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REDUCTION KINETICS OF CYTOCHROMES *b*

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SUMMARY

1. Durohydroquinone (H_2DQ), a specific reductant for the second phosphorylation site, has been used in the study of the fast reduction kinetics of cytochromes *b*.

2. In ubiquinone-extracted mitochondria, H_2DQ reduces cytochrome(s) *b* in a fast reaction, the rate and extent of which decreases upon reincorporation of ubiquinone.

3. In cytochrome *c*-extracted mitochondria, H_2DQ reduces both cytochromes *b*. After ATP addition, with cytochrome b_T reduced by endogenous substrate, H_2DQ reduces cytochrome b_K at a slow rate whereas ubiquinone readily accepts reducing equivalents. In the presence of uncoupler, H_2DQ reduces cytochrome b_K at a faster rate while ubiquinone behaves as a less efficient electron acceptor. Under these conditions, cytochrome b_T is not reduced by H_2DQ.

4. Cytochrome c_1 reduction can be resolved free of cytochrome *c* interference in cytochrome *c*-extracted mitochondria. Cytochrome c_1 reduction is 4 times faster in the presence of uncoupler than in the presence of ATP.

5. Addition of cytochrome *c* to cytochrome *c*-extracted mitochondria stimulates the rate of cytochromes *b* reduction.

6. In intact mitochondria, ATP addition causes 20 % reduction of cytochrome b_T and speeds up the initial rate of cytochromes *b* reduction by a factor of 10.

7. Addition of antimycin A to intact mitochondria results in 80–100 % reduction of cytochrome b_K by endogenous substrate. ATP added to antimycin A-inhibited mitochondria produces oxidation of cytochrome b_K and simultaneous and variable reduction of cytochrome b_T. This latter effect is reverted by the addition of an uncoupler.

INTRODUCTION

Application of the potentiometric technique to the studies of the mitochondrial respiratory chain has led to identification of two cytochromes *b* (b_T and b_K) with differing midpoint potential values, one of which (cytochrome b_T) was capable of undergoing a change in its midpoint potential upon the addition of ATP[1,2]. Simultaneous independent evidence for the existence of two cytochromes *b* came from the

Abbreviations: UQ, ubiquinone; DQ, duroquinone; H_2DQ, durohydroquinone; MOPS, morpholinopropane sulfonate.

kinetic data in which it was possible to resolve in time, in tightly-coupled mitochondria, slowly oxidizable cytochrome b_T from more rapidly oxidized cytochrome b_K[2,3]. Along these lines, SLATER and his collaborators[4-6] reviving the earlier suggestions of CHANCE[7] and SLATER AND COLPA-BOONSTRA[8], put forward a tentative mechanism of energy coupling in which they assumed, on the basis of antimycin A and ATP-induced spectral shifts in the 560–565-nm region, the existence of two cytochrome *b* species, both of which were involved in the coupling phenomena.

Most of the kinetic studies on the respiratory chain have been carried out using a pulse technique in which the anaerobic mitochondria are rapidly mixed with suitable oxidants (*e.g.* oxygen, ferricyanide). Differences in oxidation times of the individual components obtained from the experimental data have aided in establishing the sequence of the carriers of the mitochondrial respiratory chain. Efforts to introduce reductants for the same purpose were handicapped by the lack of appropriate substances which would interact with the carriers at a rate sufficiently fast to permit their use in kinetic studies. However, recent investigations of RUZICKA AND CRANE[9-11] and of ourselves[12,13] demonstrated that durohydroquinone (2,3,5,6-tetramethylbenzhydroquinol (H_2DQ)) which intercepts the respiratory chain at ubiquinone–cytochromes *b* level can be used in pulse studies to establish the kinetic parameters that describe the multienzyme system of the mitochondrial respiratory chain.

It was found[12] that the time course of redox cycles produced by H_2DQ addition could be used as a criterion to determine the involvement of the carriers in the chain. In addition, the concentrations of reductant necessary to complete the reduction of each carrier in a blocked system could serve as an indirect estimation of its redox potential.

The present work focuses our attention on the use of reductant pulses in the study of the kinetics of the particular region of the respiratory chain (cytochromes *b*, c_1 and ubiquinone) under a variety of experimental conditions. This investigation complements the data obtained with oxygen pulses and affords us a direct comparison of both approaches.

MATERIALS AND METHODS

Pigeon heart mitochondria were isolated in 0.225 M mannitol–0.075 M sucrose–0.2 mM EDTA medium by the method of CHANCE AND HAGIHARA[14]. Cytochrome *c*-depleted mitochondria were prepared essentially according to the procedure of JACOBS AND SANADI[15]. The mitochondrial pellet was suspended in 0.015 M KCl and incubated at 0° for 30 min, with occasional stirring. After centrifugation for 10 min at 8000 × *g* the sediment was washed twice in 0.15 M KCl and once in mannitol–sucrose–EDTA medium and finally suspended in the same medium. This method afforded over 90 % decrease in the amount of cytochrome *c* estimated by the succinate + KCN reduced – oxidized spectra and resulted in a marked diminution of oxygen uptake in the absence of added cytochrome *c* (5–10 % of the State 3 rate).

Ubiquinone extraction was carried out in the acetone–water (96:4, v/v) mixture as described by LESTER AND FLEISCHER[16]. Reincorporation of ubiquinone to the depleted mitochondria was performed by a modification of the method of ERNSTER *et al.*[17] in which extracted mitochondria suspended in 0.225 M mannitol–0.075 M sucrose–20 mM Tris–HCl (pH 7.4) medium were mixed with an equal volume of

acetone containing ubiquinone-35 (UQ_7) (through the courtesy of Professor K. Folkers, University of Texas) at concentrations of 5–20 nmoles UQ_7 per mg mitochondrial protein. After an incubation of 20 min the preparation was diluted 5 times with mannitol–sucrose–Tris medium, centrifuged, washed and finally suspended in the same medium. Oxygen uptake was measured in 0.225 mannitol–0.075 M sucrose–40 mM KOH–morpholinopropane sulfonate (MOPS) (pH 6.8) (mannitol–sucrose–MOPS) medium using a Clark oxygen electrode.

Kinetic experiments were carried out using stopped flow technique[18]. A solution of 5.6–7.0 mM H_2DQ in 30 % *N*,*N*-dimethylformamide containing 5 mM EDTA was delivered from the side syringe giving a 1.25 % dilution per discharge of the flow apparatus. The light path of the observation chamber is 6 mm.

Split-beam spectra and titration experiments were performed in a Model 356 Perkin–Elmer spectrophotometer. Cytochromes *b* ($b_K + b_T$) were recorded at 560–575 nm and 562–575 nm (ε_{mM} red–ox = 22 cm^{-1}) or at 430–410 nm (ε_{mM} red–ox = 180 cm^{-1}). Ubiquinone was measured at 285–305 nm (ε_{mM} ox–red = 6 cm^{-1}) and H_2DQ was recorded at 270–285 nm (ε_{mM} ox–red = 16 cm^{-1}). Protein was determined by the biuret method[19].

RESULTS

H_2DQ oxidation by intact pigeon heart mitochondria

H_2DQ, whose spectral properties are shown in Fig. 1, is oxidized by intact pigeon heart mitochondria at a rate of 21 natoms oxygen per min per mg protein which is approx. 1.3 times faster than the oxidation rate of succinate at this pH value and in mannitol–sucrose–MOPS medium (Fig. 2). In order to exclude participation of

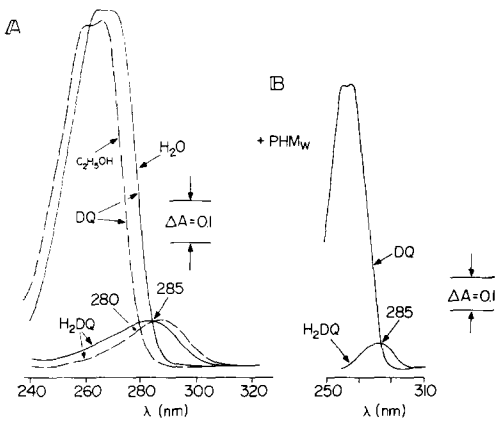


Fig. 1. Spectral properties of the redox pair DQ–H_2DQ. A. ———, in water solution, 46 μM DQ (maximum at 263–271 nm) and 46 μM H_2DQ, reduced by KH_4Br (maximum at 283–284 nm), showing an isosbestic point at 285 nm; – – –, in ethanol solution, 46 μM DQ (maximum at 259–266 nm) and 46 μM H_2DQ, reduced by KH_4Br (maximum at 286–288 nm), showing an isosbestic point at 280 nm. Similar spectra are observed in methanol, propanol and butanol. Light path, 1 cm. B. Differential spectra, DQ 98 μM DQ added to washed pigeon heart mitochondria (PHM_W) (1.4 mg/ml in mannitol–sucrose–Tris medium) *vs.* washed pigeon heart mitochondria. H_2DQ after addition of 5 mM succinate and 5 mM glutamate to sample and reference cuvettes allowing enough time (approx. 10 min) to reach anaerobiosis and full DQ reduction. Light path, 5 mm. (Experimental data from R. OSHINO AND A. BOVERIS, unpublished, Expt. 333.)

the energy-linked reduction of NAD^+ elicited by H_2DQ[9,11,12], the reaction is carried out in the presence of 5 μM rotenone. The oxidation rate is unaffected by ATP and stimulated 5-fold by the addition of 15 μM pentachlorophenol. Addition of ADP + P_i evokes typical State 4 to 3 transition with acceleration of oxygen uptake in the presence of ADP and return to State 4 rate when ADP is exhausted. The calculated ADP:O ratio of 1.7 indicates that both phosphorylation sites II and III are operative during H_2DQ oxidation. Oxidation of H_2DQ is, as reported previously[12], 94 % inhibited by the addition of antimycin A. Thus, H_2DQ intercepts the respiratory chain between the rotenone and antimycin A sensitive sites.

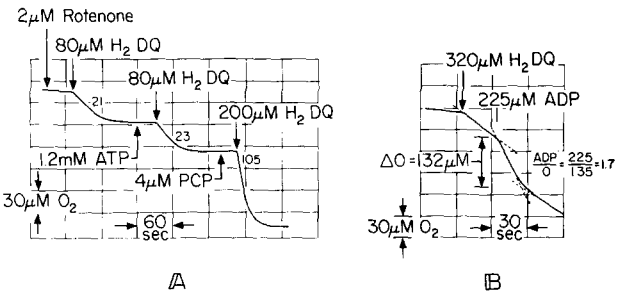


Fig. 2. Oxygen uptake supported by H_2DQ in pigeon heart mitochondria. A. Rates in State 4, State 4 + ATP and State 3 (+ pentachlorophenol, PCP). Numbers near the trace indicate oxygen consumption in natoms oxygen per min per mg protein. 2.4 mg pigeon heart mitochondria per ml in mannitol–sucrose–MOPS (pH 6.8). B. ADP:O ratio. 1.4 mg pigeon heart mitochondria per ml suspended in 2 μM rotenone, 5 mM phosphate, mannitol–sucrose–MOPS (pH 6.8). Expt. 4031.

Reaction of H_2DQ with the respiratory chain

In order to use H_2DQ effectively in the kinetic study of the respiratory chain determination of the reaction order with respect to H_2DQ and mitochondrial protein as well as identification of the possible rate-limiting steps seems indispensable. The order of the reaction depends primarily on the oxidation rate and on the initial H_2DQ concentration. At about 100 μM H_2DQ, in the range of the usually employed concen-

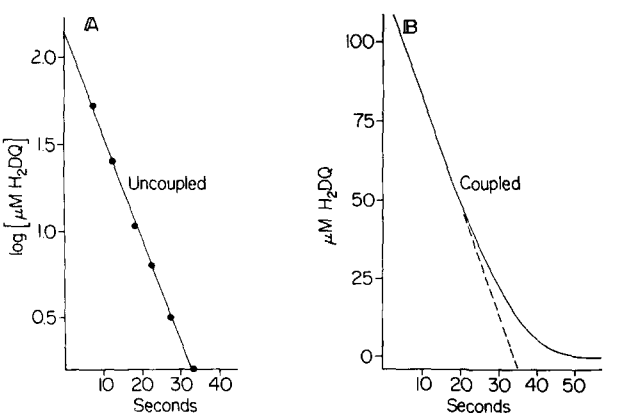


Fig. 3. Order of the oxidation reaction with respect to $[H_2DQ]$ in (A), uncoupled mitochondria (0.3 μM S-13, 0.6 mg protein pigeon heart mitochondria per ml) and (B) coupled mitochondria (0.9 mg protein pigeon heart mitochondria per ml). Mannitol–sucrose–MOPS (pH 7.3). Expt. 4019.

trations, the reaction is of first order with respect to H_2DQ in the uncoupled state (Fig. 3A) and in coupled mitochondria at high protein concentration. In coupled mitochondria at low protein concentration (Fig. 3B), and in inhibited systems, *i.e.* in cytochrome *c* extracted or antimycin A supplemented mitochondria, the reaction remains of zero order with respect to H_2DQ for a variable time, usually measured in minutes, until the H_2DQ concentration falls to levels in which it becomes critical. It was found experimentally that the concentration of H_2DQ that gives half maximal oxidation rate, $[H_2DQ]_{50\%}$, follows approximately the equation:

$$[H_2DQ]_{50\%} = 8\ \mu M + \alpha[\text{maximal oxidation rate } (\mu M/\text{min})]$$

values for α are: 0.03 min for coupled or inhibited mitochondria and 0.12 min for uncoupled mitochondria.

In order to establish whether either ubiquinone or cytochromes *b* were the actual point of intercept of the respiratory chain by H_2DQ mitochondria were either depleted or enriched in their endogenous ubiquinone content and then pulsed with H_2DQ. The rate of H_2DQ oxidation, measured in the presence of added cytochrome *c* (4 μM) was found to be almost independent of the ubiquinone level and was essentially the same in ubiquinone-depleted (210 nmoles/min per mg protein) and in ubiquinone-reincorporated mitochondria (310 nmoles/min per mg protein). Reduction of cytochrome(s) *b* by H_2DQ was recorded in a rapid flow apparatus at 430–410 nm as illustrated by Fig. 4. When ubiquinone extraction is nearly complete (ubiquinone below 1.2 nmoles/mg protein; Trace a) the entire cytochrome(s) *b* reduction occurs in a rapid phase at a rate of 11.6 μM/sec. On the other hand, with an increase in the amount of ubiquinone to a near normal level (6.0 nmoles/mg protein; Trace b), the reduction of cytochrome(s) *b* consists of an initial rapid phase at 11.6 μM/sec, better described as an initial jump, and a slow phase at a rate of 0.06 μM/sec. At higher levels of ubiquinone incorporated (21 nmoles/mg protein; Trace c) no initial jump is observed and only a slow reduction of cytochrome(s) *b* is recorded at a rate of about 0.12 μM/sec.

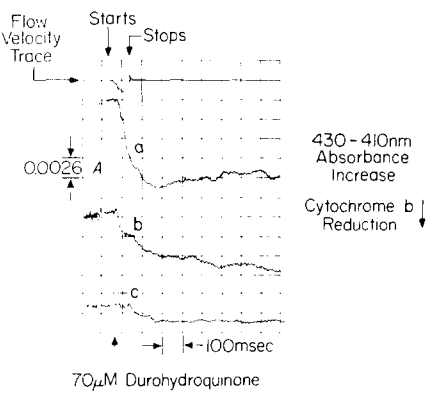


Fig. 4. Reduction of cytochrome(s) *b* in ubiquinone-extracted and ubiquinone incorporated pigeon heart mitochondria. A. UQ-extracted pigeon heart mitochondria (<1.2 nmoles UQ per mg protein). b and c, UQ_7-incorporated pigeon heart mitochondria (6.0 and 21 nmoles UQ per mg protein). 0.9 mg protein per ml mannitol–sucrose–MOPS (pH 6.8).

Reaction of H_2DQ with cytochrome c depleted mitochondria

Kinetics of reduction of cytochromes *b* can be studied in a convenient way in a system modified in such a way that it becomes less complex than the intact mitochondria. Such an opportunity is offered by mitochondria depleted of their endogenous cytochrome *c* in which the rate determining step of the overall electron transfer is across the cytochrome *c* site. Hence the reactions on the oxygen side of the cytochrome *c* have only negligible effect on the kinetic changes of cytochromes *b*, c_1 and ubiquinone which are induced by the addition of H_2DQ.

Extraction of cytochrome *c* from pigeon heart mitochondria reduces the rate of H_2DQ oxidation in State 4 to about 1/3 of the value observed in intact mitochondria. This oxidation rate is only slightly stimulated by pentachlorophenol (from 8.6 to 13.4 natoms oxygen per min per mg protein). Addition of a pulse of 150 μM H_2DQ results in a reduction–oxidation cycle approx. 3 times longer than in the intact mitochondria; thus, the absorbance change can be followed easily at room temperature. The spectrum recorded under such conditions (Fig. 5A) exhibits a broad asymmetrical peak with maximum at 552 nm and a shoulder at about 561 nm. The main peak at 552 nm corresponds to the reduced cytochrome c_1 which amounts close to 100 % of the dithionite-reducible material at this wavelength. The shoulder at about 561 nm corresponds to the reduction of both cytochromes *b* which are usually seen (*cf.* Fig. 5B, Trace 3; Fig. 15, Traces 3 and 5) as a broad absorption peak with a maximum at about 562 nm. In this particular case, the reduction of the pigment absorbing at 552 nm precludes an exact positioning of the absorption maximum.

Of the two cytochromes *b*, according to the description of SATO *et al.*[20,21], one of them, designated here as cytochrome b_K ($E_0' = +30$ mV)[1] exhibits a maximum absorption peak at 561 nm and the other one, designated as cytochrome b_T, has a split peak with maxima at 565 and 558 nm. This latter cytochrome b_T is able to exhibit a low redox potential ($E_0' = -30$ mV) in the absence of energy sources and a high redox potential (+245 mV) in the presence of ATP[1,2,20,21]. Oxidation of the substrate, *i.e.* H_2DQ (Fig. 5A-2) with generation of endogenous ATP drives cytochrome b_T to

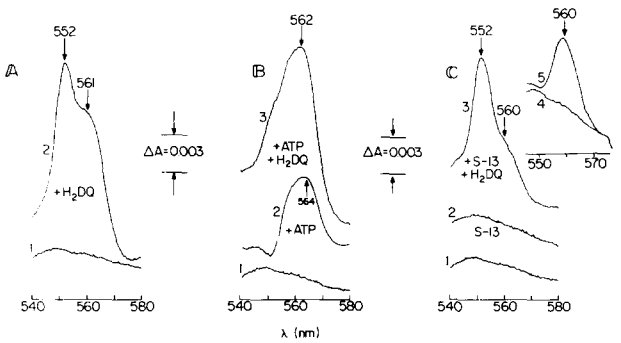


Fig. 5. Cytochrome reduction by H_2DQ in cytochrome *c*-extracted pigeon heart mitochondria. (3.4 mg/ml mannitol–sucrose–MOPS (pH 6.8)). A. (1), baseline + 2 μM rotenone; (2), (1) + 100 μM H_2DQ. B. (1), baseline + 2 μM rotenone; (2), (1) + 1.2 mM ATP; (3), (2) + 100 μM H_2DQ. C. (1), baseline + 2 μM rotenone; (2), (1) + 4 μM S-13 (5-chloro-3-*tert.*-butyl-2′-chloro-4′-nitrosalicylanilide); (3), (2) + 100 μM H_2DQ; (4), new baseline with 5.1 mg pigeon heart mitochondria per ml; (5), (4) + 6 μM S-13 + 150 μM H_2DQ recorded *versus* a reference cuvette supplemented with 4 mM ascorbate and 1 mM KCN. Expt. 4020.

its high potential form. Alternatively, energization of the mitochondrial membranes can be induced by the addition of ATP, prior to H_2DQ (Fig. 5B). ATP addition does not change the rate of oxygen consumption (11.4 natoms oxygen per min per mg protein) but produces marked changes in the reduction level of the respiratory carriers. In these cytochrome *c*-extracted mitochondria, due to the existence of a rate limiting step at the cytochrome *c* site, reducing equivalents from endogenous substrate produce a partial reduction of cytochrome c_1. Upon addition of ATP, oxidation of cytochrome c_1, indicated by the trough at 552 nm, occurs simultaneously with the reduction of the high-potential cytochrome b_T, as shown by the appearance of the 564 nm absorbance peak (which has an asymmetrical shape due to the absorption band at 557 nm, not resolved under these experimental conditions). The source of electrons for high potential cytochrome b_T are both cytochrome c_1 *via* reversed electron transfer and endogenous substrate *via* cytochrome b_K. This absorbance increase that occurs prior to the addition of H_2DQ accounts in most cases for over 40 % of the total absorption at 562–575 nm. Reduction by endogenous substrate seems to be favored at this relatively low pH. Subsequent addition of a pulse of H_2DQ (Trace 3) reduces the remaining oxidized cytochromes *b*. The overall spectrum show both cytochromes *b* reduced with a maximum at 562 nm. Reduction of cytochromes *b* under these experimental conditions accounts for 100 % of that obtained with succinate – ATP and over 90 % of the amount reducible by dithionite.

If an uncoupler is added to the mitochondria (Fig. 5C) in which high potential cytochrome b_T was reduced by prior addition of ATP the 564-nm peak disappears (Trace 2) indicating oxidation of cytochrome b_T. The recorded spectrum follows the baseline. Addition of the reductant (Trace 3), brings about a strong increase in absorbance with a maximum at 552 nm and a shoulder at about 560 nm. The former accounts for 100 % of the total cytochrome c_1 and the latter represents reduction of cytochrome b_K, identified by its absorption maximum at 560 nm in Trace 5, which is a difference spectrum of a mitochondrial suspension under conditions similar to those of Trace 3 *versus* a sample containing cytochrome c_1 reduced by ascorbate and cyanide.

Durohydroquinone as succinate[20,21] is unable to reduce the low-potential form of cytochrome b_T under uncoupled conditions.

The sequence of events described above can be summarized as follows: (a) addition of H_2DQ to coupled mitochondria produces reduction of both cytochromes *b*, cytochrome b_K and cytochrome b_T, the latter presumably raised to its high-potential

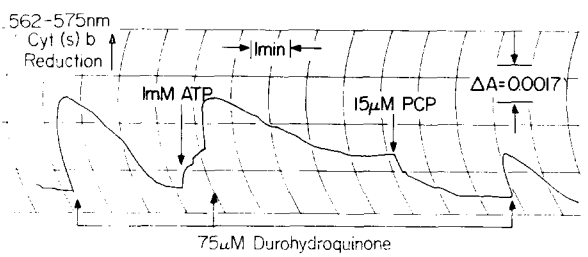


Fig. 6. Reduction of cytochromes *b* by H_2DQ and endogenous substrate in cytochrome *c*-extracted pigeon heart mitochondria (2.4 mg protein per ml in mannitol–sucrose–MOPS (pH 6.8)). Trace obtained from the slow-time scale recorder of the stopped flow apparatus. PCP = pentachlorophenol. Expt. 211.

form by internally generated ATP; (b) after oxidation of the pulse of reductant, addition of ATP results in reduction of the high-potential cytochrome b_T by endogenous substrate; (c) a pulse of reductant produces a reduction–oxidation cycle of cytochrome b_K, whereas the high-potential form of cytochrome b_T is kept reduced by the endogenous substrate, (in the absence of ATP, cytochrome c_1, the carrier with the highest potential [+230 mV] is reduced by the endogenous substrate); (d) addition of an uncoupler brings about oxidation of cytochrome b_T; and (e) only cytochrome b_K is reduced under uncoupled conditions by the addition of H_2DQ. These five situations are faithfully shown as absorption changes at 562–575 nm by the actual experimental tracing of Fig. 6 taken from the slow time-scale recorder of the flow apparatus. The baseline was not reset during the interval of the experiment. Pentachlorophenol was used instead of S-13 in this experiment implying that these effects do not depend on a particular uncoupler.

Several interesting points require consideration: (1) The absorbance increase induced by ATP and attributed to reduction of cytochrome b_T accounts for about 50 % of the total absorbance change, H_2DQ reduces the remaining 50 % due to reduction of cytochrome b_K. (2) Oxidation of H_2DQ through the respiratory chain results in a cycle of increase and decrease of absorption at 562–575 nm and the trace returns to the level observed in the presence of ATP. (3) Uncoupler induces oxidation of high-potential cytochrome b_T upon the transition to the low-potential form. The experimental trace returns to the original baseline recorded at the beginning of the test. (4) A subsequent pulse of H_2DQ results in an absorbance increase which accounts for about 50 % of the total signal at these wavelengths which is identified with cytochrome b_K by its absorption maximum at 560 nm (Fig. 5C-5). Thus, in the cytochrome *c*-depleted mitochondria, both in the presence of ATP and in the presence of PCP addition of H_2DQ results in reduction of cytochrome b_K. Cytochrome b_T in the presence of ATP is reduced prior to addition of H_2DQ while in the presence of uncoupler is reduced only to a negligible extent.

As mentioned above in the cytochrome *c*-depleted mitochondria under conditions of H_2DQ pulse all the carriers on the oxygen side of cytochrome *c* are oxidized, while those on the substrate side are reduced to varying degrees. The steady-state reductions of individual components are governed by the reaction rate between H_2DQ and the primary acceptor in the respiratory chain on the one hand, and by the reaction rates between the carriers within the chain, on the other. This can be tested by the titration of the depleted membranes with increasing amounts of H_2DQ (Fig. 7). In these profiles, the slope of the titration curves gives the efficiency of the carrier as electron acceptor. In the presence of ATP, reducing equivalents are distributed among high-potential cytochrome b_T (0.01 nmole reduced per mole H_2DQ; abbreviated 0.01), ubiquinone (0.52) and cytochrome c_1 (0.01). Cytochrome b_T reduction is completed at about 16 μM H_2DQ, and only thereafter reduction of cytochrome b_K (0.005) occurs. It is remarkable that in the energized state of the mitochondrial membrane, ubiquinone is the main acceptor by a ratio of 50 to 1. In the presence of uncoupler a different distribution of the reducing equivalents is observed. Only cytochrome b_K (0.005) is reduced of the two cytochromes *b*, ubiquinone (0.06) greatly diminishes its efficiency as electron acceptor, while electrons seem to accumulate in cytochrome c_1 (0.03) in accordance with the spectral data of Fig. 4.

Although these measurements lead to determination of the steady-state levels

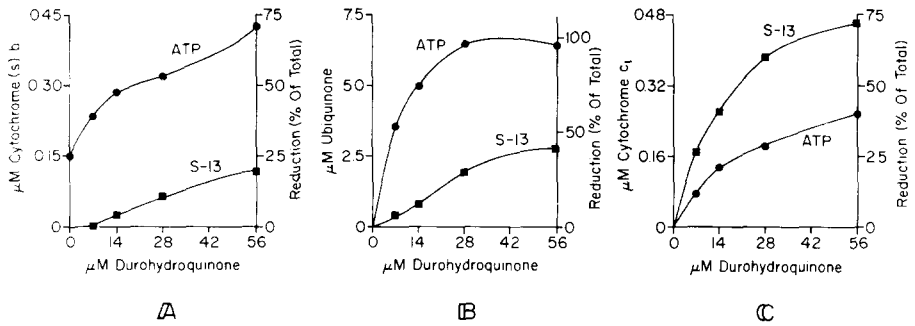


Fig. 7. Titration of cytochromes *b*, ubiquinone and cytochrome *c* in cytochrome *c* extracted pigeon heart mitochondria. Cytochromes *b* (562–575 nm) and cytochrome c_1 (552–540 nm) titrations: 1 mM ATP or 2 μM S-13. Ubiquinone (285–305 nm, 5 mm light path) titration: 0.5 mM ATP or 2 μM S-13. In all cases 1.8 mg/ml cytochrome *c*-extracted pigeon heart mitochondria and 3 μM rotenone in mannitol–sucrose–MOPS (pH 6.8). Expt. 4024.

of the reduced components they do not allow us to obtain the actual, initial reduction rates. These can only be obtained by the use of a rapid flow technique, as presented in Figs. 8, 9 and 10. Fig. 8 illustrates the initial rate of cytochrome c_1 reduction by H_2DQ in the presence of ATP (A) and pentachlorophenol (B). The initial reduction rate of cytochrome c_1 is approx. 4 times faster in the presence of pentachlorophenol (5.2 μM/sec; half-time approx. 80 msec) than in the presence of ATP (1.4 μM/sec; half-time approx. 300 msec). It deserves mentioning that the amount of cytochrome c_1 reduced by H_2DQ is bigger in the presence of uncoupler than in the presence of ATP.

The reduction rate of both cytochromes *b*, since both b_K and b_T, are reduced by H_2DQ in the absence of ATP and uncoupler (Figs. 5A and 6) is shown in Fig. 9A. The trace shows a biphasic reduction with an initial rate of 1.1 μM/sec and after 200 msec a second slow phase at a rate of 0.04 μM/sec.

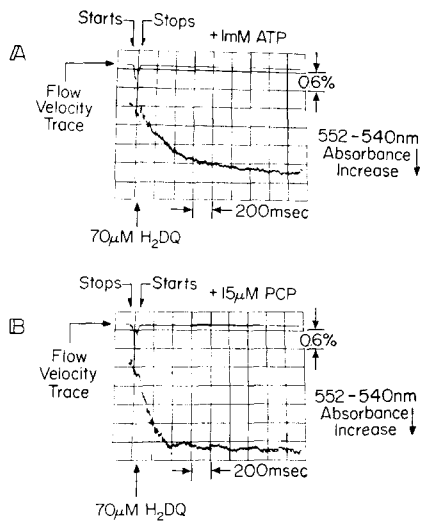


Fig. 8. Reduction of cytochrome c_1 by 80 μM H_2DQ in cytochrome *c*-extracted pigeon heart mitochondria, 3.0 mg/ml, in mannitol–sucrose–MOPS (pH 6.8). A. + 1.0 mM ATP. B. + 15 μM pentachlorophenol (PCP). Expt. 277.

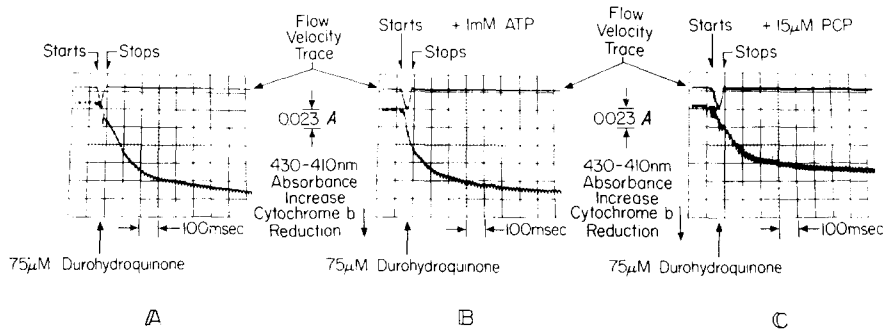


Fig. 9. Reduction of cytochromes *b* by H_2DQ under the experimental conditions of Fig. 6. PCP = pentachlorophenol. Expt. 211.

An elegant opportunity to study the effect of the energy state of the mitochondrial membranes on the reduction rates of cytochrome b_K, free of cytochrome b_T interference, is provided by the ATP-supplemented and the uncoupled cytochrome *c*-extracted mitochondria.

In the presence of ATP, Fig. 9B, cytochrome b_K is reduced initially at a slow rate, 0.25 μM/sec and after 300 msec there is a speeding-up of the rate to 0.45 μM/sec. This faster, second rate can be explained by a change in the reduction level of ubiquinone, which can act as a pool able to retain cytochrome b_K partially oxidized until ubiquinone had become reduced.

In the presence of uncoupler, Fig. 9C, cytochrome b_K is reduced at a faster initial rate, 1.0 μM/sec, followed by a second, slower, linear phase, at 0.2 μM/sec.

The effect of cytochrome c on the reaction of H_2DQ with mitochondria

It was demonstrated some time ago that addition of cytochrome *c* to the depleted rat liver mitochondria restores both the oxygen uptake and coupled electron flow[15]. It has been found recently (M. ERECIŃSKA AND B. CHANCE, unpublished) that the cytochrome *c* added to depleted mitochondria behaves under conditions of rapid flow measurements as endogenous cytochrome *c*. The half-time of the cytochrome *c* oxidation in the "reconstructed" membranes was found to be identical to that of control mitochondria. Addition of cytochrome *c* to the depleted mitochondria removes the rate determining step at the cytochrome *c* site and increases the amount of cytochrome c_1 present in oxidized form. Thus faster oxidation of H_2DQ occurs. Under such conditions the reduction rate of cytochromes *b* recorded in Fig. 10B is biphasic with the initial slope corresponding to 1 μM cytochrome *b* reduced per sec which can be compared with 0.45 μM cytochrome *b* reduced per sec (Fig. 10A) in the depleted mitochondria. The biphasicity of the trace is even more pronounced in the presence of 1 mM ATP (Fig. 10C); the initial rapid reduction indicated by the steep downward deflection of the trace which accounts for approx. 50 % of the total absorbance change and is completed within 20 msec of the mixing flow time. This fast phase is followed by a much slower one (half-time of approx. 300 msec) which accounts for the remaining 50 % of the absorbance change. It is suggested that the fast phase that is under control of ATP corresponds to the reduction of high-potential cytochrome b_T. Under these conditions cytochrome b_T at +245 mV could successfully compete with

ubiquinone (+60 mV) for the reducing equivalents and become the most efficient electron acceptor.

Reaction of H_2DQ with intact mitochondria

Since the "reconstructed" mitochondria behave in many respects as normal, non-depleted membranes, it seemed worthwhile to compare directly both systems. Fig. 11 illustrates the spectral changes recorded upon addition of 150 μM H_2DQ to intact mitochondria. A broad asymmetrical peak with maximum at 562–564 nm and shoulders at 557 and 550 nm corresponds to reduction of both cytochromes *b* and cytochromes $c + c_1$. The amount of cytochrome $c + c_1$ reduced in the steady state is about 10–15 % of the dithionite-reducible cytochromes and is 6 times lower than the steady-state level of cytochrome c_1 reduced in the cytochrome *c*-depleted membranes.

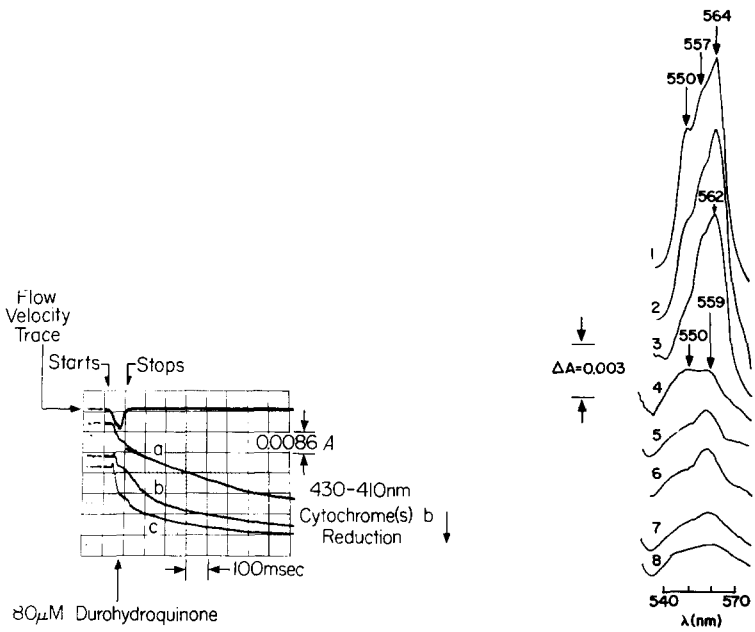


Fig. 10. Reduction of cytochromes *b* by H_2DQ in cytochrome *c* extracted pigeon heart mitochondria, 2.3 mg protein per ml, 1.2 μM cytochrome *c* (b, c) and 1.2 mM ATP (c). Mannitol–sucrose–MOPS (pH 6.8). Expt. 262.

Fig. 11. Cytochrome reduction by a pulse of H_2DQ. 170 μM H_2DQ was added to the sample cuvette and the spectra numbered 1–8 were recorded successively every 50 sec. Differential spectra *vs.* an oxidized sample. Pigeon heart mitochondria, 1.6 mg/ml, in mannitol–sucrose–MOPS (pH 6.8), 4 μM rotenone. Temperature, 6–8°. Expt. 4008.

Since H_2DQ is subsequently oxidized through the respiratory chain spectra retaken at constant intervals enable us to obtain a dynamic picture of the changes taking place. Traces 2–4 of Fig. 11 recorded 50–150 sec after addition of H_2DQ show gradual disappearance of the 564-nm absorbance peak and exposure of the 560-nm peak. This suggests that the oxidation of cytochrome b_T is preceded by the change in its midpoint potential from +245 to −30 mV, after exhaustion of the substrate.

Subsequent oxidation of cytochrome b_K is illustrated in Fig. 5 by the disappearance of the 560-nm absorbance peak.

Kinetic changes followed by rapid flow techniques are presented in Figs. 12 and 14. As in the case of cytochrome *c*-depleted membranes supplemented with exogenous cytochrome *c*, the kinetic traces recorded in the intact mitochondria exhibit similar biphasicity. The initial rapid reduction phase is greatly speeded up in the presence of 1 mM ATP, and corresponds to a rate of cytochrome *b* reduction of about 18 μM cytochrome *b* reduced per sec. Both in the absence and presence of ATP the rapid phase is followed by a slow increase in absorbance (half-time approx. 300 msec) to a steady state.

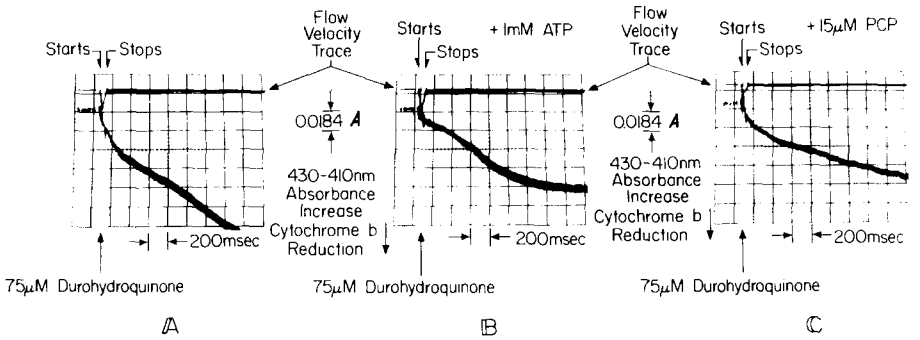


Fig. 12. Reduction of cytochromes *b* in intact pigeon heart mitochondria by H_2DQ recorded in the stopped flow apparatus. A. 5 μM rotenone + 75 μM H_2DQ. B. 5 μM rotenone and 1.0 mM ATP + 75 μM H_2DQ. C. 5 μM rotenone and 15 μM pentachlorophenol (PCP) + 75 μM H_2DQ. Pigeon heart mitochondria, 2.3 mg/ml, in mannitol–sucrose–MOPS (pH 6.8). Expt. 211.

Addition of pentachlorophenol lowers the steady-state reduction of cytochromes *b* from 80 % of the amount reducible with dithionite in the absence of pentachlorophenol to about 25 % in its presence. No rapid phase of reduction is observed and the initial rate drops to 2.3 μM cytochrome *b* reduced per sec.

H_2DQ oxidation in antimycin A blocked system

Addition of antimycin A to the mitochondrial suspension in amounts which block electron transfer in over 85 % (0.2 nmole per mg protein) causes an increase in absorbance with a maximum at 560.5 nm (Fig. 13-2) due to reduction of cytochrome b_K by the endogenous substrate. This absorbance increase accounts usually for about 80–100 % of the total cytochrome b_K. Addition of a pulse of H_2DQ results in an additional increase in absorbance caused by reduction of the remaining portion of cytochrome b_K and cytochrome b_T. Kinetic tracing obtained by rapid flow techniques shows that the latter reaction occurs with the half-time of about 100 msec and a rate of 1.2 μM cytochrome *b* reduced per sec (Fig. 14B).

Addition of 1 mM ATP to the antimycin A inhibited system causes disappearance of the 560-nm peak and concomitant appearance of a double peak with maxima at 565 and 557 nm (Fig. 13-3). These spectral changes can be interpreted as oxidation of cytochrome b_K and concomitant reduction of cytochrome b_T by ATP in the aerobic, antimycin A inhibited system, implying that the potential change of cytochrome b_T from low to high potential has occurred under these experimental

conditions. The previous addition either of oligomycin or uncoupler prevents this ATP-induced change of absorption. The amount of high-potential cytochrome b_T reduced accounts for about 50 % of the total cytochrome b_T absorbance, but in some cases (absence of rotenone), it is possible to reduce nearly 50 % of dithionite-reducible cytochrome *b*, as is shown in Fig. 15-2. Under such conditions, the reduction recorded after addition of H_2DQ corresponds mainly to the reduction of cytochrome b_K.

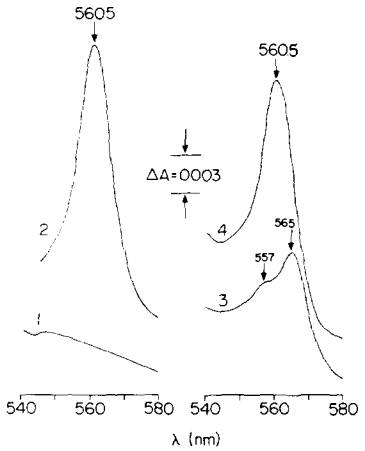


Fig. 13. Effect of antimycin A, ATP and the uncoupler S-13 on the reduction of cytochromes *b* by endogenous substrate. Pigeon heart mitochondria, 3.9 mg/ml, in mannitol–sucrose–MOPS (pH 6.8). (1) baseline + 3 μM rotenone; (2) (1) + 0.8 μM antimycin A; (3) (2) + 1.2 mM ATP; (4) (3) + 4 μM S-13. Expt. 4009.

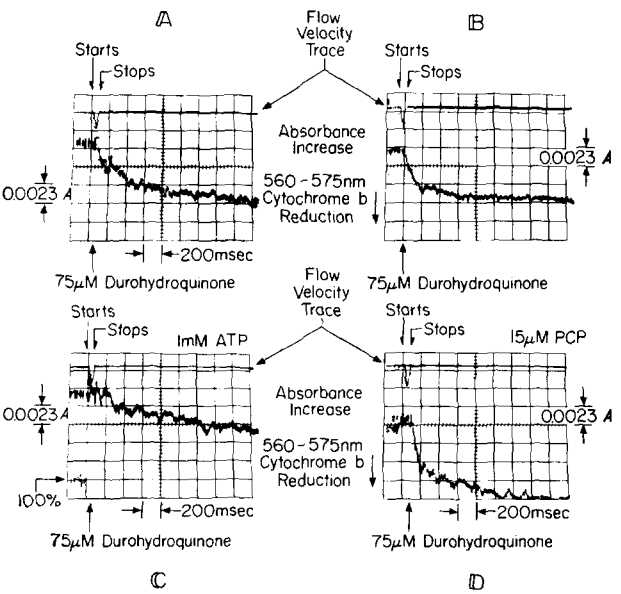


Fig. 14. Reduction of cytochromes *b* by H_2DQ in the presence of 1 mM antimycin A. (B–D). Pigeon heart mitochondria, 2.3 mg/ml, suspended in mannitol–sucrose–MOPS (pH 6.8). PCP = pentachlorophenol. Expt. 175.

Kinetic traces (Fig. 14C) obtained from the rapid flow measurements show this reaction to occur with a half-time of about 1 sec and at a rate of 0.5 μM cytochrome *b* reduced per sec. Total cytochrome *b* reduced shows a maximum at 562 nm, characteristic of reduction of both *b* cytochromes (Fig. 15-3). Addition of uncoupler to the antimycin A-blocked system unsupplemented with ATP results in no spectral change. If, however, ATP and antimycin A are present, uncoupler causes disappearance of the 565–557-nm peak and a concomitant appearance of the 560-nm peak (Figs. 13-4 and 15-4). This reflects oxidation of cytochrome b_T and reduction of cytochrome b_K, resulting in a situation analogous to the one observed in the presence of antimycin A only. Accordingly, addition of H_2DQ causes reduction of cytochrome b_T which, as shown in Fig. 14D, occurs with a half-time of about 70 msec at a rate of 1.6 μM cytochrome *b* reduced per sec. Titration of the antimycin A-supplemented pigeon heart mitochondria with H_2DQ was performed in the presence of ATP and in the presence of uncoupler (Fig. 16). In the presence of ATP, high-potential cytochrome b_T, which

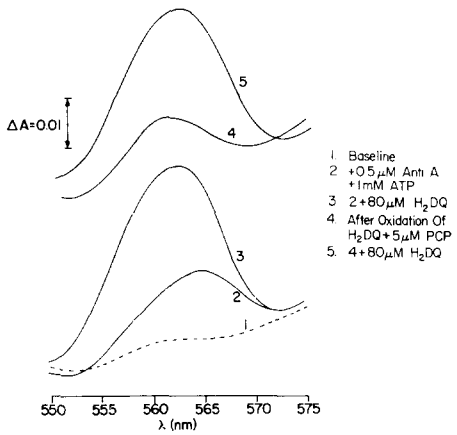


Fig. 15. Absolute spectra of the cytochromes *b* reduced by durohydroquinone in the presence of antimycin A. Pigeon heart mitochondria, 4.2 mg/ml, in mannitol–sucrose–MOPS (pH 6.8). PCP = pentachlorophenol. Expt. 4002.

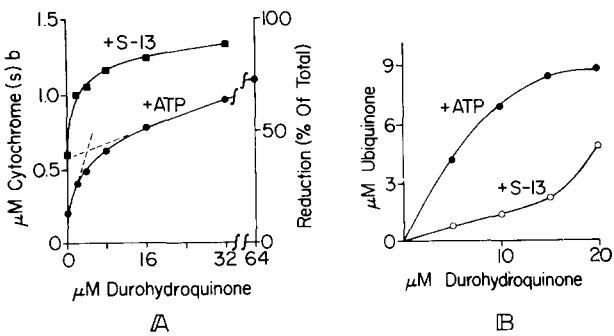


Fig. 16. Titration of cytochromes *b* and ubiquinone by H_2DQ in pigeon heart mitochondria in the presence of antimycin A. Cytochromes *b* titration (562–575 nm), 3 μM rotenone, 0.8 μM antimycin A, 1.2 mM ATP or 6 μM S-13. Pigeon heart mitochondria, 3.5 mg protein per ml. Expt. 4037. Ubiquinone titration (285–305 nm, 5 mm light path), 2 μM rotenone, 0.6 μM antimycin A, 0.5 mM ATP or 2 μM S-13. Pigeon heart mitochondria, 1.9 mg protein per ml. Expt. 4010.

is about 30 % reduced prior to H_2DQ addition is reduced at a ratio of 0.15 nmole cytochrome b_T per nmole H_2DQ and completes its reduction at 4 μM H_2DQ; ubiquinone reduced at a ratio of 0.82 nmole/nmole H_2DQ is fully reduced at 18 μM H_2DQ.

In the presence of an uncoupler, cytochrome b_K is fully reduced even before H_2DQ addition; cytochrome b_T, reduced at a ratio of 0.24 nmole/nmole H_2DQ completes its reduction at 3 μM H_2DQ whereas ubiquinone reduction lags behind (0.15 nmole/nmole H_2DQ) being still half oxidized at 20 μM H_2DQ. It must be noted that in the energized state of the mitochondrial membranes, ubiquinone can accept over 80 % of the total reducing equivalents supplied to the system, whereas in the energy-depleted membranes it only accepts about 15 % of these reducing equivalents.

The kinetic data obtained in the three types of preparation are summarized in Table I. Column I gives the type of cytochrome *b* reduced prior to H_2DQ addition (Column II) the type(s) of cytochrome *b* reduced by H_2DQ, Column III presents the initial rates of reduction (in μM/sec) and Column IV the amount of cytochromes *b* reduced under various conditions. The data of the first three columns have been discussed in the text. Results of Column IV indicate that cytochromes b_T and b_K are present in approx. 1:1 ratio in amounts of 0.18 nmole/mg protein.

DISCUSSION

The rates with which most of the known reductants react with the respiratory chain are at least an order of magnitude slower than the slowest reaction within the respiratory system. It has been observed that under conditions of oxygen-pulse experiment the reduction rates of the carriers recorded upon the exhaustion of oxygen are much slower than the oxidation rates. These different rates of oxidation and reduction could not be fitted into a simple straight-chain model of the respiratory chain system consisting of a sequence of irreversible first-order reactions[22]. Thus, availability of a fast reductant reacting with the respiratory chain carriers in the presence of oxygen and giving high electron flow seems to offer new opportunities in the study of the intercarrier reactions.

There are two main reasons that make H_2DQ an extremely useful tool in kinetic studies of the respiratory system. Firstly, it reacts with the chain rapidly enough to be used in fast kinetic measurements, secondly, it reacts specifically with cytochromes *b*. The specific interaction of H_2DQ with cytochromes *b* is substantiated by the following data: (i) reduction of cytochromes *b* in ubiquinone-depleted mitochondria, (ii) comparable rates of H_2DQ oxidation in ubiquinone depleted and in ubiquinone-containing mitochondria, (iii) the shift of the isosbestic point of H_2DQ to 285 nm (as it is observed in water and in contrast to alcohol solutions where it is located at 280 nm, Fig. 1) suggesting that H_2DQ is in the water phase and interacts with the surface proteins rather than with the hydrocarbon core of the membrane, (iv) the finding of RUZICKA AND CRANE[11] that the oxidized form, DQ, interacts with a compound located on the oxygen side of ubiquinone.

Any study on cytochromes *b* is complicated by the fact that cytochrome *b* is not a single substance but a mixture of at least two species, cytochromes b_K and b_T. The b_T and b_K components have spectral properties distinguishable enough to be seen in steady-state measurements[20,21] when either one can be reduced separately but overlap of the spectral is too strong to measure accurately the kinetics of a single

TABLE I

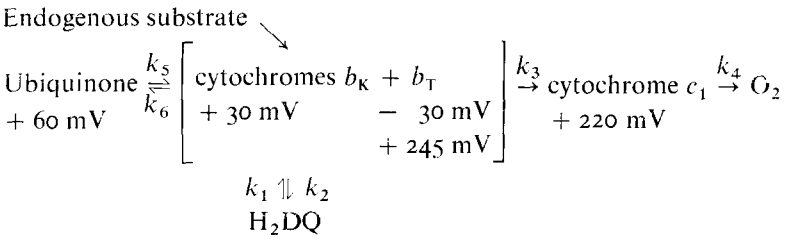
REDUCTION OF CYTOCHROMES *b* BY H_2DQ (70–90 μM) IN THE STOPPED FLOW APPARATUS

Preparation	*Cytochrome b reduced by endogenous substrate prior to H_2DQ pulse*	*Cytochrome b reduced by H_2DQ pulse*	*Initial rate (μM/sec)*	*Maximal reduction (nmoles/mg protein)*	*Expt. No.*
UQ-extracted washed pigeon heart mitochondria					
(<1.2 nmoles UQ per mg protein)	None	b_K?	11.6	0.060	3366 —
+ UQ_7 (6.0 nmoles UQ per mg protein)	None	b_K?	11.6 –0.06	0.045	3366 —
+ UQ_7 (21 nmoles UQ per mg protein)	None	b_K?	0.12	0.011	3366 —
Cytochrome c-extracted washed pigeon heart mitochondria					
No additions	None	100% (b_K and b_T)	1.1 –0.54	0.22–0.22	211–262
+ 1.2 mM ATP	b_T	b_K	0.25–0.25	0.12–0.11	211–262
+ 15 μM pentachlorophenol	None	b_K	1.0 – —	0.12– —	211– —
+ 1.0 μM cytochrome *c*	None	80% (b_K and b_T)	— –1.20	— –0.19	— –262
+ cytochrome *c* + ATP	15% b_T	70% (b_K and b_T)	— –8.3	— –0.17	— –262
Intact washed pigeon heart mitochondria					
No additions	None	80% (b_K and b_T)	1.9 –2.9	0.31–0.29	211–205
+ 1.2 mM ATP	20% b_T	70% (b_K and b_T)	18.0 –29.0	0.24–0.20	211–205
+ 15 μM pentachlorophenol	None	b_K?	1.2 –2.3	0.16–0.16	211–205
+ antimycin A (1 μM)	b_K	b_T	1.2 –0.9	0.12–0.26	175–213
+ antimycin A + ATP	b_T	b_K	0.5 –0.2	0.18–0.22	175–213
+ antimycin A + pentachlorophenol	b_K	b_T	1.6 –1.7	0.18–0.22	175–213

species when both cytochromes are being oxidized or reduced at nearly the same rates. Under conditions of oxygen-pulse advantage has been taken of the fact that in coupled mitochondria cytochrome b_T is oxidized at a slower rate than cytochrome b_K, thus a certain degree of kinetic resolution could be achieved[2,3]. The availability of specific reductant of cytochromes *b* offered another way to get a deeper insight into the properties of the different cytochromes *b*.

We will focus our attention first on the cytochrome *c* depleted mitochondria in which some of the kinetic phenomena become clearer. This seems to be further justified by the fact that cytochrome *c* depletion does not affect the energy coupling phenomena *per se* as evidenced by the finding that cytochrome b_T is able to respond with a shift of its midpoint potential upon the addition of ATP[1,2]. Furthermore, in contrast to antimycin A, cytochrome *c* depletion does not affect the very immediate carrier of the cytochromes *b* but leaves cytochrome c_1 to react freely in the redox reactions thus affording a good model system for the kinetic studies.

The reduction rate of cytochrome *b* observed upon addition of H_2DQ is at least the result of three reactions presented graphically in the following scheme: (i) rate of reaction of H_2DQ with cytochromes *b*; (ii) rate of equilibration of cytochromes *b* with ubiquinone; (iii) rate of equilibration of cytochromes *b* with cytochrome c_1.



According to this scheme a pulse of H_2DQ reduces cytochromes *b* in a transient state during which they equilibrate with ubiquinone and are oxidized by cytochrome c_1. This sequence of events can be expressed equally well in the form of chemical equations (both cytochromes *b* are treated as one substance):

$$H_2DQ + b^{3+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} b^{2+} + DQ \tag{1}$$

$$b^{2+} + c_1^{3+} \xrightarrow{k_3} b^{3+} + c_1^{2+} \tag{2}$$

$$c_1^{2+} + O_2 \xrightarrow{k_4} c_1^{3+} + H_2O \tag{3}$$

$$b^{2+} + UQ \underset{k_6}{\overset{k_5}{\rightleftharpoons}} b^{3+} + UQH_2 \tag{4}$$

These chemical equations yield the following differential equations;

$$\frac{d[H_2DQ]}{dt} = -k_1[H_2DQ][b^{3+}] + k_2[DQ][b^{2+}] \tag{5}$$

$$\frac{d[b^{2+}]}{dt} = k_1[H_2DQ][b^{3+}] - k_2[DQ][b^{2+}] - k_3[b^{2+}][c_1^{3+}] - k_5[b^{2+}][UQ] + k_6[UQH_2][b^{3+}] \quad (6)$$

In the steady state or at the point of maximal reduction, $d[b^{2+}]/dt$ is set to zero and Eqn. 6 after rearranging becomes:

$$\frac{[b^{2+}]}{[b^{3+}]} = \frac{k_1[H_2DQ] + k_6[UQH_2]}{k_2[DQ] + k_3[c_1^{3+}] + k_5[UQ]} \quad (7)$$

which establishes that the percentage of cytochromes *b* reduction in the steady state or the maximal reduction in the fast transients is: (i) directly proportional to $[H_2DQ]$; (ii) inversely proportional to the rate of electron transfer between cytochromes *b* and cytochrome c_1; and (iii) inversely proportional to the rate of electron equilibration between cytochromes *b* and ubiquinone.

Eqn. 6 can be simplified by setting [DQ] to zero and neglecting both reverse reactions between cytochromes *b* and DQ or ubiquinone. For conditions in which equilibration of cytochromes *b* with ubiquinone is allowed, the observed values of $d[b^{2+}]/dt$ are of the order of 0.2–1.0 μM e^- per sec per mg protein with half-reduction times of the order of 100–200 msec (data from Table I in the absence of ATP). These rates are approximately one order of magnitude smaller than those calculated for the forward rate of Reaction 1, 16 μM e^- per min per mg protein (calculated from dDQ/dt in the uncoupled state where b^{2+} is nearly zero). This consideration may imply that the equilibration between cytochrome *b* and ubiquinone (Reaction 4) is comparable to or faster than the reduction of cytochrome(s) *b* by H_2DQ (Reaction 1). In fact, in the cases in which ubiquinone is not reduced, as in the ubiquinone depleted mitochondria, the rate of $d[b^{2+}]/dt$ (13 μM e^- per min per mg protein; half time of the order of 10 msec) can account for 80 % of the reducing equivalents coming from H_2DQ (Reaction 1). After addition of ATP to normal mitochondria, the very fast rates of db^{2+}/dt, 7.5–12 μM e^- per sec per mg protein with half-reduction times of about 10–20 msec, suggest that cytochrome *b* is preferred as electron acceptor to ubiquinone under such conditions, accepting 47–75 % of the supplied electrons.

Independent kinetic measurement of the rate of cytochrome *b* reduction by ubiquinone (M. ERECIŃSKA, unpublished data) indicate these reactions as extremely fast with half-reduction times of about 7 msec.

On the basis of these considerations, interpretation of the slowing down of the initial rate of cytochrome b_K reduction by H_2DQ in the presence of ATP (as compared to the rate recorded in the presence of an uncoupler) seems to indicate that ATP affects the cytochromes *b*–ubiquinone reaction directly by enhancing the rate of electron transfer towards ubiquinone reduction. In this connexion, CHANCE[23] has previously reported that the reduction of ubiquinone from a kinetic standpoint depends entirely upon the energy state of the mitochondria with higher reduction in the energized state.

As a minimal hypothesis, it is possible to assume that the overall electron transfer through a phosphorylation site is determined by only one rate-limiting step, most probably that one at which energy conservation occurs. Clear evidence coming from the fast kinetic studies points it out to be the electron transfer between cyto-

chromes b_T and c_1; (i) faster rate of c_1 reduction in the uncoupled state; (ii) faster rate of cytochrome b_T reduction in the presence of ATP; (iii) as additional evidence we can consider high reduction of b_T and low reduction of c_1 in the coupled state as compared with the uncoupled state, as shown by the titration experiments; and (iv) undetectable reverse rate in the b–c_1 reaction in the uncoupled state, as demonstrated by the oxygen-pulse technique[24]. As a consequence, it seems reasonable to postulate that the slow energy conserving reaction occurs after the electron transfer reaction between reduced cytochrome b_T and oxidized cytochrome c_1.

However, our experimental observations indicate that the high-potential oxidized form of cytochrome b_T is readily reducible by H_2DQ. This might imply that either the energy transfer occur independently of the redox state of cytochrome b_T or that the reduction occurs prior to energy transfer. The latter statement is at variance with recently proposed schemes for the mechanism of oxidative phosphorylation at Site II (refs. 1 and 25).

The rate-limiting reaction determines a tendency of the components located on the substrate side of the reaction to approach equilibrium, supported in these experiments by the fact that in the presence of ATP the most reduced component is cytochrome b_T, with the highest potential (+245 mV), followed by ubiquinone (+77 mV at pH 6.8)[26] whereas cytochrome b_K (+30 mV) reduction lags behind.

Abolition of the energy-transducing reaction in the uncoupled state results in irreversibility and non-equilibrium situation, conditions under which the thermodynamic potentials cannot describe the degree of reduction of the individual components.

The ready reducibility of cytochrome b_T in the aerobic, uncoupler-supplemented pigeon heart mitochondria in the presence of antimycin A seemed at first difficult to reconcile with the fact that antimycin prevented the ATP-induced midpoint potential shift of cytochrome b_T (ref. 27). It has been, however, reported recently that electron transfer across the antimycin A-sensitive site is able to raise the midpoint potential of cytochrome b_T in an uncoupler insensitive manner thus resulting in its reducibility by substrate[27,28]. This finding explains our experimental observations.

An apparent discrepancy arises, however, in the case of ATP supplemented mitochondria in which we observed oxidation of cytochrome b_K with concomitant reduction of cytochrome b_T. Two explanations are possible: (1) that in the aerobic system ATP does raise cytochrome b_T midpoint potential from −30 to +245 mV even in the presence of antimycin A; (2) that ATP activates oxidation of endogenous substrates and that the activated electron flow is responsible for the potential change of cytochrome b_T. Our experimental data do not allow us to distinguish between both possibilities.

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