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IRON–SULPHUR PROTEINS IN THE SUCCINATE OXIDASE SYSTEM

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SUMMARY

1. Succinate reduces at least three different species of iron–sulphur protein, detectable by EPR spectroscopy at 82°K, in sub-mitochondrial particles or preparations of Complex I + II + III. Two of these species (symbolized as Fe–S_8 and Fe–S_{P_1}) are rapidly reduced by succinate in non-phosphorylating particles, the third (Fe–S_{P_2}) is slowly reduced.

2. The reduction of Fe–S_8 is unaffected by addition of 2-thenoyltrifluoroacetone (TTFA), or by extraction of the dried particles with pentane. This species probably belongs to succinate dehydrogenase.

3. Reduction of the other rapidly reduced species (Fe–S_{P_1}) is inhibited by addition of TTFA or by pentane extraction. Re-incorporation of Q-10 or 'P' (ALBRACHT, VAN HEERIKHUIZEN AND SLATER, *FEBS Lett.*, 13 (1971) 265) restores the rate of reduction.

4. Antimycin blocks about one-half of the EPR signal of the slowly reduced species (Fe–S_{P_2}).

5. All three iron–sulphur species reducible by succinate have similar EPR spectra with $g_z = 2.02$, $g_y = 1.94$ and $g_x = 1.92$.

6. NADH also reduces these iron–sulphur species as well as those associated specifically with NADH dehydrogenase.

7. The bleaching of a pigment absorbing at 460 nm brought about by addition of succinate to pentane-extracted particles is largely (62 %) due to reduction of succinate dehydrogenase (flavin and Fe–S_8). Fe–S_{P_1}, Fe–S_{P_2} and cytochrome *b* contribute to the extent of 15 %. The component responsible for the remainder (23 %) has not been identified. Its reduction is inhibited by TTFA.

8. A model of the respiratory chain is proposed that takes account of the recently discovered electron carriers.

INTRODUCTION

The experiments described in this paper arose out of an investigation of the pigments absorbing at 460–510 nm in respiratory-chain preparations. In 1967, CHANCE

Abbreviations: TTFA, 2-thenoyltrifluoroacetone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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et al.[1] proposed that two flavoproteins absorbing in this region are bleached on adding NADH to beef-heart mitochondria. The higher-potential flavoprotein (fp_{D_2}) was supposed to be reducible by succinate as well as by NADH, whereas the lower-potential component (fp_{D_1}) is reducible only by NADH. It was further suggested that the site of action of rotenone is between the two flavoproteins. HATEFI[2,3] also concluded that Complex I contains two carriers absorbing in this region, one before and one after the rotenone block. In disagreement with CHANCE *et al.*[1], however, HATEFI[3] believes that the carrier on the oxygen side of the rotenone block in the respiratory chain is an iron–sulphur protein, not a flavoprotein.

CHANCE *et al.*[1] and HATEFI[3] agree that both carriers are on the substrate side of ubiquinone. In a previous publication[4], however, we showed that the bleaching, measured at 465 nm with 510 nm as the reference wavelength, is completely insensitive to rotenone after extraction of the ubiquinone with *n*-pentane, suggesting that the carrier whose reduction is blocked by rotenone is beyond ubiquinone, and is therefore presumably an impurity in the Complex-I preparation. One-third of the rotenone-sensitive but antimycin-insensitive NADH-induced decline of $A_{465-510\,nm}$ in sub-mitochondrial particles from beef heart (the Keilin and Hartree heart-muscle preparation) is accounted for by reduction of endogenous ubiquinone which also absorbs at 465 nm. We further calculated that 88–94 % of the rotenone-sensitive bleaching of Complex I by NADH could be accounted for by ubiquinone also present in this preparation. In a personal communication, Dr. Hatefi drew our attention to the fact that this calculation must be in error, and, on re-examination of our data it was indeed found that a mistake of a factor of 10 had been made in the calculation of the contribution of ubiquinone to the observed bleaching in Complex I. (No error was made, however, in the calculation relating to heart particles). Thus, only 9 % of the rotenone-sensitive bleaching in Complex I is accounted for by ubiquinone, leaving 91 % unidentified. In the present paper the possibility is examined that the unidentified pigment is an iron–sulphur protein acting between ubiquinone and oxygen in the NADH oxidation chain.

Since it reduces the higher-potential pigment but not that with the lower potential[1], succinate was used as electron donor. The characteristic EPR spectrum of reduced succinate dehydrogenase, with lines at $g = 2.02$, 1.94 and 1.92 in addition to $g = 2.00$ free-radical signal, has been known since the pioneer studies of BEINERT AND SANDS[5,6] in 1960. Already in 1961, ZIEGLER[7] and DOEG[8] proposed an iron (non-haem) protein linking succinate dehydrogenase with ubiquinone in Complex II as the site of action of the inhibitor 2-thenoyltrifluoroacetone (TTFA). HATEFI *et al.*[9] have recently split Complex II into a ferroflavoprotein (succinate dehydrogenase) and an iron–sulphur protein with a function similar to that proposed by ZIEGLER[7] and DOEG[8]. No EPR measurements were carried out in these studies[7-9].

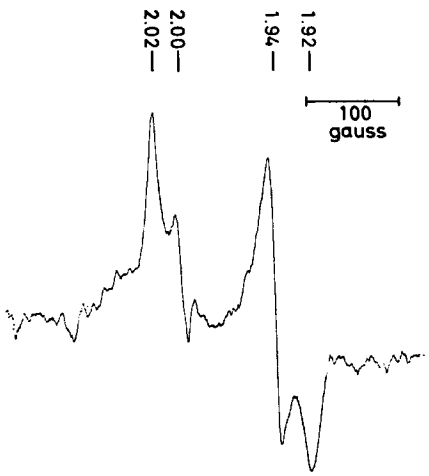
In this paper, evidence is presented for the existence in the succinate oxidation pathway, between the TTFA block and the cytochromes, of two Fe–S proteins with EPR spectra similar to that of the succinate dehydrogenase Fe–S moiety. Since one of these proteins is present in Complex I, its reduction by NADH is sensitive to rotenone and requires ubiquinone, and it absorbs at 460 nm, it has the properties of the unidentified pigment. However, its absorbance coefficient and concentration are too low to account for more than a small fraction of the rotenone-sensitive bleaching by NADH, or the TTFA-sensitive bleaching by succinate.

Although these experiments have not solved the identity of the missing pigment, they have provided new information on the components of the succinate oxidase system and, together with other experiments in this laboratory, have stimulated us to propose a new model of the respiratory chain.

RESULTS

Fe–S components reducible by succinate

Isolated Complex I + III (ref. 10) usually contains small amounts of succinate dehydrogenase. Fig. 1 shows the EPR spectrum of a preparation of Complex I + III reduced with 20 mM succinate and immediately frozen in liquid nitrogen. Lines with g values at 2.02, 2.00, 1.94 and 1.92 are clearly visible. The height of the $g = 1.94$ line was the same when the suspension was incubated with succinate for up to 15 min at 25° before freezing (Fig. 2). In the presence of TTFA, the height of the line found after immediate freezing was only 5 % that obtained in the absence of TTFA. Since TTFA has no effect on the $g = 1.94$ line in isolated succinate dehydrogenase (unpublished observations), 95 % of the $g = 1.94$ line in Complex I + III appearing with succinate is not due to succinate dehydrogenase but to iron–sulphur proteins located in the respiratory chain after the dehydrogenase. Inhibition by TTFA was incomplete, and the intensity of the $g = 1.94$ line finally reached after 8-min incubation was not affected by TTFA.



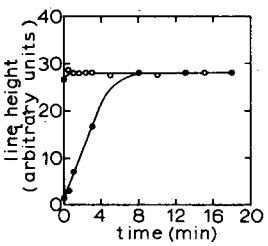


Fig. 1. EPR spectrum of Complex I + III reduced with succinate. Complex I + III (41 mg/ml), suspended in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine, was mixed with 20 mM succinate and immediately frozen in liquid nitrogen. Spectrum measured at 83°K, modulation amplitude 6.3 gauss, power 80 mW.

Fig. 2. Effect of TTFA on the rate of appearance of the $g = 1.94$ line of Complex I + III reduced with succinate. Complex I + III (41 mg/ml), suspended in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine, was mixed with 20 mM succinate and incubated at 25° for the time indicated and then frozen in liquid nitrogen, and the EPR spectrum measured as in Fig. 1. The top *minus* trough is taken as the line height. (○—○), without inhibitor; (●—●), with 40 nmoles TTFA/mg protein.

In Fig. 3A the effects of TTFA and antimycin on the succinate-induced signals in non-phosphorylating sub-mitchondrial particles (Keilin and Hartree heart-muscle preparation[11]), are shown. Ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and cyanide were added to reduce the copper of cytochrome oxidase which gives a strong EPR line with $g = 1.99$ (trough)[12]. The kinetics of the appearance of the line at $g = 1.94$ are biphasic, about 65 % of the line appearing virtually instantaneously, and the remaining in about 2 min at 10 °C. Antimycin has no effect on the rapid phase, but blocks about half of the line appearing in the slower phase. TTFA blocks rather more than one half of the rapidly appearing line (*cf.* Figs. 4A and 4B).

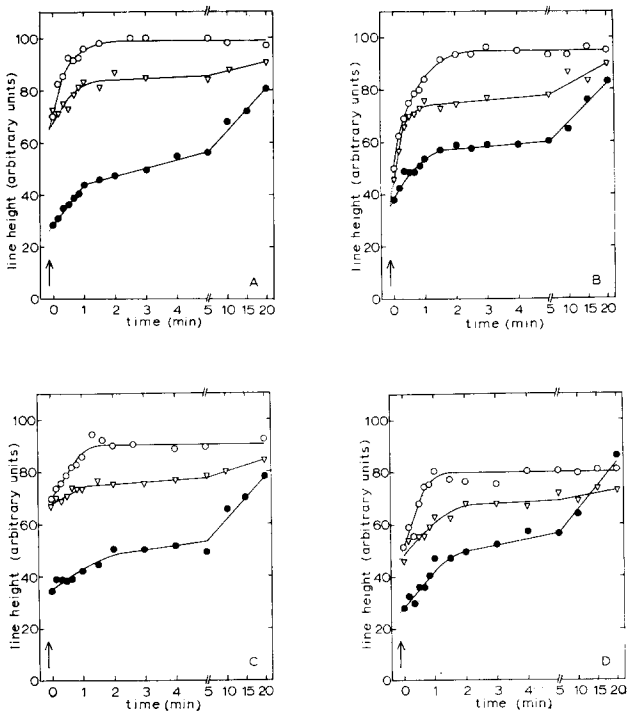


Fig. 3. Effect of TTFA and antimycin on the appearance of the $g = 1.94$ line in lyophilized, pentane-extracted and P- and Q-10-reconstituted heart-muscle particles with succinate as substrate. The preparations (48 mg/ml), suspended in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 8.0), were mixed with 2 mM KCN, 4 mM Tris–HCl buffer (pH 8.0), 0.18 mM TMPD, 2 mM ascorbate and 20 mM succinate and incubated at 10 °C. A, lyophilized heart-muscle particles; B, lyophilized particles extracted 9 times with pentane; C, P-reconstituted particles; D, Q-10-reconstituted particles (6.3 nmoles Q-10/mg protein). (○—○), no inhibitor; (▽—▽), antimycin (0.6 nmole/mg protein); (●—●), TTFA (33 nmoles/mg protein).

Pentane extraction of dried particles removes the ubiquinone and an unidentified substance, called 'P', that restores succinate oxidation but not NADH oxidation in pentane-extracted particles[13]. Q-10 restores the oxidation of both succinate and NADH[14]. In the experiment described in Fig. 3B, the pentane extraction was not quite complete, and the particles still oxidized succinate at a rate of 5 nmoles/min

per mg of protein. The TTFA-insensitive component was still rapidly reduced by succinate in these particles, but the other component rapidly reduced in the unextracted particles in the absence of TTFA (Fig. 3A) was only slowly reduced. Thus P and/or Q is necessary for reduction of the latter component but not for that of the TTFA-insensitive rapidly reducible component. P- and Q-reconstituted heart-muscle preparations[13] (Figs. 3C and 3D) behaved in all respects like the unextracted preparations.

The effect of extraction of Complex I + II + III (ref. 10) with pentane and of the addition of inhibitors is shown in Fig. 5. Ascorbate, TMPD and cyanide were omitted in this experiment, because this preparation is deficient in cytochrome oxidase. The kinetics of the reduction of unextracted preparation in the presence of TTFA (Fig. 5A) or of pentane-extracted preparation in the absence of inhibitor (Fig. 5B) suggests, however, that some residual oxidase was present and that complete reduction occurred only on exhaustion of the oxygen.

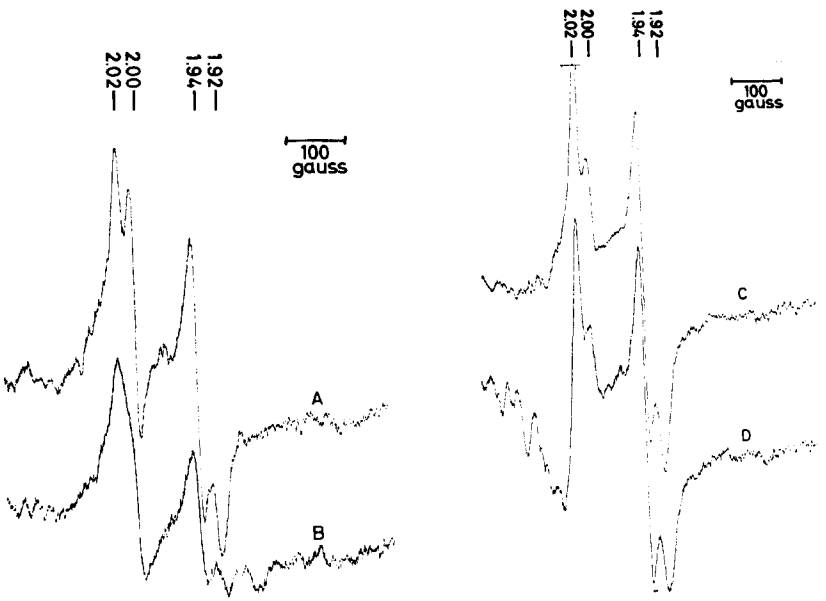


Fig. 4. Effect of TTFA on the EPR spectra of lyophilized heart-muscle preparations reduced with succinate. Conditions as in Fig. 3. Trace A, no inhibitor, frozen immediately after mixing with succinate; Trace B, TTFA (33 nmoles/mg protein) present, and frozen immediately after mixing with succinate; Trace C, no inhibitor, frozen after 20 min standing with succinate at 10 °C; Trace D, TTFA present, frozen after 40 min standing with succinate at 10 °C. Spectrum conditions: temperature, 82 °K; modulation amplitude, 16 gauss; power, 80 mW.

The experiments shown to date suggest that at least three components contribute to the $g = 1.94$ line in sub-mitochondrial particles (Complex I + II + III + IV) and Complex I + II + III, and two in Complex I + III. The properties of the three components and the approximate proportions in which they appear in the three preparations are listed in Table I. All three preparations contain two components rapidly reduced by succinate, only one of which is sensitive to TTFA and requires P and/or Q for its reduction. Preparations of Complex I + II + III and of Complex I + II + III + IV contain in addition a slowly reduced component (No. 3), sensitive to antimycin.

The effect of TTFA on the reaction with pentane-extracted Complex I + II + III suggests that reduction of this component is inhibited by TTFA. Since removal of (P + Q) is incomplete, and the reduction of this component is slow, the requirement for P and/or Q for its reduction is uncertain. It is possible that the slowly reduced component in preparations of Complex I + II + III + IV contains in reality two components, only one of which is sensitive to antimycin (see Fig. 3A). Component 1 is probably part of succinate dehydrogenase itself. Components 2 and 3 correspond to the non-haem iron[7,8] or iron–sulphur[9] components proposed by previous authors.

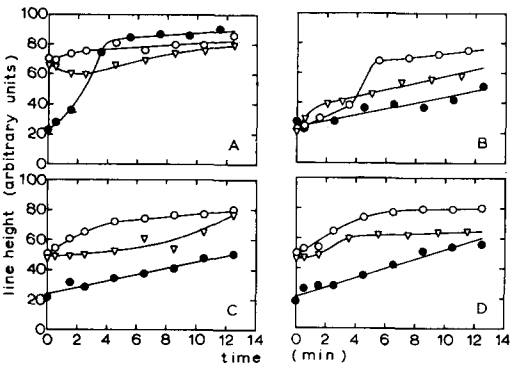


Fig. 5. Effect of TTFA and antimycin on the rate of appearance of the $g = 1.94$ line in lyophilized, pentane-extracted and P- and Q-10-reconstituted Complex I + II + III with succinate as substrate. Complex I + II + III (36.5 mg protein/ml), suspended in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine was mixed with 20 mM succinate and frozen in liquid nitrogen after various periods at 22 °C. A, lyophilized preparation; B, lyophilized preparation extracted 4 times with pentane; C, P-reconstituted preparation; D, Q-10 (Sigma)-reconstituted (7 nmoles Q-10/mg protein). (○—○), no inhibitor; (▽—▽), antimycin (4 nmoles/mg protein); (●—●), TTFA (44 nmoles/mg protein).

TABLE I

COMPONENTS CONTRIBUTING TO $g = 1.94$ LINE WITH SUCCINATE AS SUBSTRATE

	Component 1	*Component 2*	*Component 3*
Kinetics of reduction by succinate	Fast	Fast	Slow
TTFA sensitivity	—	+	+
Antimycin sensitivity	—	—	+
Requirement for P and/or Q	—	+	?
Proportions in I + III*	5	95	0
Proportions in I + II + III**	28	41	31
Proportions in I + II + III + IV***	32	34	34

* Calculated from Fig. 2.

** Calculated from Fig. 5 (mean of unextracted, P-reincorporated and Q-10-reincorporated preparations).

*** Calculated from Fig. 3 (mean of unextracted, P-reincorporated and Q-10-reincorporated preparations).

The antimycin-sensitive Component 3 was also found in phosphorylating Mg-ATP sub-mitochondrial particles[15].

Since the relative intensities of the three lines of the EPR spectrum of the iron-sulphur proteins (at $g = 2.02$, 1.94 and 1.92) are similar when Component 1 (Fig. 4B),

Components 1 and 2 (Fig. 4A) and Components 1, 2 and 3 (Figs. 4C and 4D) are reduced, it appears that all three components have a similar EPR spectrum.

Fe–S components reducible by NADH

The effect of pentane extraction on the EPR spectrum obtained on adding NADH to heart-muscle particles is shown in Fig. 6. The shoulder at $g = 1.92$ seen in unextracted particles is missing in the pentane-extracted particles. Moreover, the height of the $g = 1.94$ line is greatly decreased by pentane extraction. It may be concluded that the iron–sulphur proteins reduced by succinate, characterized by the line at $g = 1.92$ (trough), are not reducible by NADH after pentane extraction, pre-

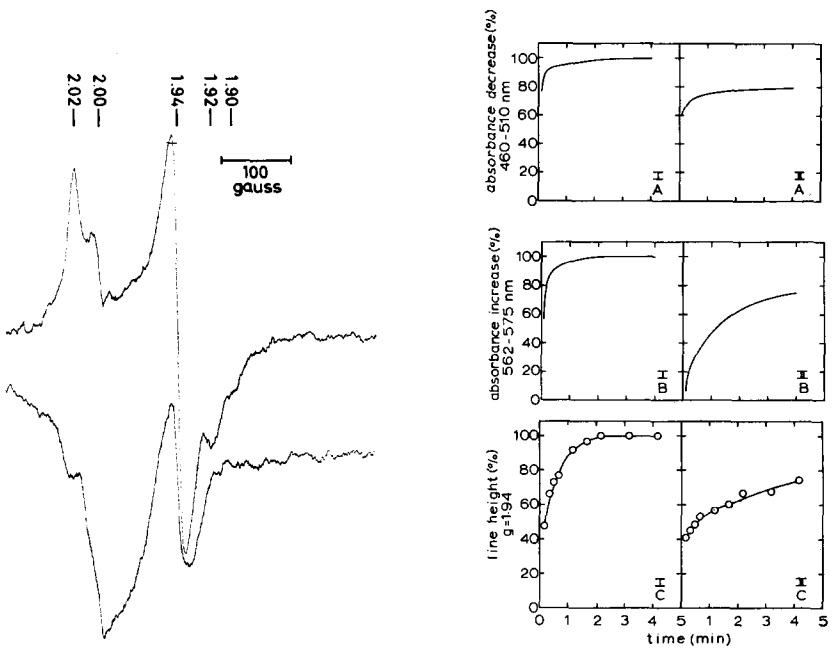


Fig. 6. Effect of pentane extraction on the EPR spectrum of 'A' particles with NADH as a substrate. 'A' particles (41 mg/ml), suspended in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 8.0), were mixed with 3 mM NADH in an EPR tube and incubated for 45 sec at 22 °C. The tube was then immersed in liquid nitrogen and stored until the EPR spectrum was recorded. Upper trace, untreated particles; lower trace, particles extracted 5 times with *n*-pentane. EPR conditions: modulation amplitude, 10 gauss; power, 80 mW; temperature, 83 °K.

Fig. 7. Comparison of the kinetics of changes at 460–510 nm, 562–575 nm and of the $g = 1.94$ line of pentane-extracted heart-muscle particles with succinate as substrate. A and B: Particles (3 mg protein/ml), suspended in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA, were mixed with 5.4 mM KCN, 5.4 mM Tris–HCl buffer (pH 8.0), 2.4 μM cytochrome *c* and 2.7 mM ascorbate. 4 mM succinate was then added and absorbance changes at the indicated wavelength pair were followed with the time in an Aminco-Chance dual-wavelength spectrophotometer. Temperature, 22 °C. C: Particles (48 mg protein/ml), suspended in 0.25 M sucrose and 50 mM Tris–HCl buffer (pH 8.0), were mixed with 5.4 mM KCN, 5.4 mM Tris–HCl buffer (pH 8.0), 2.4 μM cytochrome *c*, 2.7 mM ascorbate and 19 mM succinate and incubated at 22 °C for the times indicated before freezing in liquid nitrogen. The EPR spectra were measured as in Fig. 3. The particles were extracted 9 times with pentane. Series I, no inhibitor; Series II, TTFA (34 nmoles/mg protein) added.

sumably due to removal of Q. They must, then, lie after ubiquinone in the NADH oxidation chain.

Contribution of Fe–S proteins to $\Delta A_{460-510\,nm}$ in particle preparations

In order to eliminate interference from the Fe–S protein associated with NADH dehydrogenase, succinate was used as substrate. The rotenone-sensitive decline of $A_{475-510\,nm}$ obtained with NADH is also found with succinate in the absence of rotenone, whereas the latter substrate does not reduce the Fe–S protein associated with NADH dehydrogenase that is reducible by NADH in the presence of rotenone[1]. Pentane-extracted particles were used in order to minimize interference by Q-10, reduction of which also causes a decline of $A_{465-510\,nm}$ (ref. 4).

The absorbance changes could be followed 5 sec after adding the succinate, during which period 75 % of the bleaching at 460 nm had already taken place. The absorbance was constant after 3 min (see Curve IA, Fig. 7). The kinetics of cytochrome *b* reduction, followed with the wavelength pair 562–575 nm, were similar to those of the bleaching. Reduction of the Fe–S proteins, Components 2 and 3 (Table I), is considerably slower in the pentane-extracted particles than the bleaching (*cf.* Curves IA and IC in Fig. 7).

TTFA markedly inhibited the reduction of cytochrome *b* as well as of Components 2 and 3 of the iron–sulphur proteins. It had little effect on the initial kinetics of the bleaching, but caused a decline of 21 % in its extent after 4 min.

The difference between the kinetics of the bleaching at 460 nm and the reduction of the Fe-S proteins in the absence of TTFA, and between the bleaching and the reduction of cytochrome *b* in the presence of TTFA, show that neither the Fe–S proteins nor the cytochrome *b* make the major contribution to the 460-nm pigment. Between 45 sec and 4 min, in the presence of TTFA, the degree of reduction of cytochrome *b* increased by 34 %, and of the Fe–S proteins, Components 2 and 3*, by 31 %. During this period the $\Delta A_{460-510\,nm}$ increased by only 5 % of the final value reached in the absence of inhibitor. Thus, maximally 15 % of the bleaching is due to reduction of these components. Another calculation shows that the contribution of the cytochrome *b* is very small. Between 45 sec and 4 min, the percentage of reduction increased from 94 % to 100 % in the absence of TTFA, and from 41 to 75 % in the presence of TTFA. Thus, the increase in the presence of TTFA is 28 % (complete reduction = 100 %) more in the presence of TTFA than in its absence. TTFA had no effect in the increment of $A_{460-510\,nm}$ during the same period.

The TTFA-insensitive rapid bleaching is probably due to reduction of the flavin and Fe–S moieties of the succinate dehydrogenase. The contribution of the dehydrogenase to the total bleaching may be calculated from the data at 10 sec in the presence of TTFA, when only 13 % of the Fe–S proteins, Components 2 and 3, and 17 % of the cytochrome *b* are reduced, and $\Delta A_{460-510\,nm}$ is 64 % of the maximal value in the absence of TTFA. The combined contribution of the Fe–S proteins and cytochrome *b* at this time can be calculated to be about 2 %, leaving 62 % for the succinate dehydrogenase.

It may be concluded, then, that 62 % of the total bleaching is due to reduction of succinate dehydrogenase (including Fe–S protein, Component 1), 15 % to Fe–S proteins, Components 2 and 3, and 23 % to unidentified TTFA-sensitive components.

* The contribution of Components 2 and 3 to the $g = 1.94$ line has been calculated from Table I.

DISCUSSION

At least three species of Fe–S proteins, all with the EPR spectrum typical for succinate dehydrogenase ($g_z = 2.02$, $g_y = 1.94$, $g_x = 1.92$)[5,6], have been shown in the present study to be present in the succinate oxidase system. NADH as well as succinate reduces these species. There is also evidence of the existence of two species of iron–sulphur associated with NADH dehydrogenase. Prolonged incubation of Complex I with NADH causes the disappearance of one-half of the $g = 1.94$ line[16,17], while simultaneously a signal ascribed to molybdenum* appears[17]. The results of BOIS AND ESTABROOK[18], who found that part of the $g = 1.94$ line remains after NADH added to respiratory particles in the presence of rotenone was oxidized by excess oxygen present (*cf.* ref. 19) are in agreement with this conclusion.

It seems then that the basic unit of both Complex I (NADH dehydrogenase complex) and Complex II (succinate dehydrogenase complex) contains multiple electron acceptors. Complex I contains the FMN of NADH dehydrogenase, and four Fe–S species. Complex II contains the covalently bound FAD of succinate dehydrogenase and three Fe–S species.

The NADH dehydrogenase complex is linked to the cytochromes *via* Q-10 (refs. 14, 20). It has recently been shown[13] that succinate oxidation is possible in the absence of Q-10 provided that a lipophilic component, 'P', extracted from Q-free particles is added. P may be replaced by Q-10 in reconstitution experiments. The data presented in this paper indicate that P is required for reduction of the Fe–S components acting on the cytochrome side of the TTFA block. It is not known whether P is an electron carrier, or acts indirectly.

Electrons from NADH and succinate enter the cytochrome chain at cytochrome *b*. Two species of cytochrome *b* have been identified on the basis of reactivity with antimycin[21] and redox potential[22]. The bc_1 segment of the respiratory chain (Complex III) contains four different electron carriers, *viz.* the two cytochromes *b* (*b* and b_i[21]), cytochrome c_1 and the Fe–S protein, with an EPR line at $g_y = 1.90$, discovered by RIESKE *et al.*[23].

Four electron carriers are also present in cytochrome *c* oxidase (Complex IV), the segment of the respiratory chain between cytochrome *c* and oxygen, namely two haem groups and two copper atoms, distinguished by their reactivity with the classical inhibitors of intracellular respiration[24]. One of these copper atoms gives EPR lines at $g = 2.17$ (peak) and 1.99 (trough) (ref. 12).

This brings the total number of metal-containing electron carriers in the respiratory chain up to 16, *viz.* 6 haemoproteins (cytochromes *b*, b_i, *c*, c_1, *a* and a_3), 8 Fe–S proteins and two coppers. In addition, the chain contains two flavoproteins, ubiquinone and perhaps P, a total of at least 19 components. This brings us to propose a new model of the respiratory chain shown in Fig. 8, a simplified version of which has been presented elsewhere[25–27]. This model features the double electron-transport chain that has been proposed for the phosphorylating chain. In non-phosphorylating particles or isolated complexes, some of the components (*e.g.* the antimycin-sensitive Fe–S protein, and cytochrome b_i) are reduced too slowly to play a role in electron transport from substrate to oxygen. The pathway of electron transfer proposed in Fig. 8 for non-phosphorylating preparations by-passes these slowly reduced com-

* See NOTE ADDED IN PROOF, p. 12.

ponents. It is also assumed that one of the Fe–S proteins associated with the NADH dehydrogenase and the Fe–S protein in Complex III[23] are also off the main electron-transport chain in these preparations. In phosphorylating preparations, cytochrome b_i is rapidly reduced, and it has been proposed that two electron-transport chains, compulsorily coupled with each other, and involving high-energy forms of cytochromes b and b_i, are the basis of the mechanism of energy conservation in Site II of the respiratory chain[21,25–27]. It is tempting to suppose that similar mechanisms, involving Fe–S are involved in Site I, and Cu and/or haem in Site III.

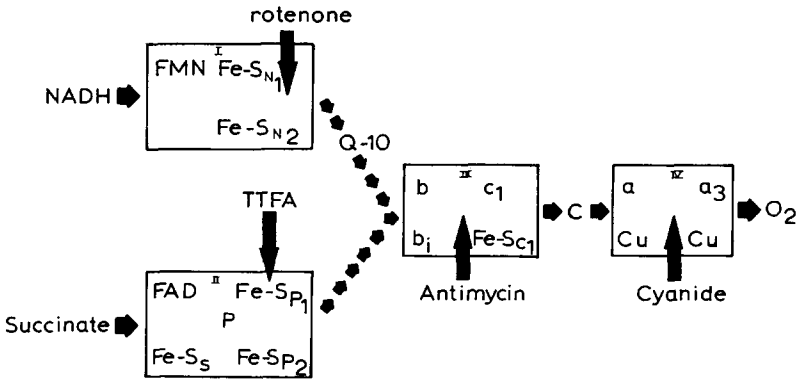


Fig. 8. Proposed model of respiratory chain in non-phosphorylating preparations. Fe–S_{N1} and Fe–S_{N2} refer to the two Fe–S proteins associated with NADH dehydrogenase*, Fe–S_S to that associated with succinate dehydrogenase, Fe–S_{P1} and Fe–S_{P2} to Components 2 and 3, respectively, identified in this study, and Fe-S_{c1} to the Fe–S protein present in Complex III.

The velocity of electron transport *via* the antimycin leak[28] or because of incomplete inhibition by TTFA is so fast in the highly concentrated preparations used in the EPR studies that it is highly unlikely that the effects of these inhibitors on the intensity of the EPR signals is simply that of cutting off the supply of electrons from substrate. Indeed, both TTFA and antimycin caused a decline in the intensity of the $g = 1.94$ line when added after ascorbate, TMPD, cyanide and succinate.

Thus, it is concluded that TTFA has a direct effect on Components 2 and 3 (indicated as Fe–S_{P1} and Fe–S_{P2} in Fig. 8) and antimycin on Fe–S_{P2}. The effect of antimycin may be the result of the same conformation change that brings about reduction of cytochrome b_i in non-phosphorylating preparations[29]. From measurements of the fluorescence quenching, BERDEN AND SLATER[30] have calculated that, in the presence of succinate, the distance between the antimycin-binding site and the iron atom in cytochrome b_i is about 2.4 nm. If Fe–S_{P2} is the direct electron donor to b_i in phosphorylating preparations, its paramagnetic centre might be sufficiently close to that of b_i and to the antimycin-binding site in Complex I + II + III so that changes in the intensity of the EPR signal results on binding of antimycin. Nevertheless, Fe–S_{P2} seems to belong to Complex II rather than Complex III, since preparations of the latter complex contain only trace amounts of Fe–S components with an EPR line at $g = 1.94$.

It is noteworthy that the blocks in Fig. 8, each containing 4–5* single-electron acceptors (in NADH and succinate dehydrogenase, the flavins also act as single-

* See NOTE ADDED IN PROOF, p. 12.

electron acceptors) corresponding to the four complexes of GREEN[31]. However, preparations of the complexes are often contaminated by components belonging to other complexes. For example, our preparation of Complex I + III contained small amounts of succinate dehydrogenase and considerable amounts of Fe–S_{P1}.

Complex I preparations contain only traces of the Fe–S proteins with the EPR signal with g values of 2.02, 1.94 and 1.92. These are revealed by reduction with $Na_2S_2O_4$ which reduces only very slowly the Fe–S proteins associated with NADH dehydrogenase. The total amount of these Fe–S proteins, like that of the ubiquinone, is, however, insufficient to account for the rotenone-sensitive bleaching of the pigment absorbing at 460 nm. Thus, this pigment still has to be identified. In pentane-extracted particles, 62 % of the succinate-induced bleaching can be ascribed to the flavin and Fe–S of succinate dehydrogenase and only 15 % to other Fe–S proteins, with 23 % as unidentified. The large contribution of the succinate dehydrogenase and the small contribution of the Fe–S proteins suggest that the unidentified pigment in both Complex I and heart particles may be a flavoprotein, as originally postulated by CHANCE *et al.*[1].

METHODS

Enzyme preparations

ATP–Mg particles were made by the method of LÖW AND VALLIN[15].

Heart-muscle particles were prepared according to the method of KEILIN AND HARTREE[11]. Complex I + III was prepared from these particles according to the method of HATEFI *et al*[10]. Complex I + II + III was the residue after the dialysis in this procedure. If used for pentane extraction all preparations were suspended in 100 mM KCl.

The lyophilized preparations were extracted with pentane according to the method of SZARKOWSKA[14]. Q-10 and/or 'P' (ref. 13) were reincorporated into the pentane-extracted preparations by stirring the dried material for 5 min at room temperature with Q-10 (Sigma) or P dissolved in *n*-pentane. The pentane was then removed in a rotary evaporator under reduced pressure. The dry powder was then homogenized in the media indicated in the legends to the figures. The amount of P added was that extracted from 5 to 8 times as much particles as used in the reincorporation.

EPR spectra

EPR spectra were obtained with a Varian E3 apparatus, equipped with a Varian nitrogen-flow system with automatic temperature control. Substrate was added to the enzyme preparation in the EPR tube and mixed for 10 sec after which the tube was immersed in liquid nitrogen. When the kinetics of the reduction were followed, the tube was removed from the apparatus after each measurement and incubated in a water bath at the indicated temperature for the indicated period before being again immersed in liquid nitrogen. Thawing was complete within 10 sec at 22 °C and 20 sec at 10 °C. The incubation times were corrected for these thawing times. Inhibitors were added 1 min before substrate. When used, ascorbate, TMPD and cyanide were added together with substrate.

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Activation analysis of Complex I, kindly carried out by Dr. J. J. M. de Goeij, Inter-university Reactor Institute, Delft, The Netherlands, showed that 0.03 ± 0.01 nmole Mo per mg dry weight is present in Complex I. This is equivalent to about 0.03 mole Mo/mole FMN. N. R. ORME-JOHNSON, W. H. ORME-JOHNSON, R. E. HANSEN, H. BEINERT AND Y. HATEFI (*Biochem. Biophys. Res. Commun.*, 44 (1971) 446) have found about the same amount by chemical analysis. These analyses make it unlikely that the EPR spectrum reported in ref. 17 is due to molybdenum. ORME-JOHNSON *et al.* have shown, by EPR spectrometry at below 25°K, the presence of 4 iron–sulphur centres associated with NADH dehydrogenase in Complex I.

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