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STUDIES ON THE ACCESSIBILITY BARRIER OF NADH TO CYTOCHROMES *b* IN PIGEON-HEART MITOCHONDRIA

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SUMMARY

1. Addition of NADH to pigeon-heart mitochondria that have been made permeable to this substrate causes complete reduction of cytochromes c_1, *c* and aa_3 upon anaerobiosis but only a small reduction of cytochrome *b*. The cytochrome *b* becomes completely reduced on addition of phenazine ethosulphate (PES), and partially on addition of ATP or OH^-. When NADH is replaced by malate *plus* glutamate or succinate *plus* glutamate, a rapid reduction of cytochrome *b* occurs upon anaerobiosis. The P:O ratios measured in the presence of malate, succinate and NADH were 3.0–3.3, 1.8–2.0 and 0.3–0.5, respectively. This suggests that the reducibility of cytochrome *b* is correlated directly with the coupling of respiration with phosphorylation, rather than with the structural integrity of the mitochondrial membrane.

2. Under anaerobic conditions the extent of reduction of cytochrome *b* is less in malate-reduced mitochondria than in succinate-reduced. This indicates that an accessibility barrier (structural or kinetic) prevents NADH dehydrogenase and cytochrome *b* coming into redox equilibrium.

3. Studies with ubiquinone- and cytochrome *c*-depleted mitochondria demonstrate that PES can reduce or oxidize cytochrome *b* by a direct interaction with the respiratory chain at or near the level of cytochrome *b*. This suggests that PES can bypass the accessibility barrier by establishing a shunt between NADH and cytochrome *b*.

4. The rate of interaction between PES and cytochrome *b* decreases with an increase of pH, so that, at high pH, PES interacts with the respiratory chain preferentially at the level of cytochromes $c_1 + c$.

5. At the optimal pH (8.5) for the energy-driven reversal of electron transport, addition of ATP to anaerobic, NADH-reduced mitochondria results in oxidation of cytochrome *c* and reduction of cytochrome *b*. In the presence of PES, on the other hand, ATP causes an appearance of a crossover point between NAD and cytochrome *b*. It is concluded that PES exerts two effects on the accessibility barrier:

Abbreviations: PES, phenazine ethosulphate; PMS, phenazine methosulphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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(1) it provides a shunt around the barrier, and (2) it exerts an effect on the barrier directly, by reducing a component located between NADH dehydrogenase and cytochrome *b* whose redox level is directly related to removal of the barrier.

INTRODUCTION

In a previous communication[1] we have reported that, in phosphorylating submitochondrial particles, there exists a certain accessibility barrier that interferes with the establishment of redox equilibrium between NADH and the low-potential cytochromes *b*-558 and *b*-566, and that the barrier can be partially overcome by the addition of ATP or OH^-, and completely by a redox mediator, phenazine methosulphate. It is of interest to know whether this barrier is a consequence of the structural re-organization of the membrane during the formation of submitochondrial vesicles, or it represents a general property of the mitochondrial inner membrane.

In the present paper, further studies on the nature of the barrier in the intact membrane of pigeon-heart mitochondria are described. Since these mitochondria have very little endogenous substrate and, like mammalian mitochondria, are devoid of an exogenous NADH oxidase system[2], it is possible to study the effect of NADH on cytochrome *b* under conditions in which mitochondria are made permeable to NADH. A preliminary report of the present studies is published elsewhere[3].

METHODS

Pigeon-heart mitochondria were prepared according to the method of Chance and Hagihara[4] modified in order to render the mitochondria permeable to exogenous NADH without loss of phosphorylating capacity. Minced and washed heart tissue was homogenized for 90 s at 0 °C in a medium containing 225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 10 mM unneutralized Tris and crystalline bacterial proteinase (Nagase and Co. Ltd, Japan; 0.5 mg or 750 P.U.N./g tissue). The final pH of the medium was 8.5. The homogenate was immediately diluted with an equal volume of an ice-cold medium containing 225 mM mannitol, 75 mM sucrose and 1 mM Tris–EDTA (pH 6.0), and the mitochondria were sedimented and washed twice by repeated centrifugation as described[4]. The total period of exposure to the proteinase (*i.e.* from homogenization to the second centrifugation step) was strictly kept to less than 20 min.

Extraction of cytochrome *c* and ubiquinone from pigeon-heart mitochondria was carried out essentially as described by Jacobs and Sanadi[5] and Szarkowska[6], respectively.

The reaction mixture used for the determination of the P:O ratio contained 250 mM sucrose, 50 mM Tris–HCl buffer (pH 7.2), 1.25 mM ADP, 1.25 mM $KH_2{}^{32}PO_4$, 2.5 mM $MgCl_2$, 50 mM glucose, 50 ng crystalline hexokinase (Boehringer und Söhne), mitochondria and substrate (4.4 mM malate *plus* 2.2 mM glutamate, 4.4 mM succinate *plus* 5 μM rotenone, or 1.3 mM NADH). The organic phosphate formed during the aerobic oxidation of substrate was separated according to the method of Nielsen

and Lehninger[7] and the specific activity of the phosphate was measured with a Unilux II (Nuclear Chicago) or Isocap/300 Liquid Scintillation Counter (Nuclear Chicago).

RESULTS

Fig. 1 shows redox changes of cytochrome *b* in mitochondria in the presence of succinate, malate and NADH. In succinate- or malate-reduced mitochondria cytochrome *b* is largely reduced in the aerobic steady state and is readily reduced further upon anaerobiosis. On the other hand, in NADH-reduced mitochondria cytochrome *b* is highly oxidized in the aerobic steady state and is sluggishly reduced upon anaerobiosis. Moreover, in succinate- and malate-reduced mitochondria, cytochrome *b* undergoes rapid redox changes in response to ADP-induced State 4 to 3 transitions as well as to de-energization caused by uncoupler, but it does not show any response in NADH-reduced mitochondria. The P:O ratios measured in the presence of malate, succinate and NADH were 3.0–3.3, 1.8–2.0 and 0.3–0.5, respectively.

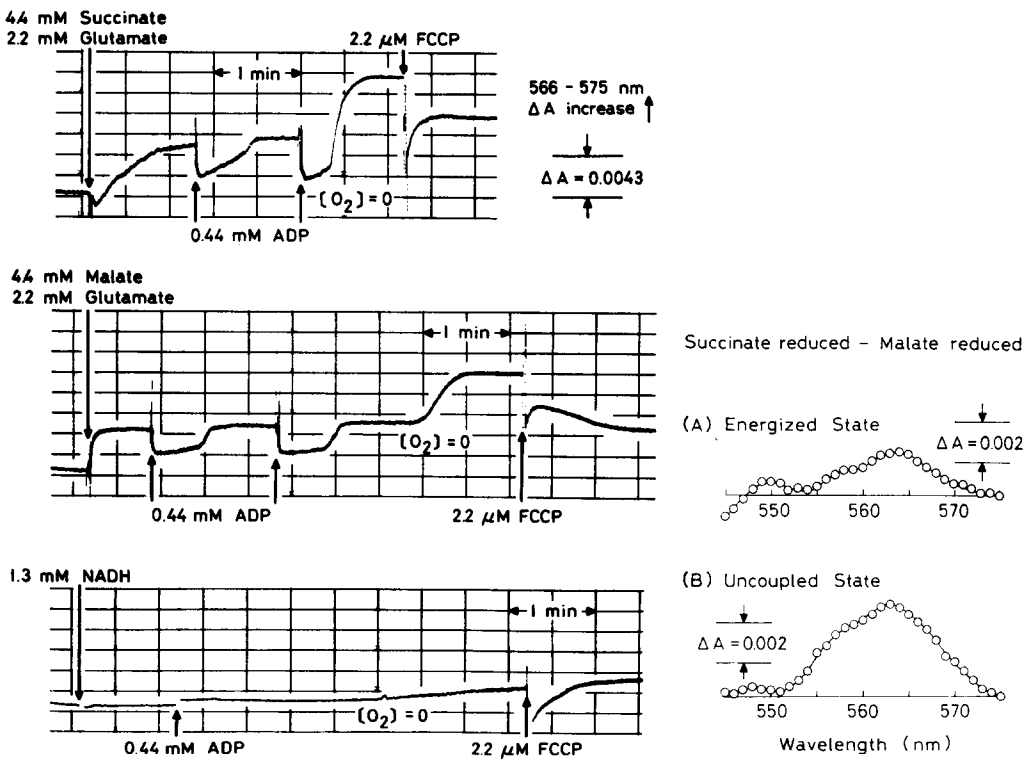


Fig. 1. (Left) Comparison of reduction of cytochrome *b* in the presence of succinate (upper), malate (middle) and NADH (lower). Glutamate was added where shown in order to avoid inhibition by oxaloacetate. The mitochondria were suspended in 225 mM mannitol, 75 mM sucrose, 50 mM Tris–acetate buffer (pH 7.0) at a final concentration of 1.14 mg protein/ml. (Right) Difference spectra of succinate-reduced *minus* malate-reduced mitochondria in the energized (upper) and uncoupled state (lower).

It is noteworthy that the total absorbance change of cytochrome *b* in succinate-reduced mitochondria is greater than in malate-reduced (see upper and middle traces). The difference spectra of succinate-reduced *minus* malate-reduced mitochondria shows a maximum at 564 nm with a shoulder at 558 nm in the energized state (Fig. 1, upper right, A) and at 563 nm with a shoulder at 558 nm in the uncoupled state (Fig. 1, lower right, B). This indicates that the extent of reduction of cytochromes *b* absorbing at 558, 562 and 566 nm is greater in succinate-reduced mitochondria than in malate-reduced, in spite of the fact that E_0' at pH 7.0 of the malate/oxaloacetate redox couple (−170 mV) is much lower than that of succinate/fumarate (+ 30 mV). Similar results were found with mitochondria from different sources (rat heart, kidney and liver, and bovine heart). Thus it is evident that the accessibility barrier between NADH dehydrogenase and cytochrome *b* observed in submitochondrial particles[1] is not the result of structural changes in the mitochondrial membrane.

Fig. 2 shows the kinetics of reduction of cytochromes aa_3, *c* and *b* in mitochondria after addition of NADH. All three cytochromes are highly oxidized in the aerobic steady state, but upon anaerobiosis both cytochromes aa_3 and *c* are readily reduced to completion. Under the identical conditions only about 13 % of the total cytochrome *b* is reduced. Addition of phenazine ethosulphate (PES) [or phenazine methosulphate (PMS), not shown] after anaerobiosis does not cause any appreciable

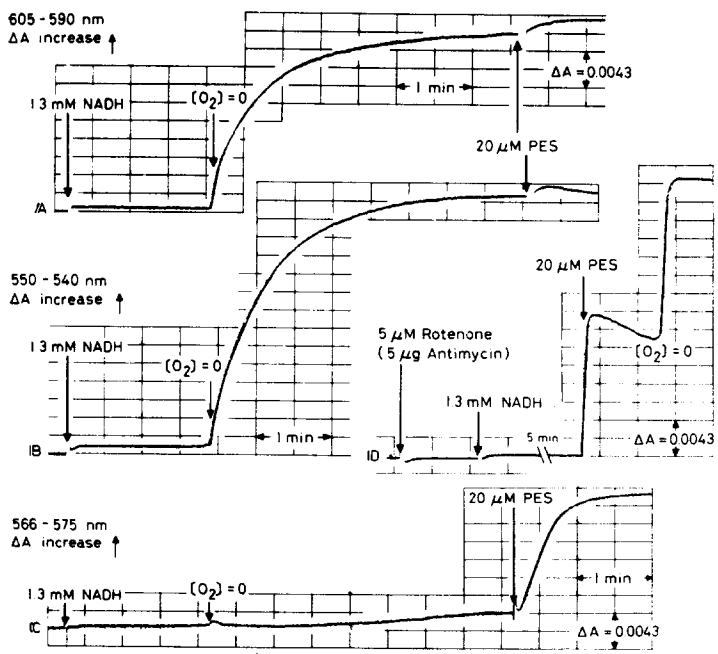


Fig. 2. Comparison of kinetics of reduction of cytochromes aa_3 (trace A), *c* (trace B) and *b* (trace C) in pigeon-heart mitochondria after addition of NADH. Trace D illustrates the inhibition of reduction of cytochrome *c* by rotenone (or antimycin) and the reversal of inhibition by PES. Experimental conditions are identical to those described in Fig. 1. Protein concentration, 1.30 mg/ml.

further reduction of cytochromes aa_3 and *c*, but causes a rapid reduction of cytochrome *b*.

Pretreatment of mitochondria with rotenone or antimycin results in inhibition of oxidation of NADH and, as shown in trace D, cytochrome *c* is not reduced, even after 5 min, unless electron transfer from NADH is shunted over the inhibition site by PES. This indicates that the reduction of cytochrome *c* by NADH is not associated with the rotenone- and antimycin-insensitive NADH–cytochrome *c* reductase[8].

In order to characterize the PES-induced reduction of cytochrome *b* observed in Fig. 2, the kinetics of reduction of cytochrome *b* in ubiquinone- and cytochrome *c*-depleted pigeon-heart mitochondria were measured in the presence of PES. As illustrated in Fig. 3, trace A, cytochrome *b* in the ubiquinone- and cytochrome *c*-depleted mitochondria is not reduced by NADH even after addition of antimycin. However, addition of PES causes a rapid reduction of cytochromes aa_3 (not shown) and upon anaerobiosis cytochrome *b* is reduced to completion over a period of approximately 5 min. The difference spectra of cytochrome *b* taken at various time intervals (Fig. 4) show that the biphasic reduction kinetics of cytochrome *b* seen

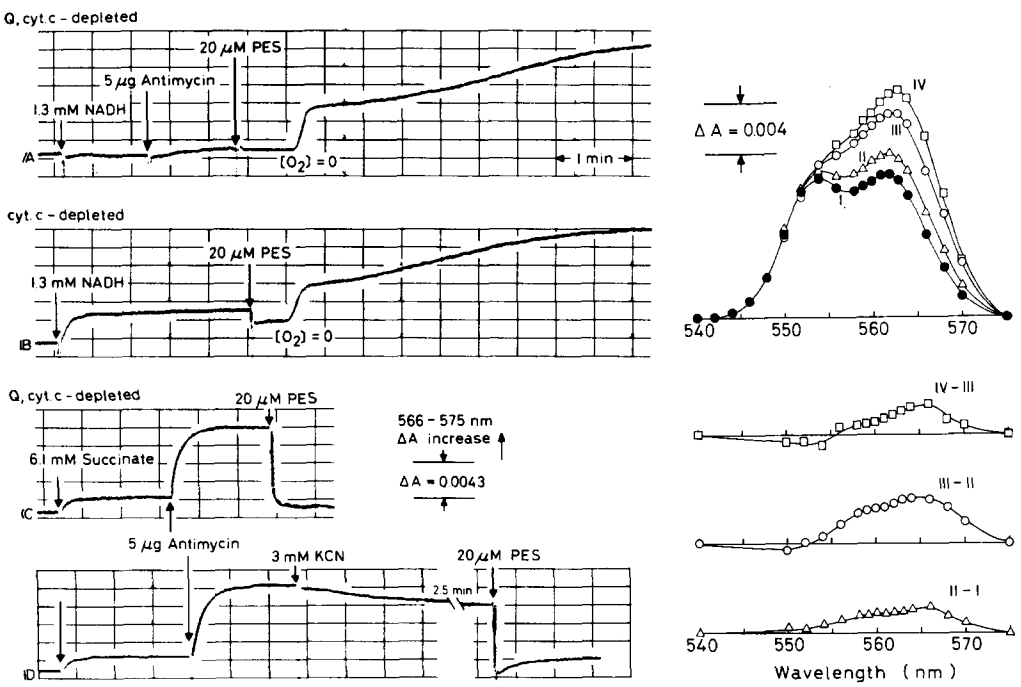
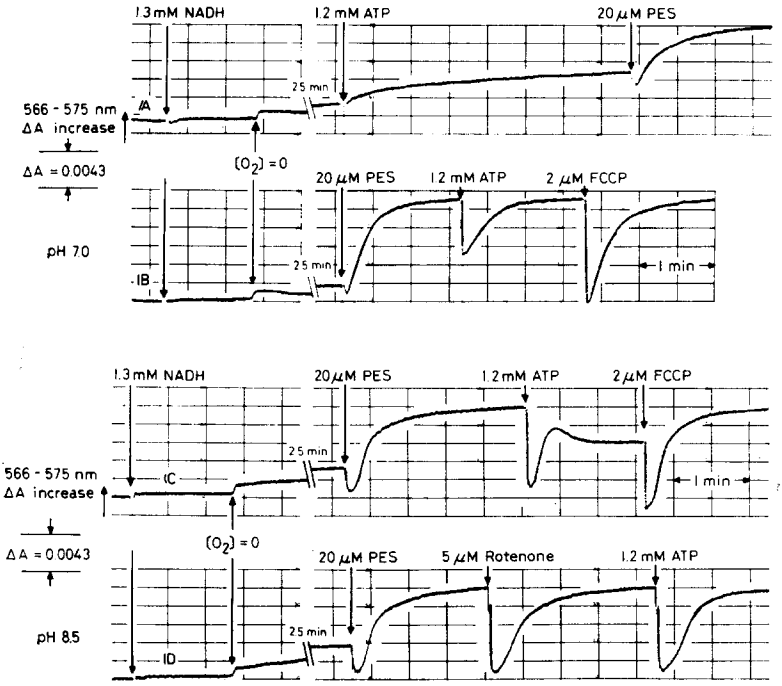
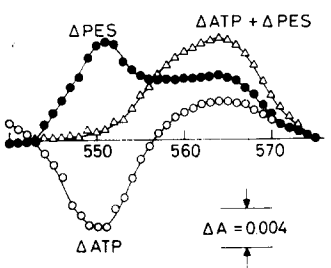


Fig. 3. Illustration of the direct interaction between PES and cytochromes *b* in ubiquinone- and cytochrome *c*-depleted pigeon-heart mitochondria. Traces A and B show the PES-induced reduction of cytochromes *b* in mitochondria depleted of both cytochrome *c* and Q (trace A) and of cytochrome *c* only (trace B). Traces C and D show the PES-induced oxidation of cytochromes *b* in mitochondria depleted of both cytochrome *c* and Q. In trace C cytochrome c_1 was oxidized before addition of PES; in trace D it was reduced. Experimental conditions are similar to those described in Fig. 1. Traces A, C and D, 1.33 mg protein/ml, and trace B, 1.52 mg protein/ml.

Fig. 4. Difference spectra of cytochromes *b* measured at various time intervals after anaerobiosis in the experiment of Fig. 3, trace A. Spectrum I, 1 min; II, 2 min; III, 4 min; and IV, 7 min after anaerobiosis.



pH 7.0



pH 8.5

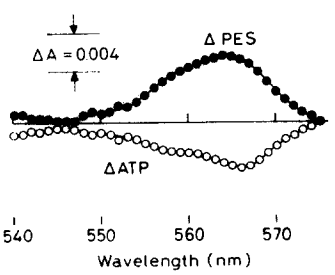


Fig. 5. (Above) Effect of pH on the reduction of cytochrome *b* by NADH after additions of ATP and PES. The abrupt absorbance decrease after each addition is due to transient oxidation of cytochromes *b* by oxygen introduced during the stirring. (Below) Difference spectra of after *minus* before addition of ATP and PES obtained in the experiment of trace A (upper) and of trace C (lower). Experimental conditions are similar to those described in Fig. 1. Protein concentration, 0.89 mg/ml.

in Fig. 3 are associated with an initial rapid reduction of cytochrome *b*-562 followed by slow reduction of cytochromes *b*-558 and *b*-566.

The kinetics of reduction of the cytochromes *b* in trace A of Fig. 3 are very similar to those of cytochrome *c*-depleted but ubiquinone-containing mitochondria shown in trace B, indicating that the PES-induced reduction of cytochromes *b* can occur without mediation of ubiquinone.

Unlike NADH, succinate is able to reduce the cytochrome *b* in pigeon-heart mitochondria even in the absence of ubiquinone when antimycin is added (trace C), just as previously shown in ubiquinone-depleted beef-heart mitochondria[9]. Addition of PES after antimycin causes a rapid oxidation of cytochrome *b*. The difference spectrum of cytochrome *b* oxidized (not given) shows a broad peak at 564 nm. The PES-induced oxidation of the cytochromes *b* can also be demonstrated when cytochrome c_1 is slowly but completely reduced by the antimycin leak[10] in the presence of KCN before the addition of PES (trace D). The difference spectrum of after *minus* before addition of PES (not given) shows that cytochrome c_1 is not oxidized by PES in the experiment of trace D. Thus, the oxidation of cytochrome *b* by PES can occur without mediation of cytochrome c_1. These results strongly suggest that the interaction between PES and the respiratory chain can occur near or at the level of cytochrome *b* and that the accessibility barrier observed in Figs 1 and 2 can be bypassed by PES.

As shown in Fig. 5, trace A, addition of ATP to anaerobic, NADH-reduced mitochondria causes partial reduction of cytochrome *b*, which becomes completely reduced by subsequent addition of PES. The difference spectra (Fig. 5, below, pH 7.0) show that ATP causes oxidation of cytochrome *c* and reduction of cytochrome *b*, whereas PES brings about re-reduction of cytochrome *c* and further reduction of cytochrome *b*. The sum of the two spectra shows the net reduction of all three cytochromes *b*-558, *b*-562 and *b*-566, with a maximum at 564 nm and shoulder at 558 nm. This experiment clearly distinguishes between the roles of ATP and PES in the reduction of cytochrome *b*: ATP induces the reduction of cytochrome *b* by *c* by an energy-driven reversal of electron transport[11], whereas PES restores the normal equilibrium between cytochrome *b* and *c* by interacting with both cytochromes and at the same time overcoming the accessibility barrier between NADH and cytochrome *b*. The same effect of ATP is obtained at pH 8.5.

When ATP is added after PES, it has hardly any effect on cytochrome *b* at pH 7.0 (trace B). However, when the experiment is carried out at pH 8.5 (optimal pH for the energy-driven reversal of electron transport over the first energy-coupling site, see Fig. 2 of ref. 1), ATP causes oxidation of cytochrome *b* (trace C). The ATP-induced oxidation of cytochrome *b* is completely inhibited by rotenone (trace D). The spectrum of the ATP effect (Fig. 5, below, pH 8.5) shows oxidation of cytochromes *b*-558 and *b*-566 but no changes in cytochromes c_1 and *c*. Whether cytochrome *b*-562 is also oxidized under these conditions is difficult to say, since the spectrum is dominated by cytochromes *b*-558 and *b*-566. However, the oxidation of cytochrome *b*-562 can be demonstrated under the same conditions if the electron flow from the third to second energy-coupling site is inhibited by antimycin.

Comparison of traces A and B with traces C and D shows that the extent of reduction of cytochrome *b* before the addition of PES is nearly doubled when the pH of the reaction mixture is increased from 7.0 to 8.5. This result is very

similar to that observed in NADH-reduced submitochondrial particles[1], and shows the increased accessibility of NADH to cytochrome *b* at high OH^-.

As illustrated in Fig. 6, additions of redox mediators with a very low E'_0 such as 2-hydroxy-1,4-naphthoquinone (−145 mV) or pyocyanine (−34 mV) do not affect the ATP-induced oxidation of cytochromes *b*. Close examination of redox changes in cytochromes *b* and *c* after addition of these two mediators indicate that, under the condition of this experiment, neither mediator interacts with cytochrome *b* to any appreciable extent.

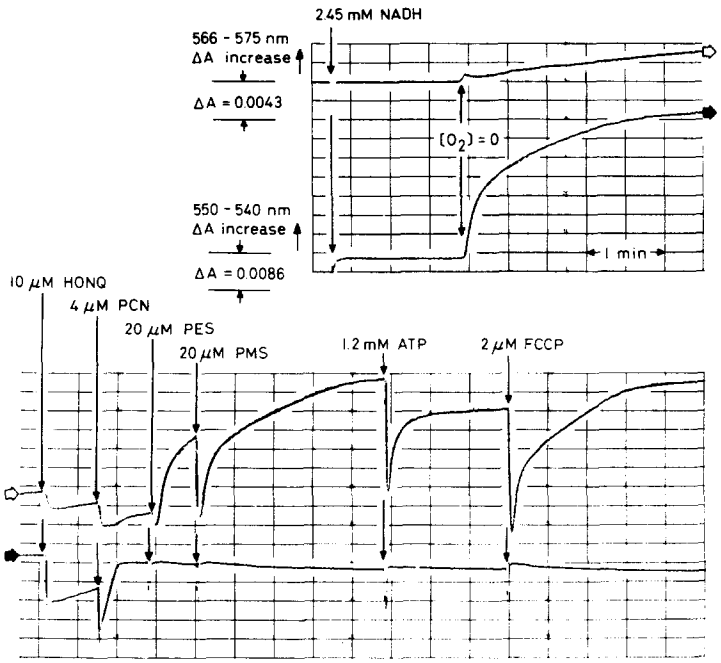


Fig. 6. Comparison of redox changes of cytochromes *b* and *c* after additions of NADH, redox mediators, ATP and FCCP. The concentrations of redox mediators (HONQ, 2-hydroxy-1,4-naphthoquinone and PCN, pyocyanine) was the same as those used in Fig. 5 of ref. 18. Experimental conditions are similar to those described in Fig. 1, except that the pH of the reaction mixture was 8.5. Protein concentration, 1.31 mg/ml.

DISCUSSION

Slater[12] showed that, although succinate and NADH are oxidized at about the same rate by Keilin and Hartree heart-muscle particles, cytochrome *b* is reduced much more slowly by NADH than succinate. The discrepancy between the rate of reduction of cytochrome *b* and oxygen by NADH led him to conclude that this cytochrome is not on the main pathway of oxidation of NADH by these non-phosphorylating particles. Chance[13] and Storey and Chance[14] observed that disruption of the mitochondria, leading to the loss of energy-conservation reactions, also results in the kinetics of oxidoreduction of the cytochrome becoming slower than those of flavoprotein and ubiquinone. They concluded that cytochrome *b* is

dislocated in the non-phosphorylating membrane in such a way that it loses its function in electron transport.

The correlation between oxidative phosphorylation and reducibility of cytochrome *b* is also found in the pigeon-heart mitochondria used in this study, that are still intact as judged by the ADP-controlled respiration and the ATP-driven reversal of electron transport. Succinate and malate, which give P:O ratios of 1.8–2.0 and 3.0–3.3, respectively, reduce cytochrome *b* both in State 4 and after anaerobiosis. On the other hand, NADH, which is oxidized without respiratory control[15] and with low P:O ratios (less than 0.5), scarcely reduces cytochrome *b*, unless ATP is added. Thus, the reducibility of cytochrome *b* appears to be correlated directly with the coupling of respiration with phosphorylation, rather than with the structural integrity of the mitochondrial membrane.

The simplest explanation of the different behaviour of NADH and malate in pigeon-heart mitochondria is that the NADH dehydrogenase molecules reduced by added NADH are different from those reduced by NADH bound to malate dehydrogenase. It is possible, for example, that NADH reacts with NADH dehydrogenase molecules near the outside of the inner membrane (*cf.* ref. 15) and that malate dehydrogenase is closer to NADH dehydrogenase near the inside of the inner membrane where cytochrome *b*-562 might also be located. This explanation is consistent with the rapid reduction by NADH of cytochrome *b*-562 in submitochondrial particles, which are 'inside-out'[16].

Since the E'_0 of *b*-558 and *b*-566 in pigeon-heart mitochondria is lower than that of *b*-562[17,18], the delay in the reduction of the former species, shown in Fig. 4, is understandable. If the potential difference is 60 mV, as reported for these mitochondria[17,18], *b*-558 and *b*-566 would be 47 % reduced when *b*-562 was 90 % reduced, provided equilibrium were reached. In the experiment shown in trace A of Fig. 3, however, *b*-566 was only 13 % reduced when *b*-562 was virtually 100 % reduced. Either the difference in redox potential is considerably greater than 60 mV (in rat-liver mitochondria, the difference is 100 mV[19]) or equilibrium between the two cytochromes is not reached. Further experiments (unpublished) showing that addition of antimycin to aerobic pigeon-heart mitochondria in the presence of NADH causes reduction mainly of *b*-562, whereas *b*-558 and *b*-566 are also reduced in the presence of the higher-potential substrate succinate (in the presence of glutamate), support the idea that equilibrium between the *b* cytochromes is not reached. Owing to the lack of accessibility of low-potential *b* cytochromes to electrons derived from NADH, the actual potential of these cytochromes must be much higher than that of the NADH/NAD^+ system, even in the presence of antimycin.

At pH 7, ATP has no effect on the redox state of cytochrome *b* in the presence of PES, indicating that at this pH the PES can react with the cytochrome *b* at least as rapidly as the ATP-driven reversal of electron transport over the first energy-coupling site. At pH 8.5, however, the rate of the ATP-driven oxidation of cytochrome *b* is greater than that of reduction by PES, so that at this pH cytochrome *b* does not equilibrate with PES. Moreover, the observation that addition of mediators with a low E'_0 does not effect the ATP-induced oxidation of cytochrome *b* at pH 8.5 clearly demonstrates that the rate of interaction between cytochrome *b* and these mediators is very slow at high pH. Thus, the overall system behaves as if the mediators interact preferentially with the respiratory chain at the level

of cytochromes $c_1 + c$. This emphasizes the need for caution in interpreting potentiometric data especially those obtained at alkaline pH (*cf.* ref. 18). In addition, these observations suggest the possibility that if the energy-dependent high-potential cytochrome *b*[17–20] is only apparent[20–22], an apparently energy-dependent low-potential cytochrome *b* would be detected potentiometrically in the presence of a mediator that reacts rapidly and preferentially with an electron carrier before the first energy-coupling site.

The effect of PES added after ATP at pH 7.0 shown in Fig. 5 is of special interest, since it mimics the inhibition by uncoupler of the energy-driven reversal of electron transport at the second (*i.e.* re-reduction of cytochrome *c*) and the first energy-coupling site (*i.e.* re-reduction of cytochrome *b*). Thus the observation is consistent with the suggestion that ATP would be rapidly hydrolyzed if the mediator reacts with both reductant and oxidant of the reaction that is linked with the synthesis of ATP[22].

The ATP-induced oxidation of cytochromes *b*-558 and *b*-566 in the presence of PES at alkaline pH requires some comment. In anaerobic heart mitochondria[23] and submitochondrial particles[24], cytochrome *b* becomes more reduced upon addition of ATP, and the crossover point appears between cytochromes *b* and *c*. Indeed, this is also found to be the case with anaerobic, NADH-reduced pigeon-heart mitochondria in the absence of PES (see Fig. 5, trace A).

This ATP-induced reduction of cytochrome *b* has evoked various speculations ranging from the activation of succinate dehydrogenase by ATP[25] to the structural dissociation of cytochrome *b* from the respiratory chain[26]. Recently, it was interpreted in terms of the ATP-induced increase of potential of cytochrome *b*[17–20,27]. This interpretation easily explains the energy-dependent reversal of electron transport from the third to the second energy-coupling site, but has difficulty in explaining that from the second to the first energy-coupling site, which requires cytochrome *b* to be in equilibrium with a low potential.

The appearance of a crossover point between cytochromes *b*-558 and *b*-566 on the one hand, and NAD, on the other, in the presence of PES (see Fig. 5, trace C) indicates that once the cytochromes *b* are brought into an equilibrium with NADH by PES, they can be readily oxidized by the energy-driven reversal of electron transport at the first energy-coupling site. Thus, it is conceivable that, in the absence of equilibrium between NADH and cytochrome *b*, the two cytochromes show a tendency to be reduced and the crossover point will appear between cytochromes *b* and *c*. It should be pointed out that although a by-pass *via* PES around the accessibility barrier is sufficient to explain the effect of PES in the forward electron transfer shown in Fig. 2, the effect of PES on the reversed electron transfer shown in Fig. 5 (trace C) cannot be explained by the same shunt mechanism since the ATP-induced oxidation of cytochrome *b* in the presence of PES is completely sensitive to rotenone, whereas the PES-induced reduction of cytochrome *b* is insensitive. Thus, PES must also exert an effect on the barrier itself, presumably by reducing a component located between NADH dehydrogenase and cytochrome *b* whose redox state is directly related to removal of the barrier. Evidence for such a possibility is provided by the observation that, even in the presence of PES and at the optimal pH for the reversed electron transfer, ATP causes reduction of cytochromes *b*-558 and *b*-566 when mitochondria are reduced with succinate. The simplest

explanation would be that the potential of the succinate/fumarate couple is too high to reduce such a respiratory component, so that succinate is unable to remove the barrier.

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