BBA 46413

OXIDOREDUCTION OF CYTOCHROME *b* IN THE PRESENCE OF ANTIMYCIN

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(Received July 12th, 1972)

SUMMARY

1. The effect of oxidizing equivalents on the redox state of cytochrome *b* in the presence of antimycin has been studied in the presence and absence of various redox mediators.

2. The antimycin-induced extra reduction of cytochrome *b* is always dependent on the initial presence of an oxidant such as oxygen. After removal of the oxidant this effect remains or is partially (under some conditions even completely) abolished depending on the redox potential of the substrate used and the leak through the antimycin-inhibited site.

3. The increased reduction of cytochrome *b* induced by oxidant in the presence of antimycin involves all three spectroscopically resolvable *b* components (*b*-562, *b*-566 and *b*-558).

4. Redox mediators with an actual redox potential of less than 100–170 mV cause the oxidation of cytochrome *b* reduced under the influence of antimycin and oxidant.

5. Redox titrations of cytochrome *b* with the succinate/fumarate couple were performed aerobically in the presence of cyanide. In the presence of antimycin two *b* components are separated potentiometrically, one with an apparent midpoint potential above 80 mV (at pH 7.0), outside the range of the succinate/fumurate couple, and one with an apparent midpoint potential of 40 mV and an n value of 2. In the absence of antimycin cytochrome *b* titrates essentially as one species with a midpoint potential of 39 mV (at pH 7.0) and $n = 1.14$.

6. The increased reducibility of cytochrome *b* induced by antimycin *plus* oxidant is considered to be the result of two effects: inhibition of oxidation of ferrocytochrome *b* by ferricytochrome c_1 (the effect of antimycin), and oxidation of the semiquinone form of a two-equivalent redox couple such as ubiquinone/ubiquinol by the added oxidant, leading to a decreased redox potential of the $QH_2/QH^{\bullet}$ couple and reduction of cytochrome *b*.

Abbreviations: DCIP, 2,4-dichlorophenolindophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BAL, 2,3-dimercaptopropanol.

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INTRODUCTION

Chance[1] originally demonstrated that both the rate and the extent of reduction of cytochrome *b* are considerably enhanced by antimycin. The increase in reducibility includes a shift of the absorption maximum of the total band of cytochrome *b* to the red. Most of this 'shift' is due to reduction of a long-wavelength cytochrome *b* species (*b*-566) not usually reduced by succinate alone[1,2], together with a shoulder at approx. 558 nm[3–5] (*b*-558). Thus the effect of antimycin when added to anaerobic mitochondria in the presence of succinate is similar to the corresponding effect of ATP[3,6–9]. The reduction of *b*-558 and *b*-566 induced by the addition of antimycin is, however, under these conditions abolished by phenazine methosulphate (PMS)[4,8] and then a small true red shift in the spectrum of the ferrocytochrome *b*-562 component is revealed[4] (see also refs 5, 9, 10). This shift is specific for antimycin and does not occur with ATP[8,9,11] or 2-heptyl-4-hydroxyquinoline *N*-oxide[5] which only induce the reduction of *b*-558 and *b*-566.

The increased reducibility of cytochrome *b* induced by antimycin has been suggested to be the result of an allosteric effect of the inhibitor, on the basis of the sigmoidal character of this effect[12] and antimycin-binding studies[13] (contrast Kröger and Klingenberg[14]).

Reduction of cytochrome *b* by substrate in the presence of antimycin appears to be greatly favoured by the presence of oxidants such as ferricyanide or O_2 (refs 3, 10, 15–19). This was recently interpreted by Rieske[18] as due to an increase in midpoint potential of cytochrome *b* when a hypothetical component (X) in the b–c_1 region is oxidized. A similar explanation was earlier proposed by Baum *et al.*[20]. Wilson and co-workers[17] interpreted similar data as indicating electron transport-generated formation of a 'high-energy' form of cytochrome *b* with raised midpoint potential, in spite of the insensitivity of these effects to uncouplers of oxidative phosphorylation and their occurrence in isolated systems.

In this paper it will be shown that the antimycin-induced extra reduction of cytochrome *b* is always due to the combined effects of the inhibitor and an oxidant (usually O_2). In contrast to Wilson *et al.*[17] and Erecińska and co-workers[19] we find that continuous electron flow or an oxidized state of cytochrome c_1 are not absolute requirements for this effect of antimycin. Evidence will be presented in favour of a simple kinetic explanation that does not involve a change in the standard state of cytochrome *b* (refs 17–20) or unidentified components of the respiratory chain (refs 18, 20).

RESULTS

The oxygen-induced reduction of cytochrome b in the presence of antimycin

In Fig. 1 it may be seen that addition of antimycin to anaerobic succinate-supplemented submitochondrial particles causes further reduction of cytochrome *b*. This is due to reduction of the *b*-566 and *b*-558 components (see Fig. 4 and refs 3–5, 16, 17, 19). From the trace it may be seen that reduction is not complete unless a sufficient amount of O_2 is added together (or after) the antimycin. Under these conditions of high succinate concentration the level of reduction equals the level after dithionite (not shown). The small decrease in $\Delta A_{565-575\ nm}$ soon after the addi-

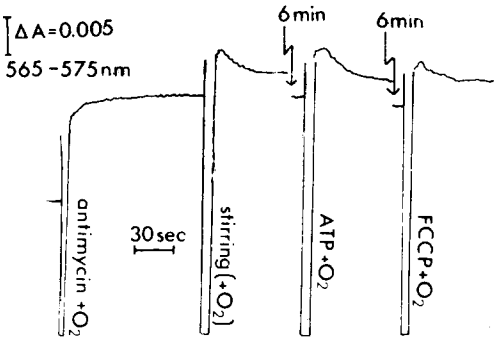

Fig. 1. Effect of oxygen in the presence of antimycin on the reducibility of cytochrome *b*. To a suspension (3 mg protein/ml) of 'A-particles' (stored a few days in liquid nitrogen) in 0.2 M sucrose, 25 mM Tris–acetate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA and 1.5 μg oligomycin, 20 mM succinate was added to reach anaerobiosis. The reaction studied was started by addition of 2 μM antimycin (together with O_2), followed by stirring and addition of 1 mM ATP and 1 μM FCCP (together with O_2) as indicated. The absorption at 565 *minus* 575 nm was followed. An upward deflection of the trace reflects an increase in this absorption difference (reduction of cytochrome *b*).

tion of O_2 is due to reduction of cytochrome $c + c_1$ at anaerobiosis. The antimycin-induced reduction of cytochrome *b* is usually quite stable after anaerobiosis under these conditions but the stability is somewhat decreased in preparations that have been frozen and thawn as is the case in this experiment (see redox level after 6 min). The decreased stability of the *b* reduction in aged preparations may be due to the increased leak through the antimycin block found in such preparations. The extra reduction of cytochrome *b*-566 on further stirring after the addition of antimycin explains the increased reduction of this component reported by Slater *et al.*[6] on addition of ATP (together with O_2) to anaerobic antimycin-treated particles.

Fig. 2 shows a similar experiment with beef-heart mitochondria. Here it is seen that the level of reduction after anaerobiosis in the presence of antimycin is less at lower succinate/fumarate ratios (varying the succinate concentration gives similar results as varying the fumarate concentration). However, even at the lowest succinate/fumarate ratio of this experiment there is still an antimycin-induced reduction after anaerobiosis that is removed only after the addition of PMS (*cf.* refs 4, 8).

Fig. 3 shows an experiment with a very low succinate/fumarate ratio (and a low concentration of succinate). Under these conditions a high level of reduction is obtained immediately after the addition of oxygen (see O_2 addition in Fig. 3) but partial reoxidation occurs already during the aerobic phase (see redox state of cytochrome $c + c_1$). Similar kinetics of cytochrome *b* have been found with NADH as substrate[21]. On anaerobiosis (reduction of cytochrome $c + c_1$) there is a further apparent oxidation to a new fairly stable redox level. As revealed from experiments not shown, this effect is only partially due to interference from the reduction of cytochrome $c + c_1$. This new level of reduction is still considerably higher than in the absence of antimycin. In the presence of PMS (*cf.* refs 4,8) an oxygen pulse gives rise to a similar response of cytochrome *b*, but on anaerobiosis the cytochrome is oxidized to the state prior to the addition of antimycin.

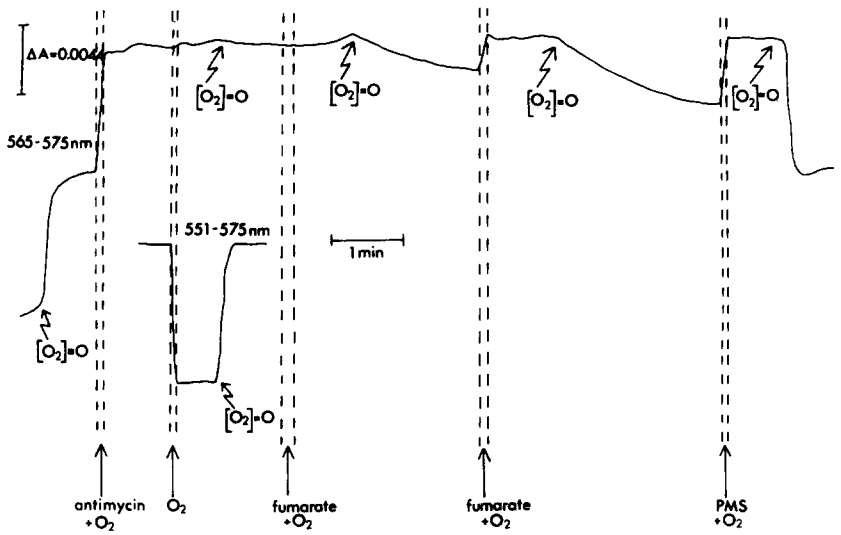

Fig. 2. The effect of the succinate/fumarate ratio, oxygen and PMS on the redox level of cytochrome *b* in the presence of antimycin. Beef-heart mitochondria (1.5 mg protein/ml) were suspended in the standard reaction mixture (see Materials and Methods) containing 5 μM rotenone, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 8 mM succinate. After anaerobiosis (see figure) 0.6 μg/ml antimycin was added together with oxygen. Subsequently the following additions were made: O_2 (stirring), 6 mM fumarate (together with O_2) twice, and 10 μM PMS (together with O_2). After the first addition of O_2 the redox state of cytochrome $c + c_1$ was followed simultaneously by alternating the measuring wavelength continuously between 565 and 551 nm. Cytochrome *b* was measured at the wavelength couple 565 *minus* 575 nm. An upward deflection of the trace indicates reduction of cytochrome *b* (or cytochrome $c + c_1$).

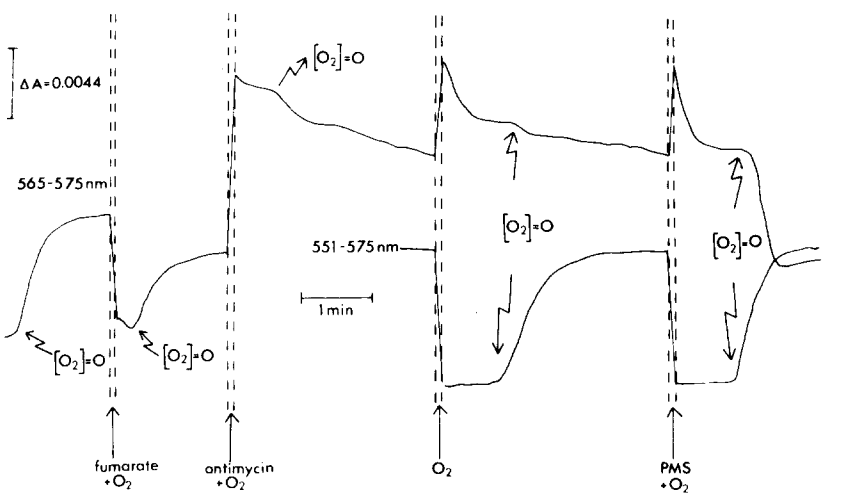

Fig. 3. The effect of O_2 and PMS on the redox state of cytochrome *b* at a low succinate/fumarate ratio. The experiment was performed under identical conditions as that of Fig. 2, except that only 0.8 mM succinate was added, and 8 mM fumarate was added after anaerobiosis (together with some O_2). The further additions indicated in the figure were: 0.6 μg/ml antimycin (together with O_2), O_2 alone, and 10 μM PMS (together with O_2). The redox state of cytochrome $c + c_1$ was followed simultaneously with the redox state of cytochrome *b* (see legend to Fig. 2) after the addition of oxygen alone (third addition shown in the figure).

Summarizing we may distinguish four levels of reduction of cytochrome *b* in the presence of antimycin:

Level 1. The aerobic highly reduced state immediately after the addition of O_2 (see Fig. 3). This level is almost independent of the succinate/fumarate ratio.

Level 2. The aerobic less reduced state (steady state) some time after the addition of oxygen (Figs 2 and 3).

Level 3. The level after anaerobiosis (quasi-equilibrium). This level is dependent on the succinate/fumarate ratio and on the rate of leak through the antimycin block, and is fairly stable in freshly prepared mitochondria or particles. At very high succinate/fumarate ratios levels 1–3 are indistinguishable (Figs 1 and 2).

Level 4. The PMS-induced level after anaerobiosis (equilibrium). This level of reduction is the same as in the absence of antimycin (*cf.* refs. 4, 8).

These data suggest that the oxidant-induced reduction of cytochrome *b* in the presence of antimycin is due to an oxidant-induced shift in redox equilibrium between components below the antimycin block that does not readily revert after exhaustion of the oxidant.

Fig. 4 shows the spectra of the O_2-induced effect in the presence of antimycin when the oxygen has been added at two different levels of reduction of cytochrome *b*. In A the typical reduction of *b*-558 and *b*-566 is seen (*cf.* refs 3, 4, 17). Since this spectrum shows the effect after exhaustion of the added oxygen, cytochrome $c + c_1$ does not contribute. This shows clearly that reduction of these carriers does not abolish the reduction of cytochrome *b* (see also Figs 1–3). In this experiment *b*-562 was already fully reduced before the pulse of oxygen. In B the oxygen was added at a very oxidized state of cytochrome *b* and here it is seen that the absorption maximum is at 563–564 nm, indicating reduction of all three cytochrome *b* components. The amount of cytochrome *b* reduced in this experiment on addition of oxygen is also considerably higher than either *b*-562 or *b*-566 *plus* *b*-558 alone, strongly suggesting that all three *b* species are indeed involved in this effect (contrast ref. 17).

Fig. 5 shows experiments performed in an anaerobic Thunberg cuvette repeatedly evacuated and filled with oxygen-free argon and finally evacuated. The sample already contained succinate before evacuation and the two side-arms contained solid KCN and antimycin dissolved in aqueous ethanol, respectively. Addition of cyanide had no effect on the cytochrome *b* spectrum (not shown). Addition of antimycin caused a little reduction of cytochrome *b* (Curve 1, Fig. 5, compare with Figs 1–3) and the shift of the spectrum of the *b* already reduced can clearly be seen[4,5,9,10]. Addition of cyanide after antimycin abolished the slight antimycin-induced reduction of cytochrome *b* (Curve 2) so that only the shift was left (Curve 3). In agreement with this, addition of antimycin in the presence of cyanide did not cause any reduction (not shown). Thus antimycin alone, without O_2, has little or no effect on the redox level of cytochrome *b* in the presence of succinate. The large reduction seen in experiments where some O_2 is added together with the antimycin (see, *e.g.* Figs 1–3) can therefore clearly be attributed to the added oxygen. That this also holds for the experiments done in the presence of cyanide[12, 13] is clear from the effect of oxygen added after antimycin and cyanide shown in Curve 4 of Fig. 5. Introduction of air results in reduction of *b*-558 and *b*-566 without any measurable change in cytochrome $c + c_1$. Although we cannot exlcude that the

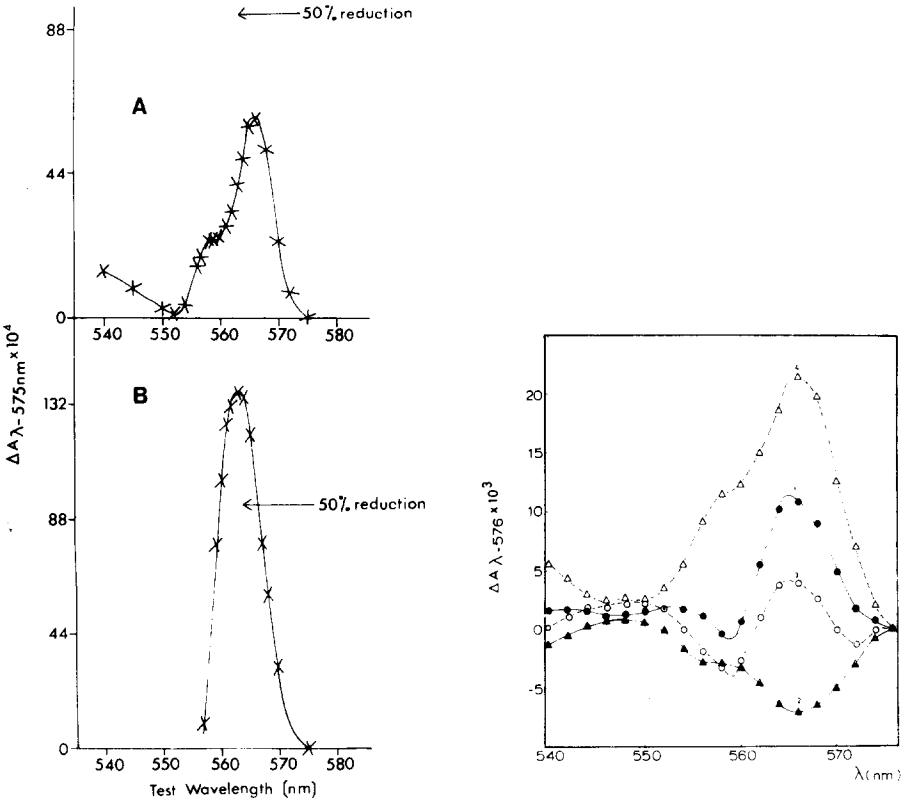

Fig. 4. Spectra of the oxygen-induced reduction of cytochrome *b*. (A) The standard reaction mixture (see Materials and Methods) was supplemented with beef-heart mitochondria (2 mg protein/ml), 5 μM rotenone, 1 μM FCCP and 4 mM succinate. After anaerobiosis 2 mM fumarate was added and then 0.86 μg/ml antimycin together with oxygen. After anaerobiosis and incubation until partial reoxidation of cytochrome *b* to a stable level had occurred (*cf.* Fig. 1) the absolute spectrum was scanned with absorption at 575 nm taken as zero. Then a pulse of oxygen was added and a new absolute spectrum taken immediately after anaerobiosis. (A) represents the latter absolute spectrum *minus* the former. The peak represents 35% of total cytochrome *b*; the other 65% was reduced already before the pulse of oxygen. (B) The standard reaction mixture was supplemented with beef heart mitochondria (2 mg protein/ml), 5 μM rotenone, 1 μM FCCP, 10 mM ascorbate and 80 μM TMPD. After anaerobiosis 0.86 μg/ml antimycin was added. Under these conditions cytochrome *b* was very little reduced as revealed from the absorption difference 565 *minus* 575 nm. Subsequent pulses of oxygen caused considerable reduction of cytochrome *b* (and oxidation of cytochrome $c + c_1$). Separate pulses were given at the measuring wavelengths indicated at a constant reference at 575 nm. The spectrum represents the extent of reduction of cytochrome *b* as a function of wavelength immediately after the oxygen pulse. Note that these mitochondria contained endogenous substrates. The peak represents approx. 75% of total cytochrome *b*.

Fig. 5. Effect of antimycin on the spectrum of cytochrome *b* in the absence of oxygen. 'A-particles' (2 mg protein/ml) were suspended in a medium containing 0.2 M sucrose, 50 mM Tris–HCl buffer (pH 7.4) and 20 mM succinate in a Thunberg cuvette. The two side-arms contained solid KCN (1–2 mg) and 10 nmoles antimycin in aqueous ethanol, respectively. The cuvette was made anaerobic by repeated evacuation and filling with oxygen-free argon and finally evacuated. Effect spectra were calculated from the values of $A\lambda_{-575\ nm}$ measured before and after each addition. Curve 1, effect of antimycin; Curve 2, effect of cyanide in the presence of antimycin; Curve 3, Curve 1 *plus* Curve 2, *i.e.* the effect of antimycin in the presence of cyanide as measured in a parallel experiment; Curve 4, effect of introduction of air in the presence of antimycin *plus* cyanide.

effect of the added oxygen is exerted *via* cytochrome *c* oxidase even in the presence of cyanide, the rate of electron flow is certainly very slow. The redox state of cytochrome *b* in the presence of cyanide, substrate, antimycin and oxygen is comparable with the redox state ('Level 3', see above) after anaerobiosis and is also abolished by PMS (not shown).

In agreement with Deul and Thorn[22] it was found that treatment of submitochondrial particles with 2,3-dimercaptopropanol (BAL) *plus* oxygen abolishes the antimycin-induced reduction of cytochrome *b* in the presence of cyanide and oxygen. Extraction of cytochrome *c* from the particles had the same effect.

The effect of redox mediators

From the previous experiments it is clear that PMS abolishes the antimycin *plus* oxidant-induced reduction of cytochrome *b* when the terminal cytochromes ($c + c_1$ and *a*) are reduced (anaerobically, Figs 2 and 3, or aerobically in the presence of cyanide), but not when they are oxidized (Figs 2 and 3). From the experiments shown in Figs 2, 3, 4A and 5 we may conclude that the reduced state of cytochrome *b* is not particularly sensitive to the redox state of the terminal cytochromes although maximal reduction of cytochrome *b* is obtained only when these cytochromes are highly oxidized (except at a high succinate/fumarate ratio, see Fig. 2). On the other hand, the releasing effect of PMS correlates with the redox state of the mediator itself, since reoxidation of cytochrome *b* occurs only under conditions where the added PMS is highly reduced. In the aerobic steady state PMS is highly oxidized, even in the presence of ascorbate, due to the extremely fast oxidation of the reduced form *via* cytochrome $c + c_1$ (unpublished observations). Under these conditions no reoxidation of cytochrome *b* is obtained even in the presence of ascorbate (not shown). Only after anaerobiosis (or inhibition of cytochrome *c* oxidase by cyanide) when the PMS becomes reduced is reoxidation of cytochrome *b* observed. This suggests that the redox state of the mediator rather than that of cytochrome $c + c_1$ may determine whether cytochrome *b* becomes oxidized or not.

As shown in Fig. 6, the increased reduction of cytochrome *b* is also not abolished by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) *plus* ascorbate in the (uncoupled) aerobic steady state where this mediator is highly reduced, but again only after anaerobiosis. Since the reduced forms of both PMS and TMPD are rapidly oxidized by the respiratory chain (*via* cytochrome $c + c_1$, refs. 23, 24), the actual redox potential of the mediator in the presence of ascorbate and oxygen is expected to attain a value in between those of the ascorbate/dehydroascorbate and ferri-/ferrocytochrome $c + c_1$ couples in the steady state. TMPD is more reduced in the steady state than PMS presumably since the midpoint potential of TMPD ($E'_0 = 260$ mV at pH 7.0, ref. 25) is considerably higher than that of PMS ($E'_0 = 80$ mV at pH 7.0, ref. 25) and the rates of TMPD- and PMS-mediated oxidation of ascorbate are about the same. We may conclude that it is the redox potential of the mediator that controls the redox state of cytochrome *b* in the presence of antimycin. Since TMPD was more than 90% reduced in the aerobic steady state in the presence of ascorbate and $n = 1$ (ref. 25) it follows that the redox potential of the mediator was below 200 mV.

Reduced 2,4-dichlorophenol indophenol (DCIP) is only very slowly oxidized by ferricytochrome $c + c_1$ in intact mitochondria (unpublished observations).

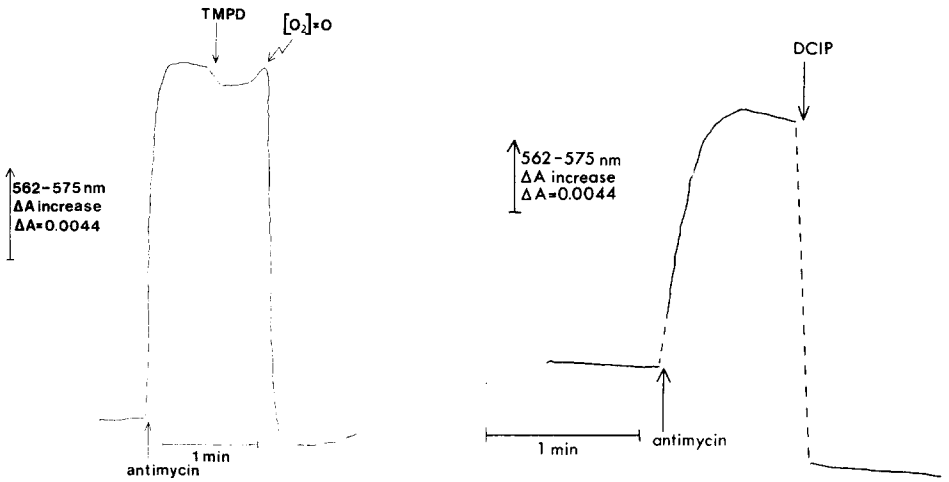

Fig. 6. Oxidation of cytochrome *b* on anaerobiosis in the presence of antimycin and TMPD *plus* ascorbate. The standard reaction mixture (see Materials and Methods) was supplemented with 5 μM rotenone, 6.7 mM ascorbate, 1 μM FCCP and beef-heart mitochondria (2 mg protein/ ml). The following additions were made as indicated in the figure: 0.83 μg/ml antimycin and 0.17 mM TMPD (with an additional 3.3 mM ascorbate).

Fig. 7. Oxidation of cytochrome *b* aerobically in the presence of antimycin and DCIP *plus* ascorbate. The standard reaction mixture was supplemented with 5 μM rotenone, 6.7 mM ascorbate and beef-heart mitochondria (1 mg protein/ml). After 2-min preincubation the following additions were made as indicated: 0.67 μg/ml antimycin and 6.7 μM DCIP.

Since DCIP is rapidly reduced by ascorbate we may expect then that the actual redox potential of this mediator would be much closer to that of the ascorbate/ dehydroascorbate couple in the aerobic ascorbate-supplemented system than is the case with PMS and TMPD. In Fig. 7 it is shown that DCIP *plus* ascorbate causes reoxidation of cytochrome *b* reduced by endogenous substrates in the presence of antimycin and oxygen (contrast TMPD, Fig. 6, and PMS). This supports the above conclusion that the steady-state redox potential of the mediator must be rather low in order to cause oxidation of cytochrome *b*. Since under the conditions of this experiment cytochrome $c + c_1$ is highly oxidized it is clear that a low redox potential of these cytochromes is not a necessary requirement for the reoxidation of cytochrome *b* (contrast refs 19, 26). The low redox potential of DCIP in the presence of excess ascorbate makes the possibility of a direct oxidation of cytochrome *b* by oxidized DCIP very unlikely. Moreover, TMPD, which is more oxidizing under these conditions (see above) and has been shown to be able to shunt reducing equivalents across the antimycin block when added in its oxidized form (in the absence of ascorbate, ref. 27) does not cause oxidation of cytochrome *b* in the presence of ascorbate and oxygen (Fig. 6).

From these data we may conclude that reduction by the mediators of a component of the respiratory chain with midpoint potential similar to or higher than that of the ascorbate/dehydroascorbate couple but lower than 200 mV results in abolition of the oxidant-induced reduction of cytochrome *b* in the presence of antimycin. The experiments of Figs 2, 3, 4A and 5 indicate that reduction of this hypo-

thetical component did not occur in the absence of redox mediator although cytochrome $c + c_1$ was very close to 100% reduced. This suggests either that the hypothetical component is not in equilibrium with cytochrome $c + c_1$ or that the midpoint potential of this component is below 170 mV (supposing cytochrome $c + c_1$ with a midpoint potential of 230 mV[28] to be more than 90% reduced). Evidence in favour of the latter suggestion is given below although we cannot exclude that the former may be also true. Since the midpoint potential of the hypothetical component is similar to or higher than (see above) that of the ascorbate/dehydroascorbate couple ($E'_0 = 80$ mV at pH 7.0, ref. 25), the data in Figs 1–3 also suggest that this component does not come into equilibrium with the succinate/fumurate couple unless a redox mediator is added. This is in agreement with the conclusion drawn above (see p. 407) that the oxidant-induced reduction of cytochrome *b* may be the result of a shift in equilibrium between components situated below the antimycin block. An oxidation of the hypothetical component by the added oxidant may thus be responsible for the observed reduction of cytochrome *b* (see Discussion).

The redox relationship between cytochromes b and $c + c_1$ in the presence of antimycin

Fig. 8 shows simultaneously recorded redox traces of cytochrome *b* and $c + c_1$ in anaerobic beef-heart mitochondria supplemented with antimycin, uncoupler and redox mediators. Ferricyanide was first added to oxidize the system. After this the redox potential above the antimycin-sensitive site (*e.g.* cytochrome $c + c_1$) was slowly and continuously lowered after addition of succinate, due to a slow flux

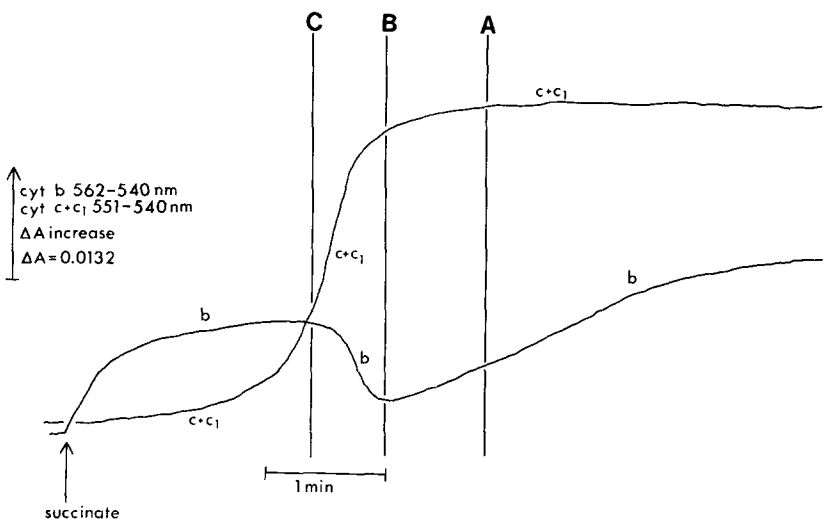

Fig. 8. Redox relationship between cytochromes *b* and $c + c_1$ in the presence of antimycin. To the standard reaction mixture was added 5 μM rotenone, 2 mM ascorbate, 1 μM FCCP, beef-heart mitochondria (2 mg protein/ml) and 18.5 μM phenazine ethosulphate. After anaerobiosis 37 μM diaminodurene and 0.9 μg/ml antimycin were added. Sufficient ferricyanide was then added to cause complete oxidation of cytochrome $c + c_1$. Finally 3.7 mM succinate was added and, as shown in the figure, the redox states of cytochromes *b* and $c + c_1$ were followed simultaneously by changing the measuring wavelength dial between 562 and 551 nm at an approximate frequency of one change every 5 s. The letters A–C refer to another experiment where ferricyanide was added to the reduced system at A as explained in the text.

of reducing equivalents from succinate to the ferri-/ferrocyanide couple. This resulted in reduction of cytochrome $c + c_1$ and a (reduction)-oxidation-reduction cycle of cytochrome *b*. Note that the immediate reduction of cytochrome *b* on addition of succinate does not occur in the absence of antimycin under conditions otherwise as in Fig. 8 (excess ferricyanide present). In the absence of antimycin cytochrome *b* becomes reduced after cytochrome $c + c_1$ in a 'normal' potentiometrical fashion corresponding to the final phase of reduction shown in Fig. 8.

If ferricyanide (or oxygen) is added to the reduced system in the presence of antimycin (*e.g.* at point A in Fig. 8) either oxidation (transition to point B) or reduction (transition to point C) of cytochrome *b* can be induced depending on the amount of oxidant added. The latter effect is identical with the anomalous reduction of cytochrome *b* on addition of oxidizing equivalents in the presence of antimycin as reported by several authors (see Introduction) and further characterized in this paper.

The experiment of Fig. 8 shows clearly that the oxidation of cytochrome *b* occurs later than the reduction of cytochrome $c + c_1$, the estimated redox potential at half-oxidation of cytochrome *b* being approx. 100–170 mV. This increases the precision of the above estimate that the midpoint potential of the hypothetical component which controls the redox state of cytochrome *b* is between 80 and 200 mV.

Aerobic redox titration of cytochrome b with the succinate/fumarate couple

Since redox mediators such as PMS abolish the antimycin *plus* oxidant-induced reduction of cytochrome *b* anaerobically[4,8] (see also Figs 2 and 3) it is to be expected that antimycin does not affect the midpoint potentials of the main cytochrome *b* components in titrations performed anaerobically in the presence of these redox mediators[4,17]. On the other hand it may be of interest to perform redox titrations in the presence of oxidant but without redox mediators, *i.e.* under the conditions where the antimycin-induced reduction of cytochrome *b* occurs. Such titrations have been carried out by Urban and Klingenberg[29] with the succinate/fumarate couple in the presence of cyanide and by Rieske[18] with the QH_2-I/Q-I couple. Since the presence of several *b* components was not generally recognized at the time of the former investigation it seemed desirable to repeat the titrations with the succinate/fumarate couple in the light of the new knowledge.

Fig. 9 shows the double logarithmic plot of a titration of cytochrome *b* with the succinate/fumarate couple in the presence of cyanide in sub-mitochondrial particles from beef heart. The experimental points are fitted by a straight line corresponding to an overall midpoint potential (E'_0 at pH 7.0) of 39 mV and a slope corresponding to an n value of 1.14. This titration is in good agreement with the recent anaerobic potentiometric titrations in the presence of various redox mediators[4,28] but the midpoint potential is lower than that found by Holton and Colpa-Boonstra[30] and Urban and Klingenberg[29] (about 75 mV at pH 7.0). The reason for this discrepancy is that these latter authors estimated the 100% reduced cytochrome *b* from the maximal reduction that can be obtained with succinate whereas we used the dithionite-reducible cytochrome *b* as the 100% value. Although the titration curve does not reveal the presence of several components, spectra taken at various points of the titration (not shown) clearly demonstrated preferential reduction of the *b*-562 species at the higher potentials and reduction of the *b*-566

and *b*-558 components at the lower potentials. The straight relationship shown in Fig. 9 then indicates that the midpoint potentials of the different species are fairly close to each other under these conditions (*cf.* refs 4 and 28).

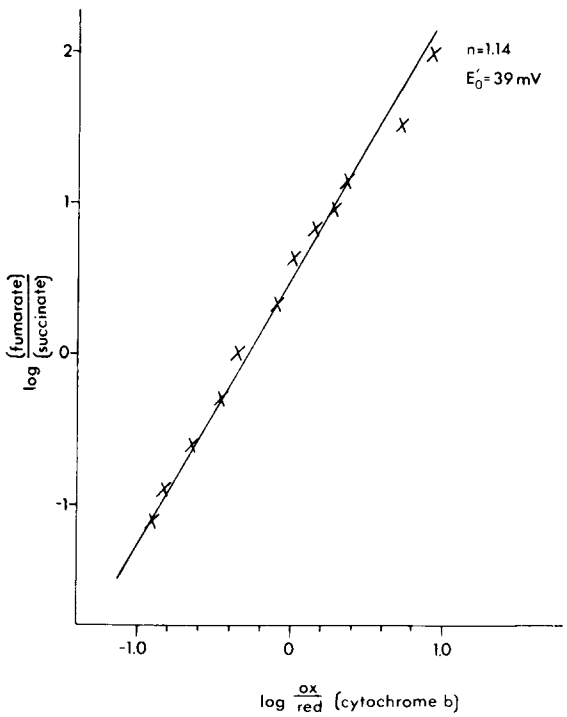

Fig. 9. Redox titration of cytochrome *b* with the succinate/fumarate couple in the presence of air and cyanide. 'A-particles' (2 mg protein/ml) were suspended in 0.25 M sucrose, 0.02 M Tris–HCl buffer (pH 7.4). After addition of 2 mM fumarate and 3 mM KCN the particles were incubated for 30 min at room temperature and the titration was performed by successive additions of succinate. After each succinate addition 5 min was allowed for equilibration, and the spectrum between 575 and 540 nm measured. 100% reduction was assumed after addition of a few grains of solid dithionite. The reduction level of cytochrome *b* was calculated from the absorption difference 563 *minus* 575 nm. The midpoint potential given in the figure (39 mV) refers to the value extrapolated to pH 7.0 assuming a midpoint potential of the succinate/fumarate couple of 24 mV at pH 7.0 (ref. 27) and a similar pH dependence for the potential of cytochrome *b* as for that of the succinate/fumarate couple (ref. 29). The figure shows the double logarithmic plot of the titration data.

Fig. 10A shows the course of a similar titration in the presence of antimycin (redox state *vs* redox potential). The system now behaves completely differently. Approximately half of the cytochrome *b* was already reduced at the highest potentials obtained with the succinate/fumarate couple (about 85 mV). On decreasing the potential further, the rest of cytochrome *b* became reduced with an apparent midpoint potential of 40 mV. This figure strongly suggests the presence of two potentiometrically distinguishable components, one of which has an apparent midpoint potential considerably higher than the limit of detection with the system used. Spectra taken during the course of the titration (not shown) demonstrated that the apparently high-potential part was due to the *b*-562 species (apparent E'_0 well

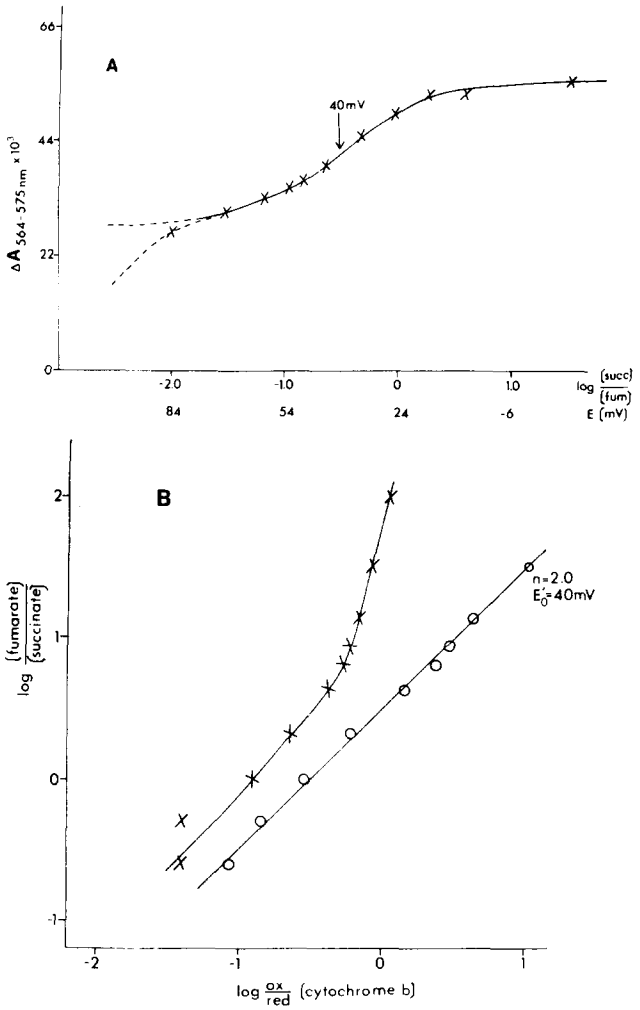

Fig. 10. Redox titration of cytochrome *b* with the succinate/fumarate couple in the presence of antimycin, air and cyanide. The experimental conditions were those reported in Fig. 9, except that before the incubation with fumarate and cyanide 1.8 μM antimycin was added. In A the redox state of cytochrome *b* (measured at 564 *minus* 575 nm) is plotted against the logarithm of the fumarate/succinate ratio. Also in this experiment the redox potential of the succinate/fumarate couple refers to pH 7.0 (see legend to Fig. 9). In B (upper curve) a double logarithmic plot of the data in A has been made. Assuming that this curve is composed of two species with equal spectral contribution at 564 *minus* 575 nm (see A), the component with lower apparent midpoint potential is resolved mathematically from the one with high apparent midpoint potential in the lower curve of B.

above 85 mV at pH 7.0) and the low-potential part (with an apparent E'_0 at pH 7.0 of 40 mV) to the *b*-558 and *b*-566 species. This suggests that the apparent midpoint potentials of all three components have been raised by antimycin under these conditions (*cf.* Figs 4 and 9). Fig. 10B (upper curve) shows the corresponding double logarithmic plot of the titration in Fig. 10 A. The sigmoidal character of this plot (the upper limb is missing) also suggests separation of two components. On the basis of Fig. 10A we have assumed that two components are present with equal contri-

bution at the wavelength couple used (564 *minus* 575 nm). With this assumption it is possible to resolve the composite curve mathematically into its two components (see ref. 31). The lower curve in Fig. 10B shows the dependence of the low-potential component on redox potential when resolved from the high-potential component. The points are best fitted by a straight line with a midpoint of 40 mV and a slope corresponding to an n value of 2.0 (*cf.* the n values given in ref. 29). The latter is an unexpected finding strongly indicating that a complication has arisen in the dependence of the redox state of cytochrome *b* on the redox potential of the succinate/fumarate couple induced by antimycin under these conditions. The n value of 2.0 may be due to necessity of reducing another one-electron carrier simultaneously with cytochrome *b* by succinate in the presence of antimycin and oxygen. A possible reason for this will be discussed below.

DISCUSSION

Wilson *et al.*[17] and Erecińska *et al.*[19] have proposed that the reduction of *b*-566 in the presence of antimycin and oxygen is the result of 'energization' due to electron flow through Site II, and therefore disappears after exhaustion of the oxygen. However, from our data it is clear that in the absence of an artificial electron carrier this reduction is quite stable after reaching anaerobiosis under favourable conditions (Figs 1 and 2). Moreover, the effect is clearly not dependent on the redox state of cytochrome c_1 (contrast Erecińska *et al.*[19]). On the other hand it is also evident that at low succinate/fumarate ratios maximal reduction of cytochrome *b* is only obtained aerobically during electron flow when cytochrome $c + c_1$ is highly oxidized. Only at high succinate/fumarate ratios the aerobic and anaerobic degrees of reduction were indistinguishable (Fig. 2). Our finding that all three spectroscopically distinguishable cytochrome *b* components are involved in the oxidant-induced reduction in the presence of antimycin (Figs. 4 and 10) is also in disagreement with the suggestion[17,19] that the effect is due to reduction and stabilization of a 'high-energy' form of an energy-transducing cytochrome *b* species (b_T). The finding that different levels of reduction of cytochrome *b* can be observed in the presence of antimycin depending on the parameters described in the Results section strongly suggests to us that we are dealing with an oxidant-induced shift in equilibrium exerted below the antimycin block. The system does not revert to the original equilibrium unless it is allowed to relax a very long time or a suitable redox mediator is added. A change in midpoint potential of any one of the components involved need not be postulated, since the effect would be the result of changes in concentration (activity) rather than in standard state.

Rieske[18] also proposed that the increased reduction of cytochrome *b* was the result of a change in standard state of the cytochrome, but in his view this is the result of the oxidized state of a hypothetical redox component (X) in the b–c_1 region rather than of electron flow. However, this mechanism would imply that the increased reducibility of cytochrome *b* should be seen in anaerobic potentiometric titrations at redox potentials above the midpoint potential of X which is about 120 mV[20]. Since this is not found (refs 4, 17, 32) we must conclude that this explanation is also unlikely. On the other hand, we agree with Rieske[18] that a component must be oxidized in order to obtain extra reduction of cytochrome

b, but since the oxidized state of that component may be the result of a shift in equilibrium, the effect cannot be seen in the potentiometric titrations carried out in the absence of oxidant and in the presence of redox mediators.

Our results with the artificial redox mediators suggest that the midpoint potential of this hypothetical component is 100–170 mV (Fig. 8), that it does not equilibrate with the succinate/fumarate couple after the addition of oxidant in the presence of antimycin, that its redox state influences the redox state of cytochrome *b*, and that it can be oxidized by oxygen (or ferricyanide) presumably *via* cytochrome $c+c_1$. These findings make it difficult to consider the respiratory chain a linear array of components in the b–c_1 region.

Erecińska *et al.*[19] made the important observation that the oxidant-induced reduction of cytochrome *b* in the presence of antimycin was accompanied by a kinetically compatible oxidation of ubiquinol. For this reason, because the semiquinone form of the ubiquinone couple has been shown to be present in the mitochondrial membrane[33], and because reduction of cytochrome *b* by ubiquinol must lead to formation of the semiquinone, we propose that the hypothetical redox component with properties as described above may be the QH·/Q couple (see Fig. 11). We do not, however, have any direct evidence in favour of this suggestion. From the point of view of the model to be proposed (Fig. 11) another two-equivalent redox couple such as flavin for instance could equally well be involved in the manner we have now suggested for ubiquinone.

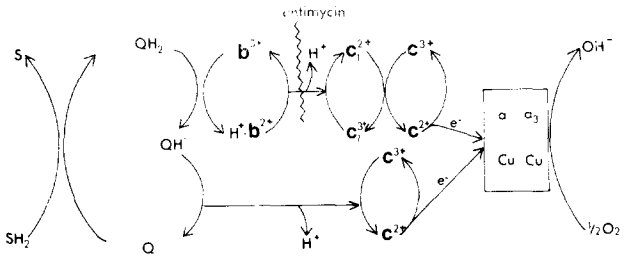

Fig. 11. A model for the mechanism of the oxidant-induced reduction of cytochrome *b*. For detailed explanation, see Discussion.

Consider the effect of a pulse of O_2 to the system depicted schematically in Fig. 11, starting anaerobically in the presence of antimycin. Before the oxygen pulse the redox state of cytochrome *b* obeys the following equilibrium:

$$K_{eq} = \frac{(H^+ \cdot b^{2+})}{(b^{3+})} \cdot \frac{(QH^\cdot)}{(QH_2)} \qquad (1)$$

This equilibrium is independent of the presence of antimycin (*cf.* the experiment of Fig. 5 where antimycin was added under anaerobic conditions and then caused only a small change in redox state of cytochrome *b*). Now the addition of O_2 will induce a rapid oxidation of the semiquinone ($QH^\cdot$) to ubiquinone (Q). This perturbation displaces the equilibrium of Eqn 1 towards reduction of cytochrome *b* at the expense of the ubiquinol (QH_2). Eventually a steady state is reached where

the rate of reduction of cytochrome *b* equals the rate of reoxidation. The redox state of cytochrome *b* is then determined by

$$\frac{(H^+ \cdot b^{2+})}{(b^{3+})} = \frac{k_1(QH_2)}{k_{-1}(QH^\bullet) + k_2(c_1{}^{3+})} \tag{2}$$

where k_1 and k_{-1} are the respective constants of the forward and backward reaction between the QH_2–$QH^\bullet$ and cytochrome *b* couples, and k_2 is the rate constant of the reaction between ferrocytochrome *b* and ferricytochrome c_1 (here the back reaction is considered negligible). Since the ($QH^\bullet$) is kept very low by the oxidant and also k_2 is very small in the presence of antimycin, cytochrome *b* will be highly reduced in the steady state, the level of reduction being dependent on the concentration of QH_2. The transient higher level of reduction of cytochrome *b* observed immediately after the addition of oxygen at low concentrations of succinate (Fig. 3) may be due to the delay in the oxidation of ferrocytochrome *b* by ferricytochrome c_1 that has been observed by Baum and Rieske[15].

After exhaustion of the oxidant (O_2) the rapid oxidation of the semiquinone stops, but the formation of semiquinone is restricted indirectly by the antimycin block due to only little production of b^{3+}. Thus a quasi-equilibrium is reached where the redox state of cytochrome *b* obeys Eqn 1. Since the concentration of $QH^\bullet$ is still lower than before the addition of oxidant, cytochrome *b* is more reduced than before the O_2 addition. In this state the redox level of cytochrome *b* will be dependent on the succinate/fumarate ratio *via* the concentration of QH_2 (Fig. 2). The addition of a redox mediator in the absence of oxidant enables equilibration of the substrate couple with the $QH^\bullet$/Q couple (PMS) which is not possible otherwise (see Fig. 11) or imposes the redox potential of the ascorbate/dehydroascorbate on this couple (TMPD and DCIP) leading to reduction of Q to $QH^\bullet$ and concomitant oxidation of cytochrome *b* (Eqn 1).

According to the scheme of Fig. 11 the hypothetical redox couple that was suggested to control the redox state of cytochrome *b* would obviously be the $QH^\bullet$/Q couple. We found (Fig. 8) that the midpoint potential of the hypothetical couple is about 100–170 mV. The midpoint potential of the $QH^\bullet$/Q couple must be higher than that of the two-equivalent QH_2/Q couple by an amount that is dependent on the stability of the semiquinone $QH^\bullet$ (see Clark[25]), *i.e.* higher than 60 mV at pH 7.0 (ref. 29). If the midpoint potential was 120 mV, within the range of our hypothetical redox couple, it would suggest that the stability constant of the semiquinone

$$K = \frac{(QH^\bullet)^2}{(QH_2)(Q)} \tag{3}$$

may be about 100, a reasonable value in a hydrophobic milieu[25]. From this would also follow that the midpoint potential of the QH_2/$QH^\bullet$ couple would be about 0 mV. Thus complete reduction of even the low-potential *b*-566 and *b*-558 components ($E'_0 = -30$ mV at pH 7.2, ref. 28) is not surprising at the high QH_2/$QH^\bullet$ ratio after a pulse of oxidant.

We conclude that the presented model closely agrees with the experimental findings if some reasonable assumptions are made and that it provides a relatively simple explanation for the oxidant-induced reduction of cytochrome *b* that is readily open for further amendments and experimental test. It also provides an interesting possibility for the arrangement of redox carriers at coupling Site II. It should be emphasized that the model is by no means complete. For instance, we have not considered the function of the different forms of cytochrome *b* since all *b* species seem to respond in a similar fashion on the "oxygen effect". We do also not imply that the reaction *e.g.* between cytochromes *b* and c_1 and between the $QH^{\bullet}/Q$ and cytochrome *c* couples need necessarily be direct. In fact putting the X of Baum *et al.*[20] as an intermediate redox carrier in the latter pathway would make our scheme very similar to the one proposed by these authors. The finding[22] that treatment with BAL inhibits the oxidant-induced reduction of cytochrome *b* in the presence of antimycin and cyanide suggests that the BAL-sensitive site[34] (the X?) may lie in this latter pathway. The fact that cyanide inhibits the "oxygen effect" only partially (Fig. 5) might imply that antimycin makes a component of the lower pathway (Fig. 11) autooxidizable[3,15] (the X?) but we cannot exclude that it is due to a minute leak through the cyanide-inhibited cytochrome *c* oxidase.

According to the scheme of Fig. 11 titration of cytochrome *b* with the succinate/fumarate couple in the presence of antimycin and O_2 is expected to result in an apparent n value of 2.0 for the $b^{3+}/H^{+}\cdot b^{2+}$ couple (see Fig. 10B) since of the two equivalents given by succinate only one is recovered in cytochrome *b*, the other being lost by the pathway of $QH^{\bullet}$ oxidation. In the absence of antimycin the second reducing equivalent is not lost, but affects cytochrome *b* *via* the b–c_1 interaction.

Finally, we would like to speculate that the release of protons at the two steps shown in Fig. 11 might have direct implications for the mechanism of energy conservation at Site II (refs 32, 35–39).

MATERIALS AND METHODS

Beef-heart mitochondria were prepared by the procedure described by Löw and Vallin[40], and 'A-particles' according to Fessenden and Racker[41]. An Aminco-Chance dual-wavelength spectrophotometer was used for measurements of redox changes in individual respiratory carriers and for difference spectra. Cuvettes of 1-cm light path were used throughout.

The standard reaction mixture consisted of 0.2 M mannitol–0.05 M sucrose–0.02 M KCl–0.05 M morpholinopropane sulphonate (pH 7.2). All measurements were carried out at room temperature (21–23 °C). The FCCP was kindly donated by Dr P. G. Heytler. All other reagents were commercially available products.

NOTE ADDED IN PROOF (Received October 10th, 1972)

Ericinska and Wilson[42] have recently reported that the oxidant-induced reduction of cytochrome *b* can be observed also in the absence of antimycin, at low temperatures and at an extended time scale. This agrees well with the presented model (Fig. 11) if it is assumed that the reaction between cytochromes *b* and c_1

is particularily temperature-sensitive. Our model requires only a relative block of this electron-transfer step in order to explain the anomalous cytochrome *b* behaviour. This block may be induced either by antimycin, by low temperatures or by ATP.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.). One of us (M. W.) wishes to thank the European Molecular Biology Organization (E.M.B.O.) for a long-term fellowship held at the University of Amsterdam. We wish to thank Professor E. C. Slater for stimulating discussions and criticism, Professor J. M. Tager for helpful suggestions, Mrs. Tuire Wikström for drawing the figures and Mr W. de Jong for excellent technical assistance.

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