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REDUCTION OF MITOCHONDRIAL COMPONENTS BY DUROHYDROQUINONE

ALBERTO BOVERIS, REIKO OSHINO, MARIA ERECIŃSKA AND BRITTON CHANCE
Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)
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SUMMARY

1. Durohydroquinone (H_2DQ) has been used to provide pulses of reducing equivalents to suspensions of pigeon heart mitochondria and submitochondrial particles.

2. H_2DQ oxidation in the presence of pentachlorophenol was 95% sensitive to antimycin A and 99% sensitive to cyanide.

3. Pulses of 150 μM H_2DQ produced reduction of cytochromes *b* (60–90% of total absorption), fluorescent flavoprotein (25% of total fluorescence) and pyridine nucleotide (60% of total fluorescence). Reduction of the latter two components was mediated through reversed electron transfer and was energy dependent.

4. Pulses of 10–30 μM H_2DQ brought about redox cycles of ubiquinone, cytochrome(s) *b* and absorbing flavoprotein (succinate dehydrogenase). Addition of antimycin A altered the kinetics of electron equilibration between ubiquinone and cytochrome(s) *b*. Antimycin A enhanced the efficiency of reduction of cytochrome(s) *b* and flavoprotein by 3–20 μM H_2DQ, but diminished the efficiency of ubiquinone reduction under the same experimental conditions. In the absence of inhibitor, ubiquinone was quantitatively the most important acceptor for the electrons arising from H_2DQ. In the presence of antimycin A, cytochrome(s) *b* was the most important acceptor for H_2DQ.

5. In the presence of antimycin A, ubiquinone did not show redox cycle changes synchronous with the redox cycles of flavoprotein and cytochromes *b*, and thus seem not to be an obligatory member for electron transfer in the main pathway from succinate dehydrogenase–cytochrome(s) *b* to oxygen. This antimycin A effect is interpreted as the result of a conformational change of cytochrome(s) *b* or an alteration of the hydrocarbon core of the membrane that ubiquinone occupies.

6. 1.2–15 μM additions of H_2DQ to cyanide-blocked mitochondria or submitochondrial particles brought about pulses of reducing equivalents which were distributed among the oxidized carriers according to the redox potential of these components. Cytochromes $a + a_3$ and *c*, absorbing flavoprotein and ubiquinone titrated in the presence or absence of uncoupler, as homogeneous pools in a sequence according to their reported potential values. Cytochrome(s) *b* titrated in the absence

of uncoupler as a heterogeneous pool and in the presence of uncoupler as a homogeneous pool in the latter case the absorbance reaching 50 % of the total.

7. In the aerobic steady state (*plus* or *minus* ATP) a pulse of 250 μM H_2DQ reduces cytochromes *b* absorbing at 562 and 557 nm. The form absorbing at 557 nm is postulated to be cytochrome b_{555}.

INTRODUCTION

Since the electron transfer reactions of the respiratory chain involve both forward and reverse constants[1], it is highly desirable to study their functional arrangements employing not only pulsed oxidants such as oxygen, but also pulsed reductants, which are the topic of this paper. While dihydrolipoamide has proved to be an effective reductant for the first phosphorylation site[2], a specific and rapidly reacting reductant functioning between the rotenone and antimycin A sensitive sites is much needed for appropriate kinetic evaluation of the function of the respiratory carriers in this span. In this paper, we report that durohydroquinone (2,3,5,6-tetramethyl benzhydroquinol) fills the need of such a reductant. The use of quinols as electron donors to the respiratory chain can be traced back as far as 1938 when KEILIN AND HARTREE[3] and STOTZ *et al.*[4] independently used *p*-benzoquinol as a reductant for cytochromes *c* and $a + a_3$. Since that time, many quinols have been used as reductants for mitochondrial preparations; of these, menadiol[5] and the reduced homologues of ubiquinone[6,7] have received the most attention. Durohydroquinone has been used by SLATER *et al.*[5] and more recently by RUZICKA AND CRANE[7] as a reductant for submitochondrial particles.

Durohydroquinone, H_2DQ, has an appropriate redox potential (E'_0 at pH 7.4, 19 mV[8]) to serve as reductant of this region of the respiratory chain. More importantly, it interacts very rapidly with mitochondrial membranes which enables the study of the response of respiratory chain components, including ubiquinone, to micromolar pulses of H_2DQ. In the present paper we have used pulses of this reductant to trace the electron pathway in the cytochrome(s) *b*–flavoprotein–ubiquinone region of the respiratory chain, both in uninhibited systems and in the presence of the respiratory inhibitors, antimycin A or cyanide. By using reducing equivalents approximately equal to those of the respiratory components, the stoichiometry of the reactions have been explored. This approach has also provided an indirect estimation of the redox potential when used in inhibited systems.

METHODS

Pigeon heart mitochondria were prepared in 0.225 M mannitol–0.075 M sucrose–0.2 mM EDTA according to the method of CHANCE AND HAGIHARA[9]. Rat liver mitochondria were prepared in mannitol–sucrose–EDTA buffer according to the procedure of SCHNEIDER[10]. Submitochondrial particles were obtained from pigeon heart mitochondria by sonication[11].

Respiratory chain components were measured with the Perkin-Elmer dual wavelength spectrophotometer Model No. 356 at the wavelength pairs indicated using the following difference extinction coefficients: flavoprotein, 475—510 nm

(10 $mM^{-1} \cdot cm^{-1}$); cytochromes *b*, 562—575 nm (22 mM^{-1} cm^{-1}); cytochrome *c*, 550—540 nm (19 $mM^{-1} \cdot cm^{-1}$); cytochrome $a + a_3$, 605—630 nm (21 $mM^{-1} \cdot cm^{-1}$). Ubiquinone was measured at 285 and 305 nm. $\varepsilon_{285-305}$ reduced—oxidized was taken as —5 $mM^{-1} \cdot cm^{-1}$ considering $\varepsilon_{275-300}$ reduced—oxidized = —10 $mM^{-1} \cdot cm^{-1}$ for ubiquinone in mitochondrial samples. The two wavelengths, 285 and 305 nm, were found to be isosbestic points of the H_2DQ/DQ redox pair in water solution and under our experimental conditions. Traces recorded at 285 and 305 nm require correction for the immediate absorption increase due to the added H_2DQ (2.1 $mM^{-1} \cdot cm^{-1}$). Absorption changes corresponding to ubiquinone reduction and oxidation can be recorded at 285—305 nm without interference from the duroquinone redox changes.

Simultaneous fluorescence and absorption flavoprotein measurements were carried out in a modified double beam spectrophotometer–differential fluorimeter in which the wavelengths used for absorbance measurement (463 and 540 nm) are also used to excite the fluorescence emission in the mitochondrial suspension[12]. Simultaneous measurement of NADH and flavoprotein fluorescence and cytochromes *b* absorbance were performed by means of a three-channel alternating fluorimeter–spectrophotometer[13].

Durohydroquinone (H_2DQ) was prepared from duroquinone (DQ) (tetramethylbenzoquinone, Aldrich Chem. Co.) in alcoholic solution by reduction with potassium borohydride, recrystallized and finally used as 50 % alcoholic solution. Protein determination was carried out by the biuret reaction[14]. Unless stated otherwise, the light path for spectrophotometric determinations was 1 cm and measurements were made at room temperature.

RESULTS

Durohydroquinone pulses in systems open to oxygen. Effect of antimycin A

Durohydroquinone is rapidly oxidized by mitochondria as shown in Fig. 1; the oxygen consumption is roughly twice as fast as the oxygen uptake with succinate as substrate in the same experimental conditions. Addition of antimycin A inhibits respiration by 95 %, the remaining oxygen uptake (antimycin A leak) is further inhibited by cyanide.

In order to determine spectrophotometrically the site of action of this quinol we have simultaneously recorded redox cycles of pyridine nucleotide, fluorescent flavoprotein and cytochrome *b* produced by H_2DQ, and we have studied the effect of inhibitors and uncouplers on these redox cycles (Fig. 2). Addition of

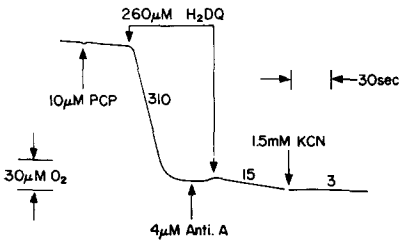


Fig. 1. Oxygen uptake supported by H_2DQ in pigeon heart mitochondria (2.1 mg/ml) suspended in mannitol–sucrose–Tris–HCl buffer. Expt. 3357. PCP = pentachlorophenol.

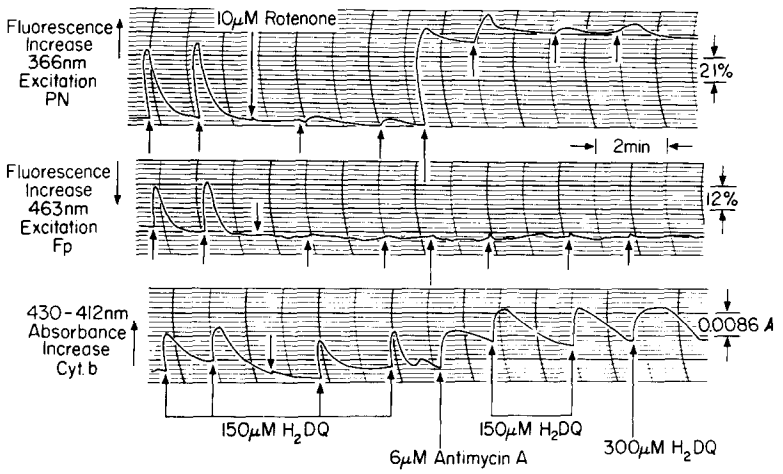


Fig. 2. Simultaneous responses of pyridine nucleotide (PN), fluorescent flavoprotein and cytochromes *b* to H_2DQ. Pigeon heart mitochondria (2.7 mg protein/ml) suspended in 0.225 M mannitol, 0.075 M sucrose and 20 mM Tris–HCl (pH 7.4). Expt. 3318.

rotenone or pentachlorophenol (not shown) inhibits or abolishes the responses of pyridine nucleotide and fluorescent flavoprotein. The addition of antimycin A results in a characteristic fluorescence increase due to the excess of antibiotic[15]. As a consequence of the respiratory inhibition, longer cycles of cytochrome(s) *b* are observed. From these data we can define the site of action of durohydroquinone as a high potential site (located between the rotenone- and the antimycin A-sensitive sites) which can reduce the low potential, highly fluorescent flavoprotein, lipoate dehydrogenase and pyridine nucleotide through reversed electron transfer.

Low concentrations (10–50 μM) of H_2DQ produce redox cycles of a mainly absorbing flavoprotein (Fig. 3) that is identified as succinate dehydrogenase [E'_0

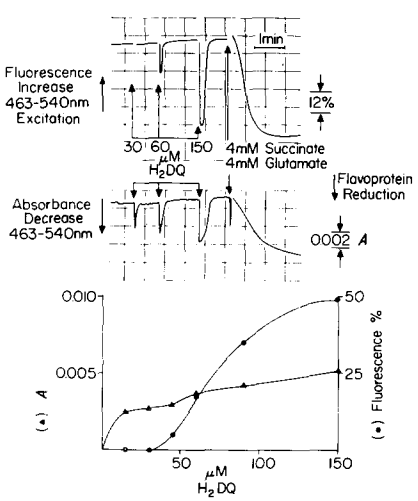


Fig. 3. Absorption and fluorescence changes of mitochondrial flavoproteins by H_2DQ pulses. Pigeon heart mitochondria (2.3 mg protein/ml) suspended in mannitol–sucrose–Tris–HCl buffer. Expt. 3324.

at pH 7.0 = −45 mV[12]]. Under these experimental conditions, cytochromes $a + a_3$, c and c_1 are maintained in the oxidized form during the oxidation of H_2DQ pulses and there is no optical interference from them. After addition of 45–150 μM H_2DQ lipoate dehydrogenase is also reduced. This phenomenon is shown in Fig. 3. Apparently, the oxidation of 10–50 μM H_2DQ does not provide the conditions required for the operation of the energy linked pathway for NAD^+ and lipoate dehydrogenase reduction.

Under conditions in which the energy linked pathway does not operate, addition of 15 μM H_2DQ produces reduction–oxidation cycles of ubiquinone, cytochrome(s) *b* and absorbing flavoprotein (succinate dehydrogenase) (Table I-A).

TABLE I

REDUCTION OF RESPIRATORY COMPONENTS BY H_2DQ PULSES

Pigeon heart mitochondria (1.8–2.0 mg/ml) suspended in 0.225 M mannitol, 0.075 M sucrose, 20 mM Tris–HCl, pH 7.4.

H_2DQ (μM)	*Additions*	*(A) Stoichiometry of reduction Reduced component/added H_2DQ (mole/mole)*		
		Ubiquinone	*Cytochrome(s) b*	*Flavoprotein*
15	—	0.30	0.01	0.01
150	6 μM pentachlorophenol	0.025	0.001	0.001
3–20	5 μM antimycin A	0.05	0.30	0.07
3–20	6 μM pentachlorophenol + 5 μM antimycin A	0.05	0.30	0.08
		(B) H_2DQ required for total reduction (nmoles of H_2DQ/mg protein)		
	5 μM antimycin A	40	1.6	1.6
	6 μM pentachlorophenol + 5 μM antimycin A	40	2.0	1.6
		(C) H_2DQ oxidation rate (nmoles H_2DQ/min per mg protein)*		
15–100	—	140	130	125
3–20	5 μM antimycin A	200	16	24

* Calculated from the plot of $t_{1/2}$ off of redox cycles *versus* H_2DQ concentration (see text).

From the ratio: (reduced component, calculated from the maximum height of the redox cycle)/(added H_2DQ) it is evident that ubiquinone is quantitatively the most important acceptor of the electrons by a factor of 30 to 1. In the uncoupled state, 10 times more reductant (150 μM) is required to observe comparable redox cycles. The release of the respiratory control mechanism elicits a faster H_2DQ oxidation (from 140 nmoles/min per mg in State 4 to 310 nmoles/min per mg in State 3_u) that keeps ubiquinone, cytochrome(s) *b* and flavoprotein far more oxidized in the aerobic state (Table I-A).

Antimycin A addition provides an inhibited system in which 3–20 μM H_2DQ

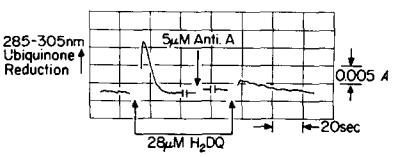
are oxidized in 10–60 sec and where electrons reach oxygen through the antimycin A leak. The following changes are observed in the antimycin A-supplemented preparation as compared with the uninhibited mitochondria (Table I-A): (a) cytochrome(s) *b* are far more reduced and become quantitatively the most important electron acceptors; (b) ubiquinone is less efficiently reduced; and (c) flavoprotein is more efficiently reduced. Effects (a) and (c) are consistent with the inhibition of electron transfer between cytochrome(s) *b* and c_1 by antimycin A.

Transients are very fast, thus, it seems that the maximum reduction of each component after the reductant pulses reflects the kinetic properties of each component rather than an equilibrium state based on the redox potentials.

Table I-B shows the H_2DQ concentrations required to obtain a maximal reduction of these respiratory components in the presence of antimycin A. Cytochrome(s) *b* and succinate dehydrogenase are fully reduced at reductant levels (1.6–2.0 nmoles/mg protein) which are lower than the ubiquinone content (5–7 nmoles/mg protein) of pigeon heart mitochondria. This result shows that the ubiquinone pool is not equilibrated with cytochrome(s) *b* and succinate dehydrogenase under these experimental conditions.

Table I-C shows values of H_2DQ oxidation estimated from the $t_{1/2}$ off[16] (the interval from half reduction to half oxidation on the redox cycle expressed in seconds) observed on the redox cycles of ubiquinone, cytochrome(s) *b* and flavoprotein after addition of H_2DQ. A plot of the quinol concentrations used to produce the cycles (y-axis) *versus* the measured $t_{1/2}$ off (x-axis) gives a straight line whose slope expresses the 50% oxidation rate in μM H_2DQ per sec. Values obtained graphically by this procedure are converted to oxidation rates by dividing by 2 (50% oxidation to 100% oxidation) and by referring them to the amount of mitochondrial protein. When these calculations are applied to the redox cycles of ubiquinone, cytochrome(s) *b* and flavoprotein observed in pigeon heart mitochondria in state 4, the obtained values (125–140 nmoles H_2DQ/min per mg; Table I-C) correspond to the recorded respiration in state 4 (130–140 natoms oxygen/min per mg). According to these observations the three components seem to undergo simultaneous redox changes.

In the presence of antimycin A, the H_2DQ oxidation rate calculated from



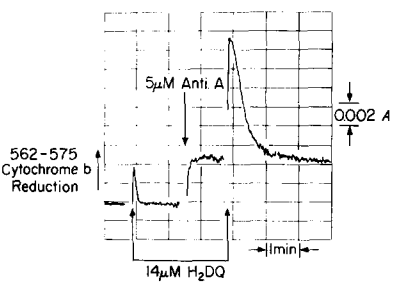


Fig. 4. Inhibition by antimycin A of ubiquinone reduction by H_2DQ pulses. The trace has been corrected for the increase in absorption at 285–305 nm due to H_2DQ addition. Pigeon heart mitochondria (1.6 mg protein/ml) suspended in mannitol–sucrose–Tris–HCl buffer. Light path, 5 mm. Expt. 3371.

Fig. 5. Effect of antimycin A on cytochrome *b* redox cycles by H_2DQ pulses. Pigeon heart mitochondria (1.8 mg protein/ml) suspended in mannitol–sucrose–Tris–HCl buffer. Expt. 3370.

the redox cycles of cytochrome(s) *b* and flavoprotein (16 and 24 nmoles H_2DQ/min per mg; Table I-C) correspond to the observed oxygen uptake (15 natoms oxygen/min per mg). On the other hand, ubiquinone redox cycles give a value of H_2DQ oxidation which is approx. 12 times greater than the recorded oxygen uptake. It should be pointed out that in this kind of calculation, a diminution in reduction rate is indistinguishable from an increase in oxidation rate. Since H_2DQ oxidation rate in the steady state can not be greater than oxygen uptake, it is concluded that this discrepancy in $t_{1/2}$ off is due to an inhibition of ubiquinone reduction by H_2DQ in the presence of antimycin A.

The inhibitory effect of antimycin A on the reduction kinetics of ubiquinone by H_2DQ which is illustrated in Fig. 4 should be emphasized since the site of action of antimycin A is located between ubiquinone and oxygen[17,18]. Fig. 5 shows, with comparative purposes the effect of the same inhibitor on the redox cycles of the cytochrome(s) *b*.

High potential flavoprotein reduced by H_2DQ

The technique of isolation of portions of the flavoprotein chain by using inhibitors that exclude an area of the chain and specific substrates that activate only a part of the flavoprotein has been successfully used by CHANCE and coworkers[12,19,20] to calculate the ratio of the observed fluorescence and absorption changes and thus to identify and characterize mitochondrial flavoproteins. Table II shows the absorption and fluorescence properties of the high potential flavoprotein reduced by H_2DQ in rat liver mitochondria. In these studies, malate–glutamate and rotenone were added to reduce completely the highly fluorescent, low potential, lipoate dehydrogenase[12]. H_2DQ reduced flavoproteins producing decreases in absorption and fluorescence with an *F/A* ratio of 4.1. On the other hand, the flavoprotein(s) reduced by succinate had a different *F/A* ratio, 5.6. H_2DQ reduced in addition to the flavoprotein reduced by succinate (succinate dehydrogenase), a flavoprotein [E'_0 at pH 7.0 = —160 mV[12]] with low *F/A* ratio, 3.2.

TABLE II

HIGH POTENTIAL FLAVOPROTEINS REDUCED BY H_2DQ

Rat liver mitochondria (2.6 mg/ml) preincubated 3 min in 0.225 M mannitol, 0.075 M sucrose, 20 mM Tris–HCl (pH 7.4), 7 μM pentachlorophenol, 0.3 mM Na_3AsO_4, 0.3 mM Na_3AsO_3 and 0.3 mM malonate. 6 mM malate, 6 mM glutamate, 6 μM rotenone and 4 μM antimycin A were added before the reductants listed in the table.

	Absorption (%)	*Fluorescence* (%)	*F/A ratio*	*Amount reduced (nmoles/mg protein)*
(a) 166 μM H_2DQ	1.6	6.5	4.1	0.27
(b) 6.6 mM Succinate	0.86	4.9	5.6	0.15
+ 166 μM H_2DQ	0.82	2.8	3.2	0.14
(c) 6 mM Choline (± previous H_2DQ)	0.25	0	0	0.04
(d) 13 μM Palmitoyl carnitine (± previous H_2DQ)	2.40	5.2	2.2	0.41

Choline and palmitoyl carnitine brought about a similar flavoprotein reduction before or after addition of H_2DQ (Table II) and thus choline dehydrogenase[21] or flavoproteins involved in fatty acid oxidation[20] are not the cause of the difference in flavoprotein reduction by succinate or H_2DQ. The possibility that the absorption change which accounts for the bulk of the extra reduction obtained with H_2DQ could be due to an interfering pigment (non-heme iron protein) is not excluded.

H_2DQ pulses in the cyanide-blocked system

Fig. 6 shows a series of spectrophotometric traces obtained successively and mounted together to illustrate the behavior of the different respiratory carriers under the same experimental conditions. Cyanide addition to pigeon heart mitochondria partially depleted of endogenous substrates brings about reduction of cytochromes $a + a_3$ and c, but cytochrome b, flavoprotein and ubiquinone remain oxidized[22]. The apparent flavoprotein reduction in Fig. 6 (absorption decrease at 475 nm with respect to 510 nm) seems to be due to cytochrome c interference, as judged by similar kinetics and in good agreement with the oxidized—reduced absorption of cytochrome c at these wavelengths (E_{ox-red} 475—510 nm = 7.0 $mM^{-1} \cdot cm^{-1}$). Cytochrome c reduction also interferes with the ubiquinone trace (285—305 nm) due to an absorption increase at 305 nm near the δ-band peak of cytochrome c at 315 nm.

Delivery of micromolar additions of H_2DQ to cyanide inhibited mitochondria produces pulses of reducing equivalents which are distributed among the oxidized carriers according to the redox potential of these components if enough time is provided for equilibration. Under the experimental conditions illustrated in Fig. 6,

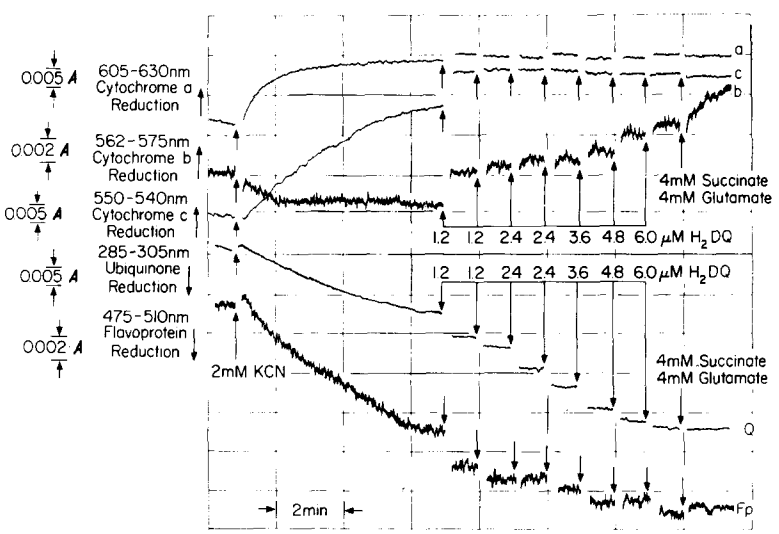


Fig. 6. Reduction of respiratory components by H_2DQ in pigeon heart mitochondria (1.8 mg protein/ml) supplemented with cyanide and suspended in mannitol–sucrose–Tris–HCl buffer. Each component was recorded in a separate experiment and mounted in the same chart paper. Ubiquinone trace was corrected for the increase in absorption at 285 – 305 nm due to H_2DQ addition. Expt. 3372. Fp. = flavoprotein.

successive additions are made at one minute intervals considered sufficiently large to reach the equilibrium. Cytochrome $a + a_3$ and cytochrome c [E'_0 at pH 7.4, 230 mV[23]] were fully reduced at 1.2 μM H_2DQ (Figs. 5–7) indicating that they are the more electropositive components of the system. An amount of cytochrome(s) b, which was about 30% of the total succinate–glutamate reducible absorption at 562—575 nm, was reduced almost simultaneously with cytochromes $a + a_3$ and c (Figs. 5–6) with a saturation level at 2.4 μM H_2DQ. A further increase in cytochrome(s) b reduction was observed from 9 to 21 μM H_2DQ. This 2-step titration shows straightforward the heterogeneous nature of the cytochrome(s) b pool and suggests the existence of two different cytochrome(s) b with different potential values.

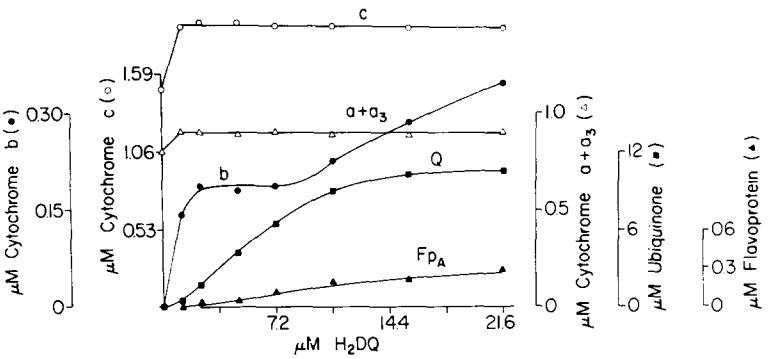


Fig. 7. Titration of respiratory components by H_2DQ in coupled state. Plot of the experiment of Fig. 6. The plot for flavoprotein (Fp_A) was corrected according to cytochrome c interference. Expt. 3372.

Ubiquinone was completely reduced at about 11 μM H_2DQ. The increase in the reductant (8.6 μM) is close to the amount of reducible ubiquinone (approx. 8 μM) in the mitochondrial sample. In other words, the stoichiometry of ubiquinone reduction by H_2DQ is 1 to 1 under these conditions. The fact that no ubiquinone seems to be reduced until cytochromes $a + a_3$ and c are fully reduced is explained by the difference (about 180 mV) in the potential of ubiquinone [E'_0 at pH 7.4, + 40 mV[24]] and those of the cytochromes.

Absorbing flavoprotein (succinate dehydrogenase) (E'_0 at pH 7.0, —40 mV[12]) completed its reduction at 21 μM H_2DQ. The H_2DQ saturation values for ubiquinone and succinate dehydrogenase show that the potential of succinate dehydrogenase is more negative than that of ubiquinone.

At the end of the traces (Fig. 6) succinate–glutamate was added to assure a full reduction of the respiratory carriers. No additional reduction was observed in any components except cytochrome(s) b. The results obtained after succinate addition deserve some comments: firstly, the absence of flavoprotein reduction indicates that H_2DQ had already fully reduced succinate dehydrogenase and, secondly, reduction of cytochrome(s) b after full reduction of succinate dehydrogenase shows that there is cytochrome b in pigeon heart mitochondria with a potential more negative than succinate dehydrogenase.

Fig. 8 shows the titration profile in the presence of cyanide and the uncoupler

pentachlorophenol. Cytochromes $a + a_3$ and c were saturated at 1.2 μM H_2DQ with most of the reduction supported by endogenous substrate before H_2DQ addition. The amount of cytochrome c titrated in the uncoupled state is slightly smaller than in the absence of uncoupler. Absorbing flavoprotein and ubiquinone show a titration pattern similar to that recorded in the absence of uncoupler.

An important difference to be noted is that cytochrome(s) b titrated as a homogeneous pool, showing a very different behavior in the absence (Figs. 6, 7) and in the presence of uncoupler (Fig. 8). The amount of cytochrome b reduced in the presence of uncoupler was significantly smaller than in the absence of uncoupler.

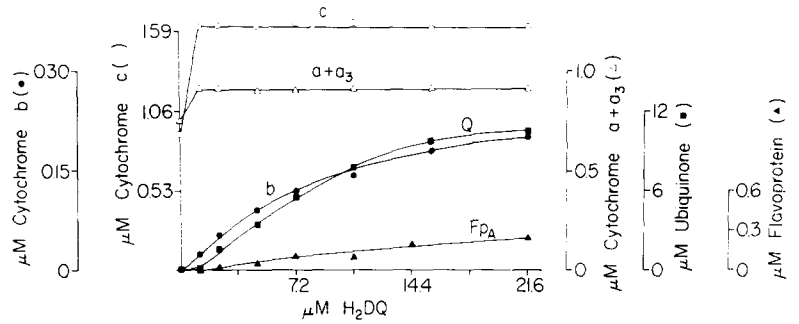


Fig. 8. Titration of respiratory components by H_2DQ in the uncoupled state. Experimental conditions as in Fig. 5 *plus* 6 μM pentachlorophenol. The plot for flavoprotein (Fp_A) was corrected according to cytochrome c interference. Expt. 3372.

Fig. 9 shows another series of spectrophotometric traces that illustrate the reduction of the respiratory carriers of submitochondrial particles obtained from pigeon heart mitochondria. The preparation was supplemented with cyanide and antimycin A (at inhibitor concentrations producing maximal effect) and titrated with H_2DQ. A trace at 270—285 nm illustrates the redox state of the added quinol, 270 nm is the absorption maximum of the oxidized form (DQ) and 285 nm is an isosbestic point for the pair H_2DQ/DQ. The first H_2DQ pulses are immediately oxidized, as is shown by the abrupt increase in the 270—285 nm trace, and the reducing equivalents are distributed among the cytochromes. Cytochrome(s) b showed redox cycles after addition of 3–6 μM H_2DQ, in which it is in a higly reduced state immediately ($\leqslant$ 1 sec) after addition of the reductant and is in an almost completely oxidized state after 2 min. Cytochromes $a + a_3$ and c were increasingly reduced by the sequential delivery of H_2DQ. These two effects, oxidation of cytochrome b and reduction of cytochromes $a + a_3$ and c show that small pulses of reducing equivalents can be effectifely oxidized through the antimycin A leak. The slight cytochrome c oxidation observed after the second to fourth H_2DQ pulses, accompanied by a decrease in the steady reduction level of cytochromes $a + a_3$, is accounted for by the cyanide leak. Artifacts at 285–305 nm and 475–510 nm producing an apparent ubiquinone and flavoprotein reduction are due to the spectral contributions of interfering cytochromes, as discussed before. After the addition of the two 15 μM H_2DQ pulses, ubiquinone and absorbing flavoprotein (succinate dehydrogenase) seem to undergo redox cycles, since steady reduction levels are

observed in the interfering cytochromes $a + a_3$ and c. Near the end of the experiment, succinate provided a continuous supply of reducing equivalents through succinate dehydrogenase which were distributed in the following way: (a) DQ was reduced at a rate of 8 nmoles/min per mg protein; and (b) ubiquinone, cytochrome(s) b and flavoprotein were fully reduced during the first minute; no further reduction was observed in cytochromes $a + a_3$ and c.

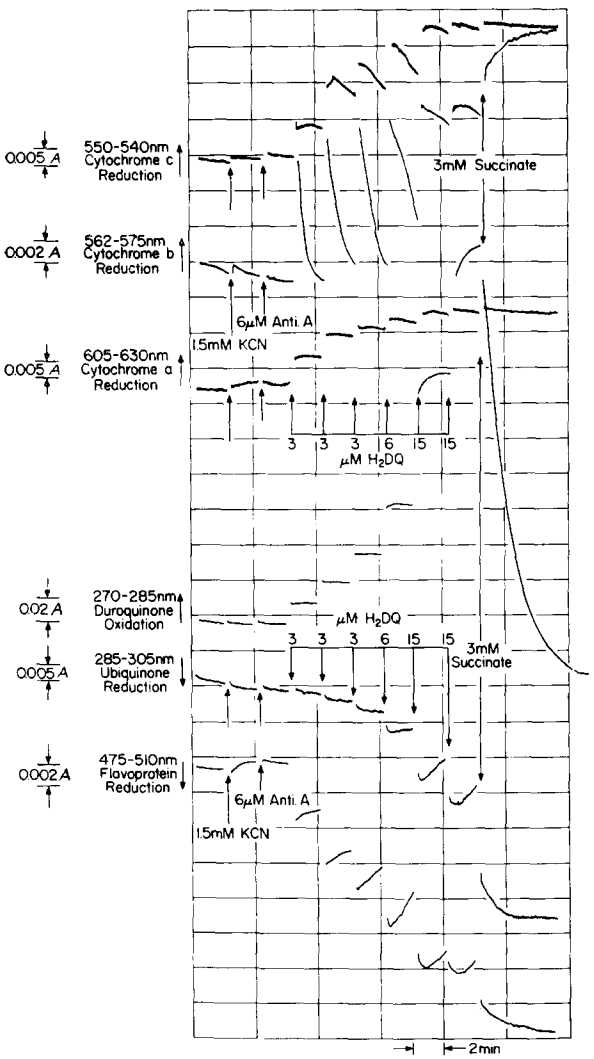


Fig. 9. Reduction of respiratory components by H_2DQ pulses and by succinate in submitochondrial particles from pigeon heart mitochondria (1.7 mg protein/ml). Each trace was obtained from a separate experiment and recorded in the same chart paper. Ubiquinone trace was corrected for the increase in absorption at 285–305 nm due to H_2DQ addition. For ubiquinone and duroquinone determinations the light path was 5 mm. Expt. 3382.

Cytochromes b reduction by H_2DQ

It was shown (Fig. 2) that the addition of 150 μM H_2DQ to pigeon heart mitochondria produces reduction cycles of cytochrome(s) *b* with simultaneous energy-linked reduction of fluorescent flavoprotein and pyridine nucleotide, which requires energization of the mitochondrial membranes. Fig. 10-A shows the spectral changes following addition of H_2DQ to pigeon heart mitochondria supplemented with rotenone. In the steady state there is an increase in absorbance in the 550–570 nm region with a main peak at 562 nm and shoulders at 557 nm and 550 nm. The main peak at 562 nm corresponds to the absorption of cytochrome(s) *b* which is 70–90 % reduced under these conditions (100 % reduction with dithionite). The shoulder at 550 nm is due to a small amount (10–15 % of total) of cytochrome *c* reduced in the steady state. The cytochrome *b* absorbing at 557 nm at room temperature is identified as the formerly reported cytochrome b_{555} (ref. 22). A sim-

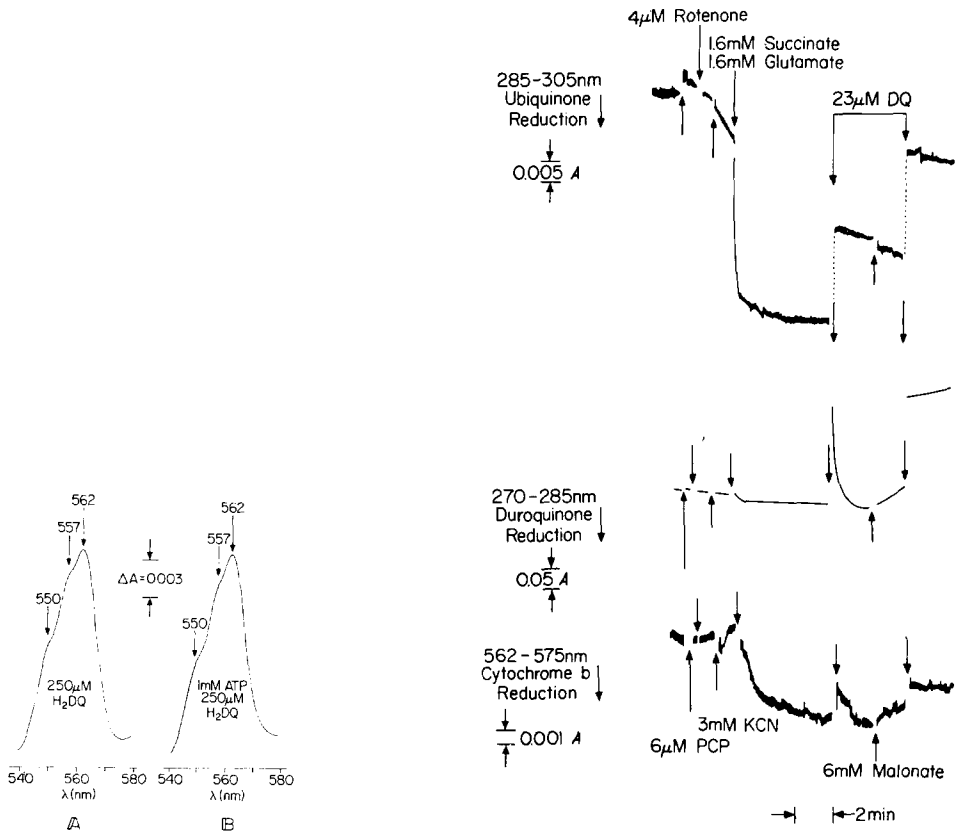


Fig. 10. Reduction of cytochromes by H_2DQ in pigeon heart mitochondria. Difference (reduced–oxidized) spectra recorded during the first minute after H_2DQ addition. Pigeon heart mitochondria (2.5 mg/ml) suspended in 0.23 M mannitol, 0.07 M sucrose, 40 mM morpholinopropane sulfonic acid–KOH (pH 6.8). Temperature, 8°. Expt. 4039.

Fig. 11. Oxidation of cytochrome *b* by DQ. Pigeon heart mitochondria (1.8 mg protein/ml) suspended in mannitol–sucrose–Tris–HCl buffer. Each trace was obtained from a separate experiment and recorded in the same chart paper. Points connecting ubiquinone trace indicate the increase in absorption due to H_2DQ addition. Light path, 5 mm. Expt. 3346.

ilar spectrum was recorded after addition of H_2DQ to mitochondria previously supplemented with ATP.

DQ as oxidant

Duroquinone can serve as electron acceptor in the cytochrome(s) *b*–flavoprotein–ubiquinone region of the respiratory chain as reported by RUZICKA AND CRANE[7,25]. Pigeon heart mitochondria supplemented with either malate–glutamate or succinate in the presence of antimycin A are not capable of reducing DQ at a measurable rate. The added quinone is kept in the oxidized form by a steady state with high oxidation and low reduction rates. Cyanide inhibits respiration by 99% and DQ is reduced by malate–glutamate or succinate–glutamate at a rate of 10–15 nmoles/min per mg protein.

Fig. 11 shows an oxidation–reduction cycle of cytochrome(s) *b* and H_2DQ production supported by succinate. Addition of 23 μM DQ oxidized 0.2 μM cytochrome(s) *b* (0.01 mole cytochrome *b*/mole DQ) that corresponds to 1/3 of the total succinate–glutamate reducible cytochrome(s) *b*. Malonate addition produces a diminution in the availability of reducing equivalents by inhibition of succinate dehydrogenase. Already formed H_2DQ is oxidized through the almost negligible cyanide leak (approx. 1 nmole O_2/min per mg protein) as indicated by the upwards slope in the 270—285 nm trace. A new addition of DQ produces a mixture of DQ and H_2DQ at a ratio of about 1 to 1 which equilibrates with the respiratory carriers producing oxidation of cytochrome(s) *b* (0.1 nmole/mg protein) but has no effect on the reduction level of ubiquinone. The changes in absorbance in the ubiquinone trace (285–305 nm) correspond to the absorption of the added DQ at these wavelengths (E_c 285—305 nm = 2.1 $mM^{-1} \cdot cm^{-1}$) and therefore there is no change in ubiquinone reduction level.

DISCUSSION

The oxygen uptake supported by H_2DQ as substrate is greater than that supported by succinate–glutamate in pigeon heart mitochondria. H_2DQ interacts with the respiratory chain at a site located between the rotenone and the antimycin A sensitive sites. In the steady state, H_2DQ produces reduction of cytochrome(s) *b*, absorbing flavoprotein and ubiquinone. Cytochrome *c* is slightly reduced in the steady state, its reduction and the reduction of cytochromes $a + a_3$ are enhanced by addition of a terminal inhibitor.

In the absence of inhibitors, H_2DQ reduces the low potential, high fluorescent flavoprotein, lipoate dehydrogenase and pyridine nucleotide through energy-dependent reversed electron transfer. The capability of H_2DQ to reduce pyridine nucleotide has been reported in a recent paper by RUZICKA AND CRANE[7] in which H_2DQ, among other quinols, showed the unique property of being an electron donor for the energy-linked reduction of NAD^+. The capacity of H_2DQ to generate energized states of the mitochondrial membranes is an aspect that should be pointed out.

A new type of cytochrome *b*, cytochrome b_T, has been recently proposed by WILSON AND DUTTON[26] and CHANCE *et al.*[23] as an energy transducer at the second phosphorylation site. SLATER *et al.*[27] have also postulated that a cytochrome of the *b* type, cytochrome b_i, is involved in the energy conservation process at the

second phosphorylation site. In agreement with its capacity to generate energized states of the mitochondrial membranes, H_2DQ was capable of reducing a form of cytochrome *b* absorbing at 557 nm. This cytochrome *b* with absorption at 557 nm at room temperature seems to be identical to the cytochrome b_{555} with maximal absorption at 555 nm at liquid nitrogen temperature formerly reported by CHANCE AND SCHOENER[22] and can be related to the energy transducing cytochrome b_T of WILSON AND DUTTON[26] and CHANCE *et al.*[23].

Titration data (Figs. 7, 8) show that in the cyanide inhibited system electrons are distributed among the oxidized respiratory components in such a way that the full reduction of each component is sequentially completed according to their redox potential. In other words, the absence of significant oxygen consumption defines a system in which the reduction level of the respiratory carriers closely approach a thermodynamic equilibrium. In this system, in the presence or absence of uncoupler, H_2DQ titrated the respiratory carriers of pigeon heart mitochondria in the following order: cytochrome $a + a_3$, cytochrome *c* ($E_0 = 230$ mV), ubiquinone ($E_0 = 40$ mV) and absorbing flavoprotein (succinate dehydrogenase, $E_0 = -45$ mV). In the absence of uncoupler, cytochrome(s) *b* show a 2-step titration. This behavior could be related to the existence of two forms of cytochrome *b* with different potentials, one with a potential near the cytochrome *c* potential and the other one with a potential similar to succinate dehydrogenase. In the presence of uncoupler, cytochrome(s) *b* titrated as a homogeneous pool in a manner similar to ubiquinone. It seems that one explanation for these different titration patterns could be provided by the potential change of cytochrome b_T reported by WILSON AND DUTTON[26] which, however, seems unlikely in the absence of energy source.

The redox cycles recorded after addition of reductant in systems open to oxygen, such as uninhibited or antimycin A-supplemented preparations, are transient states in which the extent of the reduction mainly depends on the kinetic constants of the system. These transient states must be considered from a thermodynamic point of view as non-equilibrium situations.

Addition of antimycin A to mitochondrial preparations, besides the well-known inhibition of electron transfer between cytochromes *b* and c_1, produces an alteration in the kinetics of equilibrium between cytochrome(s) *b* and ubiquinone (Table I and Figs. 4, 5) that can be summarized by saying that the antibiotic alters the reactivity of either ubiquinone or cytochrome(s) *b* so that cytochrome(s) *b* is reduced more rapidly than the quinone. This result is difficult to reconcile with a linear sequence that includes both components, unless ubiquinone were postulated to be located on the oxygen side of the antimycin A block, an extremely unlikely possibility that seems to be eliminated by the oxygen pulse titration of the components on the oxygen side of the antimycin A block[28]. The increase in the reduction rate of cytochrome *b* in the presence of antimycin A as shown by CHANCE[29] in submitochondrial particles, could not be demonstrated in our experimental conditions utilizing intact mitochondria. A modification of cytochrome *b* potential by antimycin A could not be demonstrated by direct potentiometric titration (D. F. WILSON AND M. E. ERECIŃSKA, unpublished results). Moreover, cytochrome *c* can be observed to be highly reduced when cytochrome *b* is almost completely oxidized (Fig. 9, second to fourth H_2DQ pulses) in submitochondrial particles supplemented with antimycin A. This latter evidence is consistent with a cyto-

chrome(s) *b* potential, in the presence of anticmyin A, much lower than that of cytochrome *c*.

We would like to advance the hypothesis that antimycin A combines with cytochrome(s) *b*[15] producing a conformational change of the cytochrome(s)[30] and a modification of the hydrocarbon core of the membrane that ubiquinone occupies, so that it inhibits the ubiquinone reduction.

The use of fast reducing equivalents pulses both in open and in blocked systems seems to be a promising technique for studying the relationships between the members of the multienzyme respiratory chain. (a) The stoichiometry of the reduction of respiratory components by fast reducing pulses could be used to establish the kinetic parameters that govern the system; (b) the agreement in the time course of redox cycles produced by suitable electron donors could be used as a criterion to elucidate the involvement of components in sequential systems; and (c) concentrations of reductant necessary to complete the reduction of each component in a blocked system, in which enough time to reach equilibrium is provided, could serve as an indirect estimation of the redox potential of each component. As a particular case, H_2DQ pulses have proved to be a useful tool to study the functional communication between the respiratory components in the span between the first and the second phosphorylation sites.

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