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FUSCIN, AN INHIBITOR OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN OX-NECK MUSCLE MITOCHONDRIA

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SUMMARY

1. The effect of fuscin on the mitochondrial oxidation of pyruvate *plus* malate, of succinate and of ascorbate *plus* tetramethyl-*p*-phenylenediamine (TMPD) and on the redox changes of succinate-reducible cytochromes *b* and *c* was investigated using tightly-coupled ox-neck muscle mitochondria.

2. Both respiration and oxidative phosphorylation were inhibited by fuscin. Fuscin, at a concentration of 20 nmoles per mg protein gave the following per cent inhibition of oxidative phosphorylation, measured by ADP/O ratio: pyruvate *plus* malate, 100%; succinate, 22%; ascorbate *plus* TMPD, 12%. Pre-incubation of the mitochondrial suspension with fuscin was necessary for maximal effect on the State 3 rate, respiratory control index and the ADP/O ratio.

3. Inhibition of State 3 rate induced by ADP with either succinate or ascorbate *plus* TMPD as substrate but not that of pyruvate *plus* malate could be released by *p*-trifluoromethoxy-carbonyl-cyanide-phenylhydrazone, an uncoupler of oxidative phosphorylation.

4. The aerobic steady state reduction of both cytochromes *b* and *c* was affected by fuscin, the former being more fuscin-sensitive.

5. The oxidation–reduction cycle of cytochrome *c* induced by ADP was inhibited by fuscin (29 nmoles per mg protein). At this concentration fuscin also inhibited 40% of the aerobic steady state reduction of cytochrome *c* and also delayed the time of reaching the aerobic steady state from 5 to 45 s.

INTRODUCTION

Fuscin, a quinonoid compound from the mould, *Oidiodendron fuscum*[1-2], inhibits the growth of Gram-positive bacteria[1]. It is also a potent inhibitor of mitochondrial respiration but has no uncoupling property or effect on oxidative phosphorylation[3]. It exhibits different inhibitory effects with animal and yeast mitochondria. With rat-liver mitochondria, fuscin prevents the reduction of cytochrome *b* by NADH and the succinate-linked reduction of mitochondrial NAD^+ but does not inhibit succinate oxidation[4]. However, with mitochondria from *Saccharomyces cerevisiae* which

Abbreviations: FCCP, *p*-trifluoromethoxy-carbonyl-cyanide-phenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

lack the first coupling site and with *Candida utilis*, both the oxidation of succinate and pyruvate are fuscin-sensitive[4]. Fuscin also prevents the reduction of cytochrome *b* in *Candida utilis* mitochondria during succinate oxidation[4].

This paper reports the effects of fuscin on the mitochondrial oxidation of pyruvate *plus* malate, succinate and ascorbate *plus* TMPD by the ox-neck muscle mitochondria. Complementary to previous observations[3-4], fuscin was found to be an inhibitor of both respiration and oxidative phosphorylation, affecting all three coupling sites of the respiratory chain.

MATERIALS AND METHODS

Reagents

Antimycin A (Type III), oligomycin and the sodium salts of ADP, ATP, malate, pyruvate, rotenone and succinate were obtained from Sigma; sodium salts of L-ascorbate, EDTA and TMPD from the British Drug Houses, and all other reagents were of analytical grade. Crystalline *Bacillus subtilis* proteinase (Nagarse) were purchased from Teikoku Chemical Co., Osaka. *p*-Trifluoromethoxy-carbonyl-cyanide-phenylhydrazone (FCCP) was kindly supplied by Dr P. Heytler and fuscin by Professor D. H. R. Barton.

Methods

The mitochondria from the ox-neck muscle were prepared as previously described[5] using *B. subtilis* proteinase. The mitochondrial pellet was washed three times before being suspended in 250 mM sucrose.

Oxygen uptake was measured polarographically with a Clark oxygen electrode (Yellow Spring Biological Oxygen Monitor (Model 53)) at 25 °C. The reaction medium (pH 7.2) contained 1.0 mM EDTA, 30 mM KCl, 6.0 mM $MgCl_2$, 75 mM sucrose and 20 mM KH_2PO_4. The ADP/O ratio and the respiratory control index were calculated from the electrode traces as described by Chance and Williams[6]. Protein was determined by Folin–phenol reagent[7] using bovine serum albumin as standard.

The effect of fuscin on the aerobic steady state reduction of cytochrome *b* and cytochrome *c*, measured at the wavelength pairs 562-575 and 550-540 nm respectively, was determined with an Aminco-Chance dual-wavelength spectrophotometer at room temperature using 10-mm light-path cells.

RESULTS

Pre-incubation of the mitochondrial suspension with fuscin was necessary in order to observe a maximal effect on the oxidation of pyruvate *plus* malate, succinate and ascorbate *plus* TMPD. Fig. 1 clearly illustrates this point using as an example the oxidation of succinate (Trace A). This system was only sensitive to fuscin if pre-incubation was carried out (Trace B). For routine analyses, a 10 min incubation period was therefore employed in all the experiments.

Effect of fuscin on NAD$^+$-linked substrate oxidation

Fig. 2 illustrates typical oxygen electrode traces of the ox-neck muscle mitochondria oxidizing pyruvate *plus* malate (Trace A) and the effect of fuscin on this

NAD^+-linked substrate oxidation (Trace B). In the absence of fuscin the ox-neck mitochondria oxidized pyruvate *plus* malate to give an ADP/O ratio of 2.98 and a respiratory control index of 8.7 (Trace A). The State 3 rate was inhibited by oligomycin and this inhibition could be relieved by the uncoupler, FCCP. With the fuscin-

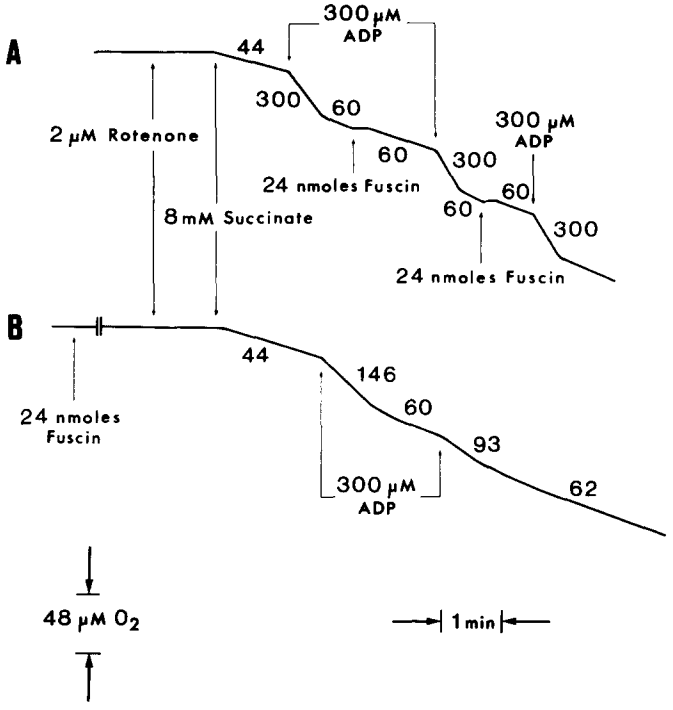

Fig. 1. Typical polarographic tracings showing the effect of fuscin on the oxidation of succinate with and without pre-incubation of the mitochondria with fuscin. The numbers adjacent to the oxygen electrode traces are respiratory activities expressed in natoms O per min per mg protein. Trace A, succinate oxidation without pre-incubation of the mitochondria with fuscin. Trace B, succinate oxidation with 10 min incubation with fuscin. The concentration of fuscin in the electrode traces is expressed in nmoles per mg protein. Total protein in A and B: 1.60 mg.

treated mitochondria (18 nmoles per mg protein), only the first addition of ADP could induce the classical State 3 to State 4 transition[6], giving a very low respiratory control index value of about 2.3 (Trace B) as compared with 8.7 (Trace A). Furthermore, the first State 3 rate was only 26 % of that observed with the mitochondria oxidizing pyruvate *plus* malate in the absence of fuscin. The subsequent addition of FCCP after the second ADP addition did not increase the rate of oxygen uptake. The result also shows that fuscin has no apparent effect on the State 4 rate.

Four parameters (State 3 and FCCP-uncoupled rates, ADP/O ratio and respiratory control index) were thus employed to study the effect of fuscin on the pyruvate *plus* malate oxidation by the ox-neck muscle mitochondria. Fig. 3 illustrates that complete inhibition was obtained with all the parameters at a concentration of 20 nmoles fuscin per mg protein. Control experiments using the same volume of ethanol instead of fuscin had no effect on the pyruvate *plus* malate oxidation.

Effect of fuscin on the succinoxidase system

The effect of fuscin on the succinate oxidation is clearly demonstrated in Fig. 4. As in the case of pyruvate *plus* malate, a marked inhibitory effect by fuscin on succinate oxidation was observed during the second addition of ADP (Trace B). Unlike that reported for the oxidation of pyruvate *plus* malate, FCCP addition stimulated

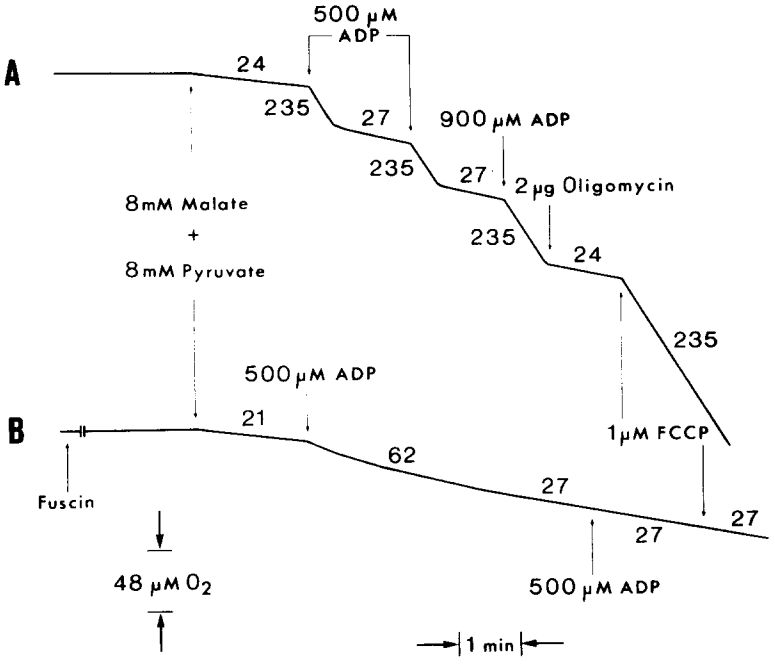

Fig. 2. Typical polarographic tracings showing the effect of fuscin on the oxidation of pyruvate *plus* malate by the ox-neck muscle mitochondria. Trace A, pyruvate *plus* malate oxidation; Trace B, pyruvate *plus* malate oxidation in the presence of fuscin (18 nmoles per mg protein). || indicates 10 min incubation with the mitochondrial suspension. Total protein in A and B: 1.81 mg.

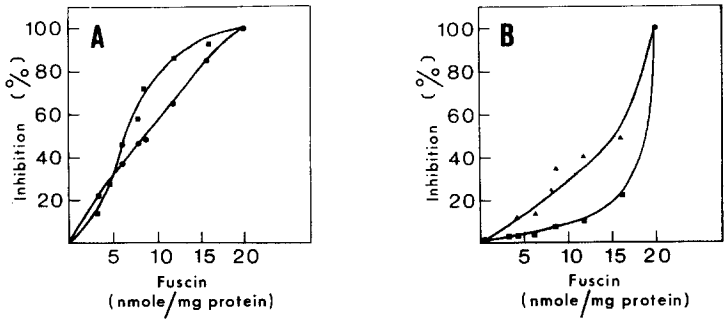

Fig. 3. Effect of fuscin on the State 3 and FCCP-uncoupled rates, ADP/O ratio and respiratory control index of pyruvate *plus* malate oxidation by ox-neck mitochondria. The data were calculated from the electrode traces as described by Chance and Williams[6]. Other experimental details as described in Figs 1 and 2. (A) ●, State 3 rate; ■, FCCP-uncoupled rate. (B) ■, ADP/O ratio; ▲, respiratory control index.

respiration, the oxygen uptake rate being greater than that observed for the first State 3 respiration (Trace B). Thus, FCCP could relieve the inhibition of the succinoxidase activity caused by fuscin. The first addition of ADP to the mitochondria previously treated with fuscin also showed a decrease in respiratory control index, from a value of 5.79 (Trace A) to 1.38 (Trace B). A decline in the State 3 rate was also observed, from 307 to 146 natoms oxygen per min per mg protein at 25 °C.

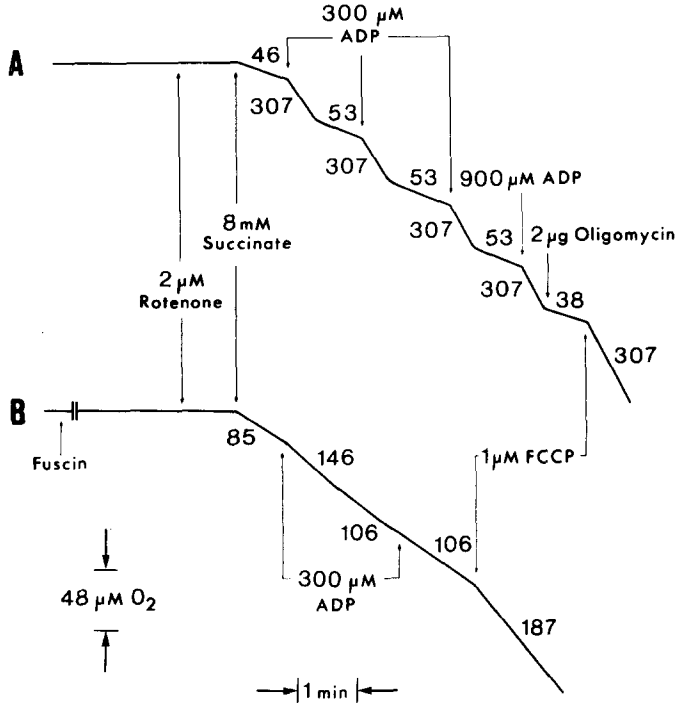

Fig. 4. Typical oxygen electrode traces illustrating the effect of fuscin on the succinoxidase system of the ox-neck mitochondria. Experimental details as described in Figs 1 and 2 except that 30 nmoles fuscin per mg protein were employed. Total protein concn: A, 1.63 mg; B, 1.54 mg.

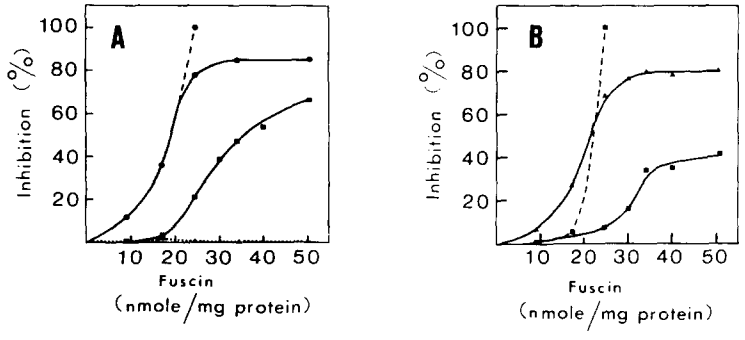

Fig. 5. Effect of fuscin on the State 3 and FCCP-uncoupled rates, ADP/O ratio and respiratory control index of succinate oxidation by the ox-neck mitochondria. Experimental details as described in Figs 1 to 3. (A) ●, State 3 rate; ■, FCCP-uncoupled rate; ▲, State 4 rate. (B) ■, ADP/O ratio; ▲, respiratory control index; – – – –, data calculated from the second State 3 to State 4 transition induced by ADP addition.

Fig. 5 shows the inhibitory effect of various concentrations of fuscin on the succinoxidase system of the ox-neck muscle mitochondria. As in the case of pyruvate *plus* malate, no apparent inhibition of the State 4 rate by fuscin was observed. Fuscin also had no effect on the oxygen uptake of aged ox-neck mitochondria which had lost their response to ADP addition. About 60 % of the State 3 rate and 8 % of the FCCP-uncoupled rate were inhibited by 20 nmoles fuscin per mg protein, a concentration which was previously demonstrated to block completely both the State 3 and the uncoupled-rates of pyruvate *plus* malate oxidation. If the calculation were based on the second ADP addition, 20 nmoles fuscin per mg protein completely blocked the State 3 to State 4 transition, giving a 100 % inhibition of the State 3 rate (Fig. 5A, - - - - - -). Both the ADP/O ratio and the respiratory control index (Fig. 5B) were also affected by fuscin.

Effect of fuscin on the ascorbate plus TMPD oxidase system

Analogous with both the oxidation of pyruvate *plus* malate and succinate, only the second addition of ADP in the presence of fuscin could prevent the classical State 3 to State 4 transition, resulting in a decline in the State 3 rate and respiratory control index (Fig. 6, Trace B). Unlike the oxidation of pyruvate *plus* malate but similar to the oxidation of succinate the subsequent addition of FCCP could still stimulate respiration, giving a rate comparable to that observed in the absence of fuscin (Fig. 6, Trace A and B).

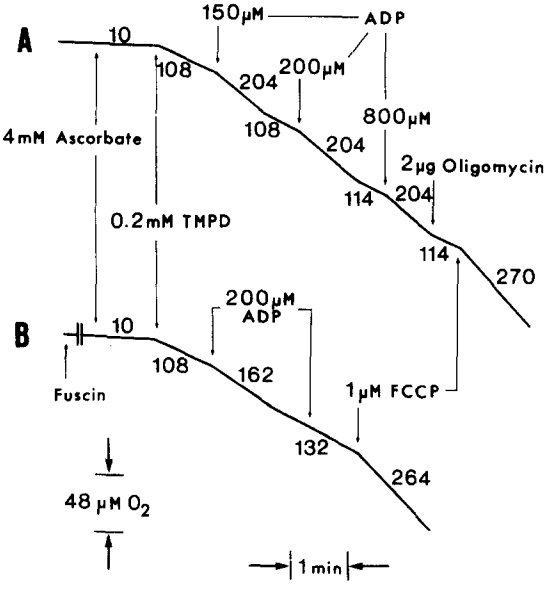

Fig. 6. Effect of fuscin on the ascorbate *plus* TMPD oxidation by the ox-neck mitochondria. Experimental procedure as described in Figs 1 and 2 except that antimycin A (0.2 μg per mg protein) was added to the reaction medium in A and B. In experiment B, 36 nmoles fuscin per mg protein were incubated with the mitochondrial suspension. Total protein concn: 1.0 mg protein in A and B.

Fig. 7 summarizes the effect of fuscin on the State 3 rate (●), ADP/O ratio (■) and the respiratory control index (▲) for the ascorbate *plus* TMPD oxidation. The data clearly illustrate that the cytochrome oxidase (EC 1.9.3.1) activity was the least sensitive to fuscin when compared with the oxidation of pyruvate *plus* malate (Figs. 2 and 3) and succinate (Figs. 4 and 5). For example, at a concentration of 20 nmoles fuscin per mg protein the following per cent inhibition in the ADP/O ratio was observed: ascorbate *plus* TMPD, 12 %; succinate, 22 %; pyruvate *plus* malate, 100 % (the calculation for both the oxidation of ascorbate *plus* TMPD and succinate was based on the second ADP addition).

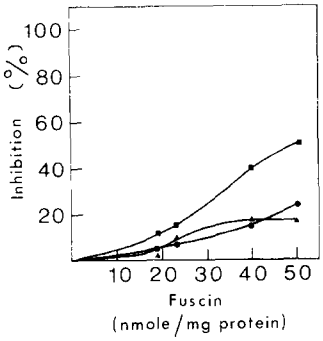

Fig. 7. Effect of fuscin on the State 3 rate, ADP/O ratio and respiratory control index of the ascorbate *plus* TMPD oxidation by the ox-neck mitochondria. Experimental details as described in Figs 1 to 3 and 6. ■, ADP/O ratio; ▲, respiratory control index; ●, State 3 rate.

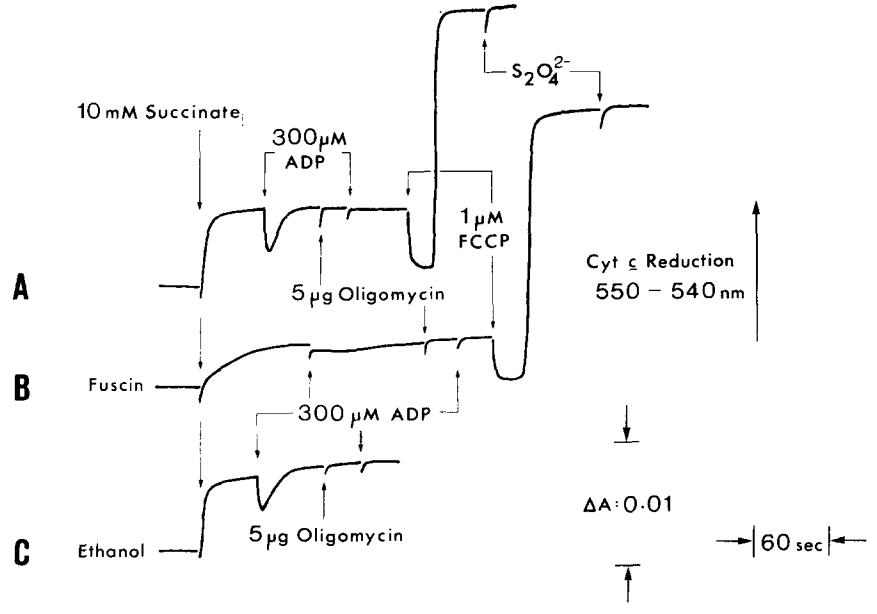

Fig. 8. Effect of fuscin on the redox states of cytochrome *c*. The experiments were carried out in 10-mm light-path cells containing 2.8 ml mitochondrial suspension (4.65 mg protein per ml). 3 μM rotenone was added in all the experiments. In experiment B, the mitochondria were incubated with 29 nmoles fuscin per mg protein for 10 min before succinate addition. The mitochondria were suspended in the same reaction medium as in the oxygen electrode experiments.

Effect of fuscin on the redox states of cytochromes b and c

Succinate was used as the electron donor to study the effect of fuscin on the redox states of both cytochromes *b* and *c*. Fig. 8 (Trace B) shows the maximal effect of fuscin (29 nmoles per mg protein) on the succinate-reducible cytochrome *c*. In the absence of fuscin (Trace A), succinate caused a rapid reduction of cytochrome *c*, the aerobic steady state was reached at about 5 s. Addition of ADP during the aerobic steady state induced an oxidation–reduction cycle of cytochrome *c*, indicating that the mitochondria were tightly coupled.

The addition of oligomycin, an inhibitor of oxidative phosphorylation, prior to the second addition of ADP completely blocked the redox changes of cytochrome *c*. When the mitochondria were pre-incubated with fuscin (Trace B), the aerobic steady state of cytochrome *c* was reached at about 45 s after succinate addition as compared with 5 s in the absence of fuscin. Furthermore, the subsequent addition of ADP during the aerobic steady state failed to induce the marked redox changes as previously observed in the absence of fuscin (Trace A), indicating that fuscin affected oxidative phosphorylation. The control experiment (Trace C) using the same volume of ethanol as fuscin (Trace B) showed no effect on the aerobic steady state of cytochrome *c* reduction nor on the oxidation–reduction cycle induced by ADP.

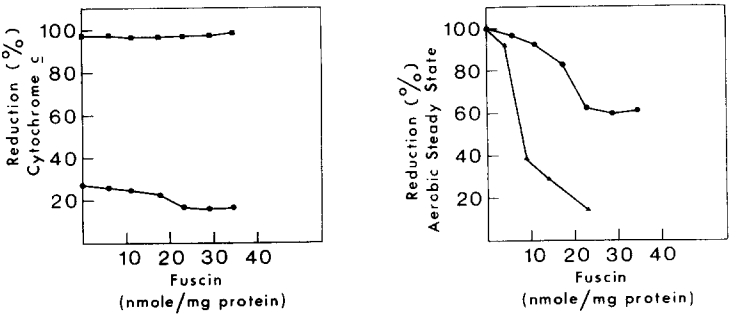

Fig. 9. Effect of fuscin on the aerobic steady state and the anaerobic state reduction of cytochrome *c* by succinate. Experimental procedure as described in Fig. 8. The % reduction of cytochrome *c* by succinate was based on the dithionite reduction assuming that dithionite gave a 100% reduction. ●, aerobic steady state; ■, anaerobic state.

Fig. 10. Effect of fuscin on the aerobic steady state reduction of cytochromes *b* and *c* by succinate. The 100% reduction of the aerobic steady states of both cytochromes *b* and *c* refers to the extent of reduction in the absence of fuscin. Other experimental details as described in Fig. 8. ●, aerobic steady state reduction of cytochrome *c*; ▲, aerobic steady state reduction of cytochrome *b*.

Only the aerobic steady state of cytochrome *c* reduction was sensitive to fuscin (Fig. 9). The effect of various concentrations of fuscin on the cytochrome *c* reduction in the aerobic steady state (●) was compared with that of cytochrome *b* (▲) using succinate as the electron donor (Fig. 10). The 100% values refer to the extent of the aerobic steady state reduction of both cytochromes *b* and *c* in the absence of fuscin. The data suggest that cytochrome *b* reduction was more sensitive to fuscin than was cytochrome *c* reduction. The effect of fuscin on cytochromes aa_3 reduction was not followed since fuscin had only a small inhibitory effect on the cytochrome oxidase activity (Figs. 6 and 7).

DISCUSSION

The above data extend further the range of inhibitory effects by fuscin, previously reported for rat-liver and yeast mitochondria[3-4]. Complementary to earlier observations[3-4], fuscin inhibited both respiration and oxidative phosphorylation with ox-neck muscle mitochondria. Pre-incubation with fuscin, not carried out by previous investigations[3-4], was essential to obtain a maximal effect. This was especially noticeable when determining the effect of fuscin on the third coupling site using ascorbate *plus* TMPD as the electron donor. In addition, fuscin also affected the aerobic steady state reduction of cytochromes *b* and *c*. The degree of sensitivity to fuscin appears to be dependent on the coupling sites of the ox-neck muscle mitochondria, with the following decreasing order of sensitivity established: pyruvate *plus* malate, succinate and ascorbate *plus* TMPD.

In comparing the data obtained with mitochondria from rat-liver[3-4] and the ox-neck muscle, it appears that fuscin has differential inhibitory effects not only with yeast mitochondria[3-4] but also with animal mitochondria. Only the oxidation of the NAD^+-linked substrate was blocked by fuscin in rat-liver mitochondria[3-4] while with ox-neck mitochondria the oxidation of all the substrates donating to the three coupling sites was fuscin-sensitive. It is also quite possible that pre-incubation of rat-liver mitochondria with fuscin would have shown up its inhibitory effect on succinate oxidation. The effect of fuscin on the cytochrome oxidase activity of yeast and rat-liver mitochondria was not determined by Demaille *et al.*[4].

It has recently been shown in rat-liver mitochondria that fuscin inhibits the transport of inorganic phosphate[8]. A similar mechanism might apply to ox-neck muscle mitochondria possibly also involving the transport of ADP. Alternatively, fuscin could interfere with the transport mechanism by binding with the mitochondrial membrane[9].

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