark oxygen electrode (10) at substrate concentrations that were not rate-limiting. Ferricyanide reductase activity which measures the primary membrane-bound dehydrogenases was assayed spectrophotometrically (11).

Protein—Protein concentration was measured by a biuret procedure (8) in the presence of 0.06% sodium deoxycholate.

Neotetrazolium Reductase—Neotetrazolium reduction was measured in the presence of 5 mM KCN by the method developed by Lester and Smith (14). Membrane fragments prepared by exposure to sonic vibration (11) were extracted by the 4% acetone method of Lester and Fleisher (15). The reduction of ferric chloride in ethanol in the presence of α,α'-dipyridyl by reduced demethyl-K_2 was performed as described (13).

Materials

Bacterial Growth—The strain of bacteria, media, harvesting procedure, and culture conditions have been described (8, 12). Essentially these conditions give maximal formation of cytochromes, respiratory flavoproteins, and demethyl-K_2.

Reagents—NHQO (Sigma Chemical Company), α,α'-dipyridyl

TABLE I

Effectiveness of demethyl-K_2 extraction procedures with H. parainfluenzae

Intact bacteria (about 10 mg of protein per ml) in 0.5 ml of 50 mM phosphate buffer, pH 7.6, were extracted by adding 19 ml of redistilled acetone and stirring with a Teflon-covered magnetic stirring bar at room temperature for 15 minutes in a stainless steel centrifuge tube. The extraction mixture was then centrifuged at 24,000 × *g* for 10 minutes and the acetone decanted. The acetone was transferred to a separatory funnel and 4 ml each of isooctane and water added. The isooctane was collected and taken to dryness under vacuum on a rotary evaporator at 40°. The quinone was redissolved in ethanol-ammonium acetate and assayed as in Fig. 1. For the extraction with isopropyl alcohol, 1 ml of bacterial suspension was added to 1 ml of 3.5 M perchloric acid and swirled in a 50-ml mixing cylinder. Deoxygenated isopropyl alcohol, 20 ml, was added, followed by 4 ml of isooctane and the mixture inverted 10 times. Then 16 ml of water were added and the isooctane layer removed, centrifuged briefly in a clinical centrifuge, and measured in the Cary model 15 spectrophotometer.

Extraction procedure	Repetitions			
	1	2	3	4
	mμmoles demethyl-K_2/10 mg protein			
A. Reproducibility and effectiveness of acetone-isooctane-water extraction				
Extraction once with acetone-isooctane-water	2.81	2.70	2.70	2.76
Re-extraction of acetone with isooctane-water	0.021	0.056	0.080	0.040
Re-extraction of bacteria with additional acetone-isooctane-water	0.070	0.056	0.037	0.024
B. Comparison of acetone-isooctane-water and isopropyl alcohol-isooctane-water methods				
Extraction with acetone-isooctane-water	1.19	1.21		
Extraction with isopropyl alcohol-isooctane-water	1.29	1.20		

(Fisher Chemical Company), neotetrazolium chloride (Sigma Chemical Company), vitamin K_1 (California Corporation for Biochemical Research), and CoQ_{10} (the generous gift of Dr. R. L. Lester) were dissolved in ethanol. Spectral grade solvents were supplied by the Fisher Chemical Company. Other reagents were as described previously (8–12).

RESULTS

With the development of accurate, reproducible techniques for the isolation of demethyl-K_2 from the bacteria, an examination of its possible function became possible.

Cellular Localization of Demethyl-K_2—If *H. parainfluenzae* is subjected to sonic vibration, the membrane-bound electron transport system which contains the primary dehydrogenases, cytochromes, and cytochrome oxidases (12) can be isolated as membrane fragments. From the experiment illustrated in Table II, 94% of the demethyl-K_2 can be isolated in the same fraction as the respiratory system.

Isolation of Demethyl-K_2 Reduced by Electron Transport—Any member of the electron transport system should be reduced when the preceding members and the following member of that system are reduced. This result is realized as illustrated in

TABLE II

Localization of demethyl-K_2 in H. parainfluenzae

Bacteria suspended in 50 mM phosphate buffer, pH 7.6, at a protein concentration of 7.2 mg of protein per ml. Demethyl-K_2 was extracted with acetone and determined by its difference spectrum as in Fig. 1. Bacterial suspension was subjected to sonic vibration for 2 minutes at less than 10°, as described (11), centrifuged at 104,000 × *g* for 30 minutes, resuspended to volume with phosphate buffer, and recentrifuged. The entire supernatant suspension was combined and extracted for demethyl-K_2 by the acetone method. The washed pellet was resuspended to volume and extracted. All the substrate provoked oxygen utilization and substrate-reducible cytochromes of the whole bacteria were found in the pellet.

Material extracted	
	mμmoles demethyl-K_2/ml
1. Intact bacteria	6.10
2. Treated bacteria	5.80
3. Supernatant suspension	0.35
4. Resuspended pellet	5.30

TABLE III

Comparison between purified demethyl-K_2 and demethyl-K_2 isolated from H. parainfluenzae with oxidized respiratory system

The absorbance was calculated on the basis of $\epsilon = 15.5 \times 10^3$ at 264 mμ and the spectra of purified demethyl-K_2 in isooctane (6) as compared to demethyl-K_2 isolated with the isopropyl alcohol method from bacteria (12.25 mg of protein).

	Absorbance	
Wave length	Predicted	Experimental
mμ	*cm^{-1}*	*cm^{-1}*
264	0.165	0.165
254	0.198	0.197
248	0.217	0.216
244	0.210	0.209

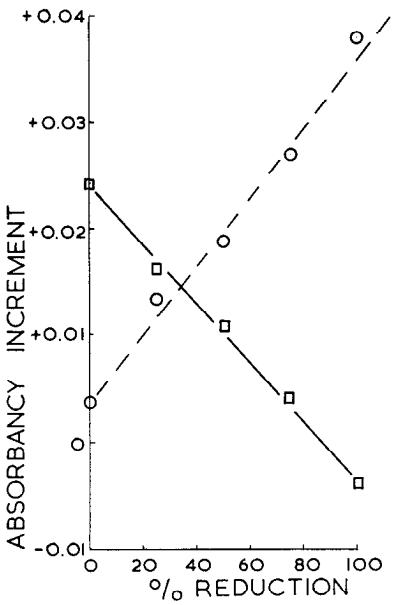

FIG. 2. Relationship between the proportion reduced and spectra of purified demethyl-K_2. Demethyl-K_2, (1.57 mμmoles per ml) reduced in ethanol-ammonium acetate by KBH_4 mixed with various proportions of oxidized demethyl-K_2 and extracted by the isopropyl alcohol procedure. □——□, indicates the difference in absorbance between the maximum at 264 mμ and the isosbestic point at 275 mμ; ○– –○ indicates the difference in absorbance between the maximum at 245 mμ and the isosbestic point at 254 mμ compared to known mixtures of reduced and oxidized demethyl-K_2.

Table V. Addition of the six substrates of the six membrane bound flavoprotein dehydrogenases (16) results in oxygen uptake, cytochrome c_1 reduction, and reduction of a substantial proportion of the demethyl-K_2. To establish demethyl-K_2 reduction, 1 ml of suspension is carefully withdrawn from the cuvette used to determine the spectra in which the bacteria have gone anaerobic in the presence of substrate. This 1 ml is carefully transferred and layered under 1 ml of 3.5 M deoxygenated perchloric acid in screw cap centrifuge tubes that have been deoxygenated. Once the possibility of enzymatic reoxidation of reduced demethyl-K_2 is stopped, the isolated demethyl-K_2 is relatively insensitive to autooxidation. Addition of several substrates leads to a less than additive rate of oxygen utilization and no increase in cytochrome or demethyl-K_2 reduction. Similar experiments have been performed with membrane fragments.

Effect of Respiratory Inhibitors on Demethyl-K Reduction—*H. parainfluenzae* was grown under conditions which produce a high level of succinic dehydrogenase, cytochromes c_1, b_1, and a_2, and demethyl-K_2. If the respiratory system were reduced in the presence of succinate, inhibitors added, and then the electron transport system reoxidized, all elements between the primary flavoprotein dehydrogenases and the site of inhibitors should remain reduced. The elements of the electron transport system between the site of inhibition and the oxidases should be reoxidized by this procedure. By use of the respiratory inhibitors of Table IV at the concentrations given the rate of oxygen utilization produced by succinate is decreased 93% by malonate, 97% by sodium secobarbital, 93% by NHQO, and 96% by KCN. There is no respiration in the absence of added

TABLE IV

Effect of respiratory inhibitors on reduction of demethyl-K_2 by succinate

The bacterial suspension (26.4 mg of protein) was allowed to go anaerobic for 10 minutes in the presence of succinate (30 mM) and reoxidized by vortex mixing for 2 minutes. A sample was withdrawn from the cuvette used to assay the cytochromes as in Table V. Demethyl-K_2 was isolated by the isopropyl alcohol technique as in Table V. The total demethyl-K_2 was 33.6 mμmoles/26.4 mg of protein. Where indicated, inhibitors were added at final concentrations of: malonate, 60 mM; sodium secobarbital, 10 mM; NHQO, 0.9 mM; and KCN, 5 mM after the succinate reduction of the cytochromes was complete and before the reoxidation by vortex mixing. Cytochrome c_1 is measured as the absorbance between the maximum at 553 mμ and a line connecting 540 and 580 mμ; cytochrome b_1 was measured as the absorbance difference between the maximum at 561 mμ and the same line; and cytochrome oxidase a_2 was measured as the absorbance difference between the maximum at 632 mμ and a line connecting 610 and 650 mμ in the absolute spectra of succinate reduced bacteria against ground glass. No correction for the effect of cytochrome c_1 on cytochrome b_1 was made. This can be done, however (12).

Inhibitor	c_1	b_1	a_2	Demethyl-K_2 reduced
	cytochromes/26.5 mg protein			%
A. Succinate reduced	0.145	0.115	0.045	76
Succinate reduced and reoxidized	<0.01	<0.01	<0.0005	0
B. Succinate reduced + inhibitor and reoxidized				
Malonate	<0.01	<0.01	<0.0005	0
Secobarbital	<0.01	<0.01	<0.0005	0*
NHQO	0.022	0.032	<0.0005	80
KCN	0.085	0.080	<0.0005	94

* Measured as the reduction of $FeCl_3$ in α,α′-dipyridyl and ethanol as described (13).

succinate. In Table IV the addition of succinate reduces cytochromes c_1 and b_1 as well as cytochrome oxidase a_2 and demethyl-K_2. Reoxidation by mixing with the vortex mixer for 2 minutes effectively reoxidizes the cytochromes and the demethyl-K_2. Reoxidation of the reduced cytochrome system in the presence of inhibitors indicates that KCN inhibits at the oxidase cytochrome a_2; NHQO inhibits between cytochromes b_1 and c_1 and the oxidase, and sodium secobarbital and malonate between demethyl-K_2 and the flavoproteins. The results of Table IV indicate that demethyl-K_2 functions between cytochromes b_1 and c_1 and the flavoprotein dehydrogenases.

Secobarbital is an effective inhibitor of the electron transport system but it interferes with the spectral assay of the quinone. The reduction of $FeCl_3$ by the reduced quinone in the presence of α,α′-dipyridyl in ethanol can effectively be used to measure the reducing equivalents of dihydroquinone formed. By use of the extinction of $\epsilon^{518} = 8.7 \times 10^3$ of the complex determined with ferrous ammonium sulfate, the method measures the proportion of demethyl-K_2 reduced enzymatically or by KBH_4 in ethanol ammonium acetate and extracted into isooctane to an accuracy of ±5%. Sodium secobarbital at less than 1 mM does not affect the assay. Unfortunately thenoyltrifluoroacetone, which is an excellent inhibitor of cytochrome reoxidation in the system used for Table IV, obscures both the spectral and $FeCl_3$ reduction.

Relationship of Rate of Electron Transport and Demethyl-K_2 Reduced—The primary dehydrogenases for L-lactate and formate can be induced by changing the growth environment of *H. parainfluenzae* (11, 16). These enzymes are the rate-limiting components of the electron transport system and can be accurately measured by their ferricyanide reductase activity (11). If cultural conditions are changed by stopping the agitation of the media, there is a drastic reduction in the oxygen concentration in the media. This change provokes increased formation of the primary dehydrogenases that are paralleled by an increase in the proportion of the cytochromes reduced by each substrate (16). The absolute amount of demethyl-K_2 reduced per bacteria also increases with the increase in the dehydrogenases. The increase in the amount of dehydrogenases is also roughly paralleled by an increase in the proportion of the total demethyl-K_2 reduced. These results are illustrated in Fig. 3.

Comparison of Rate of Electron Transport and Rate of Demethyl-K_2 Reduction—In order to demonstrate enzymatic reduction of demethyl-K_2 great care must be taken to prevent reoxidation of the electron transport system. This can be done by slowly adding 1 ml of bacterial suspension that was allowed to go anaerobic in the presence of substrate under the surface of deoxygenated 3.5 M perchloric acid in deoxygenated screw cap test

TABLE V

Oxygen uptake cytochrome c_1 and demethyl-K_2 reduction with substrates with H. parainfluenzae

Bacteria were incubated in 50 mM phosphate buffer, pH 7.6, at 25° for 10 minutes at concentration of 12.5 mg per ml (protein). Cytochrome c_1 measured as the absorbance increment between 553 mμ and a line connecting 580 and 540 mμ in the absolute spectrum measured against ground glass. Demethyl-K_2 was isolated by the isopropyl alcohol method after carefully removing 1 ml from the bottom of the cuvette used to measure the cytochromes and adding it under 1 ml of deoxygenated 3.5 M perchloric acid in deoxygenated screw cap tubes. After gentle mixing under a stream of deoxygenated CO_2-N_2 the suspension was washed into deoxygenated 50-ml mixing cylinders containing 4 ml of deoxygenated isooctane with 20 ml of deoxygenated isopropyl alcohol. The cylinder was then gently mixed and 16 ml of deoxygenated water was added and again mixed and stoppered under CO_2-N_2. After 10 minutes the isooctane was removed and assayed as in Table I. The total demethyl-K_2 measured 2.51 mμmoles/10 mg of protein ±2%.

Substrate	Rate of oxygen utilization	Cytochrome c_1 reduced per 10 mg of protein	Demethyl-K_2 reduced
30 μmoles/3 ml	*mμmoles/sec/10 mg protein*	*A/10 mg protein*	%
TPNH	0.52	0.112	16
D-Lactate	1.10	0.098	25
Succinate	2.84	0.090	35
DPNH	5.68	0.102	38
L-Lactate	11.40	0.102	58
Formate	27.8	0.118	61
$Na_2S_2O_4$		0.135	99
Succinate + DPNH	6.29	0.101	40
Succinate + DPNH + formate	32.00	0.114	62

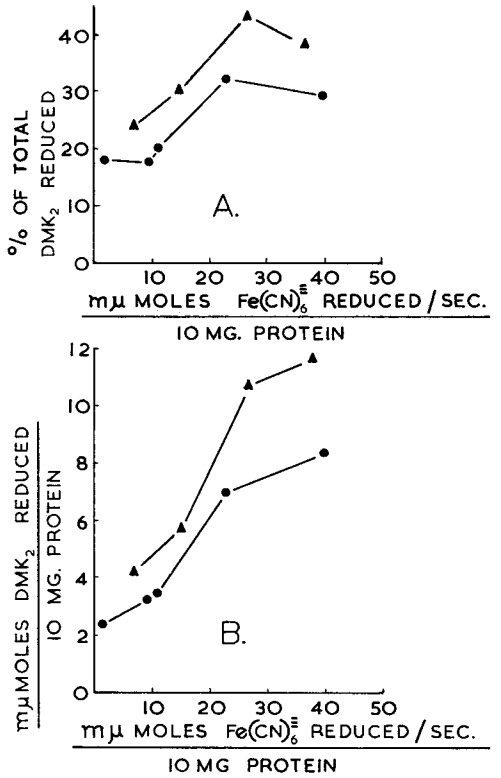

FIG. 3. Per cent of the total demethyl-K_2 (*DMK*$_2$) (*A*) and absolute amount of demethyl-K_2 (*B*) reduced by L-lactate and formate compared to the rate of ferricyanide reduction. The demethyl-K_2 and ferricyanide reduction produced in the presence of L-lactate is plotted at 10 times the actual rate of ferricyanide reduction. The demethyl-K_2 and ferricyanide reduction is produced in the presence of formate. Ferricyanide reduction rates were measured as described (11) with substrate concentrations that are not rate-limiting. Demethyl-K_2 was isolated by the isopropyl alcohol method.

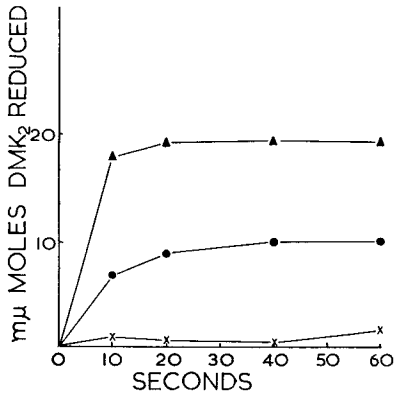

FIG. 4. Rate of formation of reduced demethyl-K_2 (*DMK*$_2$) by *H. parainfluenzae* at 20°. Bacteria (435.0 mg of protein/6 ml) were suspended in 50 mM phosphate buffer, pH 7.6, and incubated for 20 minutes at 20°. They were then aerated on a vortex mixer for 2 minutes and succinate (10 mM final concentration) was added. Aliquots, 1 ml, were quickly pipetted into 1 ml of 3.5 M perchloric acid in deoxygenated screw cap centrifuge tubes and the demethyl-K_2 was isolated as in Table IV. ×——× indicates experiments with succinate, ●——● indicates experiments with succinate in the presence of 5 mM KCN, and ▲——▲ indicates experiments with succinate in the presence of 0.24 mM NHQO. The total demethyl-K_2 was 42.5 mμmoles/72.5 mg of protein.

TABLE VI

Rate of demethyl-K_2 reduction in presence of inhibitors and succinate compared to rate of oxygen utilization at 20°

Rate of demethyl-K_2 reduction was taken from Fig. 3 as the *minimum* possible rate. Rate of oxygen uptake was measured with the oxygen electrode (10) with 21.7 mg of protein/3 ml in the presence of 10 mM succinate in 50 mM phosphate buffer, pH 7.6, at 20°. KCN, 5 mM, and NHQO, 0.24 mM, were added. The rate of oxygen utilization in the presence of inhibitors was measured at concentrations greater than 150 μM to avoid the complications discussed previously (10). To calculate electron flux, rates of oxygen uptake were multiplied by 4 and rates of demethyl-K_2 reduction were multiplied by 2.

Substrate	*Minimum* rate of demethyl-K_2 reduction	Oxygen uptake	Demethyl-K_2 reduction	Oxygen reduction
	mμmoles/sec/75.1 mg protein		*mμmoles electron flux/sec/75.1 mg protein*	
Succinate		9.2		36.8
Succinate + KCN	1.4	0.034	2.8	1.28
Succinate + NHQO	3.52	0.037	7.04	1.30

TABLE VII

Removal of demethyl-K_2 from membrane fragments of H. parainfluenzae

Membrane fragments of *H. parainfluenzae* were prepared by subjecting the bacteria to sonic vibration for 2 minutes as described (11), and the membrane fragments were collected by centrifugation and washed in 50 mM phosphate buffer, pH 7.6. Then 8 ml of membrane fragments (11.4 mg of protein) were added to 200 ml of ice cold redistilled acetone and stirred for 15 minutes in stainless steel centrifuge bottles. The extracted fragments were collected by centrifugation, washed twice with 200 ml of 10 mM Tris buffer, pH 7.6, containing 0.88 M sucrose at 0°. The membrane fragments were suspended in 16 ml of Tris-sucrose. Demethyl-K_2 was isolated by the isopropyl alcohol method from 1 ml of extracted and unextracted particles. The acetone was taken to dryness with a rotary evaporator under vacuum at 40° and the demethyl-K_2 assayed as in Fig. 1.

	Demethyl-K_2	Protein
	mμmoles/ml	*mg/ml*
Unextracted particles	1.71	11.4
Recovered from the acetone	1.34	
Recovered from the extracted particles	0.43	9.7

tubes. To study the kinetics of demethyl-K_2 reduction the rapid manipulations necessary even at reduced temperatures preclude this careful technique and consequently an accurate measure of the rate of demethyl-K_2 reduction cannot be performed. If the rate of electron transport is reduced by lowering the temperature to 20° and by adding KCN and NHQO which block electron transport between oxygen and demethyl-K_2, then the *minimum* rate of electron transport mediated by demethyl-K_2 is adequate to account for the over-all rate of electron transport. Data illustrating this conclusion are given in Fig. 4 and Table VI. The blocking of the reoxidation of enzymatically reduced demethyl-K_2 by inhibiting electron transport between demethyl-K_2 and oxygen slightly increases the proportion of demethyl-K_2 reduced as illustrated in Table

TABLE VIII

Restoration of rate of neotetrazolium reductase in presence of formate by demethyl-K_2 and 2-methyl-1,4-naphthoquinone with acetone-extracted membrane fragments of H. parainfluenzae

Membranes were prepared and extracted as in Table VII. The reduction of neotetrazolium was measured by using 0.3-ml bacterial membrane preparation containing 5 mM KCN, with demethyl-K_2 or 2-methyl-1,4-naphthoquinone added in 0.05 ml of ethanol, and incubated for 10 minutes at 30°. Then 1.5 μmoles of neotetrazolium chloride in 0.05 ml of ethanol were added and 1 minute later the reaction started with the addition of 30 μmoles of formate in 0.05 ml. The final volume was 0.5 ml. The reaction was stopped by adding 2.50 ml of a mixture containing 14 mg of Triton X-100, 80 mg of formaldehyde, 400 μmoles of potassium formate, pH 3.5. The formazan was then extracted by adding 3 ml of ethyl acetate and mixing with a vortex mixer for 3 minutes followed by a brief centrifugation. The extent of reduction was measured as the increase in absorbance in a 1-cm cell at 505 mμ compared to an ethyl acetate blank. No formazan could be detected in the protein which remained at the interface. The rates were calculated from measurements at 0, 5, 10, and 15 minutes. For faster rates the reaction was linear for 10 minutes and for slower rates the reaction was linear for 15 minutes.

Particles	Demethyl-K_2 extracted	DMK_2 added Demethyl-K_2	Absorbance change*
	mμmoles	*mμmoles/0.5 ml*	
A. Unextracted	1.68		0.055
B. Extracted			
Control (ethanol)		0	0.000
Demethyl-K_2		0.177	0.004
Demethyl-K_2		0.355	0.024
Demethyl-K_2		1.77	0.040
Demethyl-K_2		3.55	0.068
2-Methyl-1,4-naphthoquinone		25.0	0.000
2-Methyl-1,4-naphthoquinone		51.0	0.012
2-Methyl-1,4-naphthoquinone		252.0	0.068
2-Methyl-1,4-naphthoquinone		504.0	0.160

* Increase in absorbance at 505 mμ (× 10) per minute per 2.9 mg of protein (30°).

IV. Malonate inhibits the oxygen utilization produced in the presence of succinate between demethyl-K_2 and the substrate reduction site. At a concentration of 60 mM malonate inhibits the oxidation of succinate by the electron transport system 92% in the preparation used for Fig. 4, yet the attempts to recover reduced demethyl-K_2 after short incubation times were no more successful than in the case of succinate alone.

In the presence of KCN or NHQO, demethyl-K_2 can be reduced fast enough to be an integral part of the electron transport system.

Specificity of Demethyl-K_2 for Restoration of Neotetrazolium Reductase Activity—Exposure of *H. parainfluenzae* to light at 360 mμ or extraction with 95% acetone-water or isopropyl alcohol reduces the oxygen utilization capacity of the electron transport system to zero. This activity cannot be restored by addition of excess demethyl-K_2. Consequently an artificial system with less stringent requirements for restoration of activity by quinone was sought. The neotetrazolium reductase system of Lester and Smith (14) provided a system of electron transport that was sensitive to inhibition by NHQO. The reaction was performed in the presence of 5 mM KCN. The substrates capable of reducing demethyl-K_2 are also capable of stimulating neotetrazolium reduction, and this reduction is NHQO-sensitive. If membrane fragments which contain the electron transport system are extracted by the 4% acetone method of Lester and Fleischer (15), neotetrazolium reductase activity is lost. If the acetone is examined, 80 to 90% of the demethyl-K_2 in the membrane can be recovered. A typical experiment is illustrated in Table VII. In this case 78% of the demethyl-K_2 was removed and 104% recovered. About 24% of the protein is also lost. The extracted particles have no substrate-stimulated neotetrazolium reductase activity. If demethyl-K_2 in ethanol is added to these extracted membranes, neotetrazolium reductase activity is restored. Adding the same amount of demethyl-K_2 removed by the acetone extraction restores 71% of the activity originally present in the unextracted particles. 2-Methyl-1,4-naphthoquinone is about 100 times less effective in restoring neotetrazolium reductase activity. The data in support of these findings are illustrated in Table VIII.

By making assumptions as to the extinction of the formazan (14), neotetrazolium is about 10% as effective an electron acceptor as the natural one. This is illustrated in Table IX, Part A. The specificity of demethyl-K_2 in the restoration of neotetrazolium reductase activity with 5 substrates is illustrated

TABLE IX

Specificity of demethyl-K_2 addition to extracted membrane fragments of H. parainfluenzae in restoring neotetrazolium reductase activity

Rate of neotetrazolium reduction was estimated by using the extinction coefficient $\epsilon^{505} = 7.0 \times 10^3$ determined by Lester and Smith (14) for the formazan and assuming the reduction is a two electron change. Particles were prepared and extracted with acetone from *H. parainfluenzae* as in Table VII and assayed as in Table VIII. Substrates were added to concentrations of 10 mM and quinones in 0.05 ml of ethanol at concentrations (microM) of: demethyl-K_2, 2.25; 2-methyl-1,4-naphthoquinone, 20.25; K_1, 5.0; CoQ_{10}, 4.6. NHQO was added at a concentration of 0.24 μM in 0.05 ml of ethanol. The demethyl-K_2 removed by acetone extraction was 1.56 mμmoles/2.8 mg of protein.

	Substrates				
	L-Lactate	D-Lactate	Succinate	DPNH	Formate
A. Comparison of rate of electron flux					
Oxygen reduction	4.96	3.72	2.80	8.56	10.9
Neotetrazolium	0.108	0.305	0.264	0.396	0.104
B. Rate of reduction of neotetrazolium (30°)*					
Unextracted particles	0.076	0.22	0.184	0.108	0.098
Extracted particles	0.000	0.000	0.000	0.010	0.000
+ Demethyl-K_2	0.084	0.180	0.158	0.132	0.074
+ Demethyl-K_2 + NHQO	0.040	0.080	0.020	0.104	0.012
+ 2-Methyl-1,4-naphthoquinone	0.014	0.010	0.006	0.018	0.006
+ K_1	0.002	0.000	0.004	0.014	0.002
+ CoQ_{10}	0.006	0.000	0.002	0.019	0.006

* Increase in absorbance (× 10) per minute per 2.8 mg of protein (30°).

in Table IX, Part B. The effectiveness of the restored neotetrazolium reductase activity with the extracted particles varies from 105% for L-lactate to 74% for formate. The activity restored to extracted particles by demethyl-K_2 is inhibited by NHQO between 23% for DPNH and 85% for succinate- and formate-stimulated reduction. Coenzyme Q_{10} and vitamin K_1 are not effective in this restoration of activity and 2-methyl-1,4-naphthoquinone can produce about 10% the activity restored by demethyl-K_2. The neotetrazolium reduction produced in the presence of DPNH is the least specific to demethyl-K_2 restoration. DPNH-stimulated neotetrazolium reductase activity in acetone-extracted particles can be restored by adding coenzyme Q, vitamin K_2, and 2-methyl-1,4-naphthoquinone to about 10% of the original activity. This activity restored by the quinones other than demethyl-K_2 to DPNH-stimulated reductase activity is not sensitive to NHQO inhibition. Neotetrazolium reduction with unextracted particles is stimulated to a great extent by added demethyl-K_2 or 2-methyl-1,4-naphthoquinone. This activity is also not sensitive to NHQO inhibition.

DISCUSSION

The following experimental evidence implicates demethyl-K_2 as a member of the electron transport system of *H. parainfluenzae*.

1. Demethyl-K_2 is found in the membrane which contains the respiratory chain (Table II).

2. Demethyl-K_2 is present in amounts adequate to be a part of the electron transport system and its synthesis is induced under growth conditions that induce the formation of the electron transport system (17).

3. Demethyl-K_2 is reduced by substrates which reduce the electron transport system (Table V).

4. Demethyl-K_2 is reduced when the electron transport system is reduced and reoxidized when the electron transport system is reoxidized. Inhibitors of electron transport fix the localization of demethyl-K_2 as between cytochromes c_1 and b_1 and the primary flavoprotein dehydrogenases (Table IV).

5. When the rate of electron transport for the oxidation of a particular substrate is increased by increasing the particle-bound flavoprotein dehydrogenase, a correspondingly greater proportion and absolute amount of demethyl-K_2 is reduced (Fig. 3).

6. The rate of demethyl-K_2 reduction is at least adequate to encompass all electron flow in the presence of NHQO or KCN (Fig. 4, Table VI). It seems very reasonable that demethyl-K_2 can reach its steady state reduction level in a very short time—fast enough to be a member of the electron transport system. The sensitivity of reduced demethyl-K_2 to reoxidation during manipulations of the reduced electron transport system suggests that reduced demethyl-K_2 can be reoxidized very rapidly.

7. Demethyl-K_2 specifically restores the neotetrazolium reductase activity of membrane preparations from which a nearly equal amount of demethyl-K_2 has been extracted. This neotetrazolium reductase activity involves all the flavoprotein dehydrogenases and at least encompasses the NHQO inhibition site (Tables VIII and IX). In mitochondria, the succinate-neotetrazolium reductase system encompasses the major portion of the electron transport system (18).

In view of these considerations the electron transport system of *H. parainfluenzae* can be diagrammatically illustrated as in Fig. 5. These studies place the active locus of demethyl-K_2 in the electron transport system between the primary dehydrogenases and cytochromes b_1 and c_1. This is the position of CoQ in mitochondria (2) and vitamin K_2 in bacteria (3, 4).

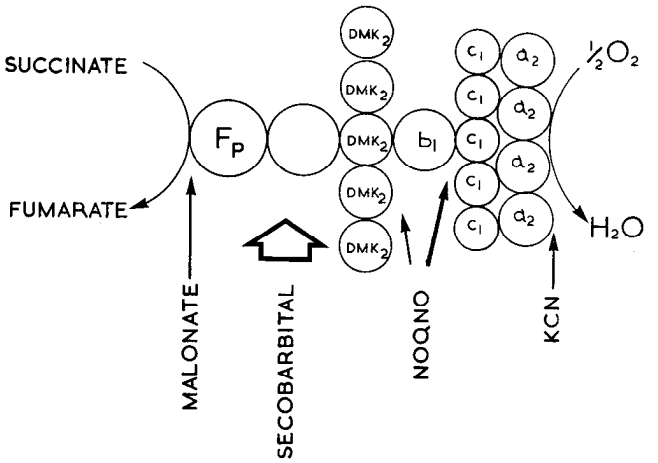

FIG. 5. Diagrammatic illustration of the electron transport system of *H. parainfluenzae*, F_p, c_1, b_1, and a_2 stand for succinic dehydrogenase, cytochromes b_1 and c_1, and cytochrome oxidase a_2. DMK_2, demethyl-K_2 and *NOQNO*, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide.

Several differences are apparent with the demethyl-K_2-*Hemophilus* system. The system has the requirement that there be no methyl substituent on the 2-position of the naphthoquinone ring and that the presence of a long isoprenoid side chain increases the effectiveness of the 2-demethyl derivative about 100-fold. All of the substrates of the electron transport system can reduce a proportion of this quinone. Adding several substrates increases the rate of electron transport without altering the proportion of either demethyl-K_2 or cytochrome reduction, suggesting the overlap with minimal competition characteristic of the cytochrome system (12). There is no evidence for other quinones (17) or the distinct pathways for each dehydrogenase as postulated for *Escherichia coli* (4).

Our studies of the electron transport system have led to the concept that the cytochrome system is formed from an interconnecting network of pigments which offer each of the six flavoprotein dehydrogenases multiple cytochrome oxidases via a widely branched cytochrome system (12). This mechanism serves to compensate very well for growth in very low concentrations of the terminal acceptor of electrons (10, 12). Demethyl-K_2 appears to be a part of this membrane-bound network.

SUMMARY

That the 2-demethyl vitamin K_2 homologues found in *Hemophilus parainfluenzae* appear to have a function in the respiratory chain system is indicated by the following facts.

1. The quinone is a part of the membrane-bound system which contains the active respiratory chain.

2. It is reduced by the substrates that reduce the respiratory chain.

3. It is reduced and oxidized with the respiratory system at a rate of reduction that is adequate to encompass all electron transport in the presence of inhibitors.

4. Inhibitors of the respiratory system can be used to place the active locus of the quinone between the primary dehydrogenases and the cytochromes.

5. Increases in the rate of electron transport by a given dehydrogenase correspond to increases in the amount of quinone that is reduced.

6. A 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide-sensitive neotetrazolium reductase system for each of the substrates of the electron transport system in the membrane is specifically restored by equimolar amounts of this quinone after the quinone has been removed from the membrane by extraction with acetone.

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