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THE EFFECT OF ATP ON THE EPR SPECTRUM OF PHOSPHORYLATING SUB-MITOCHONDRIAL PARTICLES

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SUMMARY

1. The addition of either ATP or antimycin to substrate-reduced phosphorylating sub-mitochondrial particles causes a decline in the intensity of the EPR signals derived from free radicals and the iron–sulphur paramagnetic signals.

2. It is suggested that antimycin induces a conformation change, resulting in the redistribution of electrons within the paramagnetic centre.

3. It is suggested that ATP, on the other hand, causes the oxidation of iron–sulphur proteins. Two species are involved, one with $g_z = 2.02$, $g_y = 1.94$ and $g_x = 1.92$, and one with $g_\perp = 1.94$ and a $g_\parallel$ of low intensity. With NADH as substrate, the main effect of ATP is to cause oxidation of the latter species; with succinate (in the presence of rotenone) the main effect is on the former species.

4. In the presence of antimycin, with NADH as substrate, ATP causes the oxidation of both species. With succinate as substrate (in the presence of rotenone) ATP has a much smaller effect on the $g = 1.94$ line in the presence of antimycin than in its absence.

5. In the presence of antimycin (but not in its absence) the addition of ATP causes the appearance of the $g = 6$ signal, characteristic of a_3 in partially reduced cytochrome aa_3, as well as the $g_\perp$ line of Cu(II). It is concluded that, when reversal of the chain is inhibited by antimycin, ATP induces a displacement of electrons within the cytochrome *c* oxidase molecule that results in a_3 being more oxidized than *a*.

6. It is pointed out that, despite the fact that 15 or 16 electron carriers have been identified in the respiratory chain, there still remain unidentified electron sinks where electrons disappear on adding ATP and reappear on adding uncoupler.

INTRODUCTION

In addition to the free-radical signal of the flavin[1] and ubiquinone[2,3] semiquinone, three sets of signals, ascribed to iron–sulphur paramagnetic centres, are observed in the EPR spectra at about 80° K of sub-mitochondrial particles treated with succinate or NADH in the absence of oxygen.

The first of these, with $g_\parallel = 2.02$ and $g_\perp = 1.94$, is observed on addition of

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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NADH to sub-mitochondrial particles[4], Complex I (ref. 5) or NADH dehydrogenase[4,6]. ALBRACHT *et al.*[7] have proposed that two Fe–S species, reducible by NADH but not by succinate, contribute to this signal at 83°K (*cf.* refs 8–11).

The second, with $g_z = 2.02$, $g_y = 1.94$ and $g_x = 1.92$, is observed in sub-mitochondrial particles on the addition of either NADH or succinate[4]. The same signal is given by succinate-reduced succinate dehydrogenase[12]. ALBRACHT *et al.*[7] have proposed that three species contribute to this signal in sub-mitochondrial particles or Complex I + II + III, two acting in the respiratory chain on the oxygen side of the 2-thenoyltrifluoroacetone block. One of these two species is sensitive to antimycin. The third species is the Fe–S moiety of succinate dehydrogenase.

The third signal, with $g_y = 1.90$, is seen clearly only in preparations of Complex III[13] although it has been observed also on addition of substrate to sub-mitochondrial particles (*e.g.* ref. 8).

The effect of the energy state on the EPR spectra in reduced phosphorylating sub-mitochondrial particles has not hitherto been studied. This became necessary as part of a study of the oxidation of ferrocytochrome *b* induced by the addition of ATP and antimycin to substrate-reduced phosphorylating sub-mitochondrial particles in the absence of oxygen or presence of cyanide[14–17]. Since this oxidation is insensitive to rotenone as well as to antimycin[15–17], the electrons derived from the ferrocytochrome *b* must reduce some component between the rotenone and antimycin blocks. Although ubiquinone seemed a likely candidate it was found that the addition of uncoupler after ATP, in the presence of rotenone and antimycin, brought about the reduction of both ubiquinone and cytochrome *b*[17]. Other possible electron acceptors for ferrocytochrome *b* under high-energy conditions are flavins and the iron–sulphur proteins. GUTMAN *et al.*[18] have shown that ATP causes the oxidation of a pigment absorbing at 470 nm (with 500 nm as reference wavelength), irreversibly reduced by NADH in rotenone-inhibited sub-mitochondrial particles, and ascribe this to the oxidation of the iron–sulphur moiety of NADH dehydrogenase. However, it seems likely that the flavin moiety makes at least as large a contribution to $A_{470-500\ nm}$ as the Fe–S (ref. 7).

The present paper describes the effect of ATP, in the presence and absence of antimycin, on the EPR spectrum of substrate-reduced sub-mitochondrial particles in the absence of oxygen.

RESULTS

EPR spectrum of succinate- and NADH-reduced phosphorylating particles

Fig. 1 shows the EPR spectrum of NADH- and succinate-reduced phosphorylating sub-mitochondrial particles, in the presence of uncoupler. The absence of a large trough around $g = 2$, characteristic of the $g_\perp$ line of the Cu (II) signal, shows that the suspension was anaerobic before immersion in liquid nitrogen. The spectra are similar to those reported by BEINERT *et al.*[4] for non-phosphorylating particles. The method used in this paper to determine the intensity of the lines is illustrated in Fig. 1. The free-radical signal at $g = 2.00$ is presumably derived from the semiquinones of flavin and/or Q. It is more intense with NADH than with succinate (Fig. 2), which is to be expected since NADH reduces the flavin of both NADH and succinate dehydrogenases, whereas the higher-potential succinate cannot reduce the NADH dehydrogenase. The line at $g = 1.94$, characteristic for the iron–sulphur proteins, is

also more intense with NADH than with succinate. The $g = 1.92$ line is, therefore, more easily resolved with succinate than with NADH as substrate. However, as to be expected from the fact that this line is characteristic of the iron–sulphur proteins associated with succinate dehydrogenase [12,7], its intensity is the same with both substrates. Surprisingly, the $g = 2.02$ line was also almost equally intense, suggesting that these iron–sulphur proteins make a much larger contribution to this line than those associated with NADH dehydrogenase.

With NADH as substrate, but not with succinate, an additional line at $g = 1.90$ is seen. If this is the iron-sulphur protein found by RIESKE *et al.*[13] in Complex III, it is strange that succinate does not reduce it.

Effect of antimycin on EPR spectrum

Antimycin added to substrate-reduced non-phosphorylating[19] or phosphorylating[15–17] sub-mitochondrial particles, in the absence of oxygen, causes the reduction of a long-wavelength *b*, ascribed by SLATER *et al.*[15–17] to a specific conformation of b_1^{2+}, the antimycin-sensitive cytochrome *b*. ALBRACHT *et al.*[7] showed that antimycin

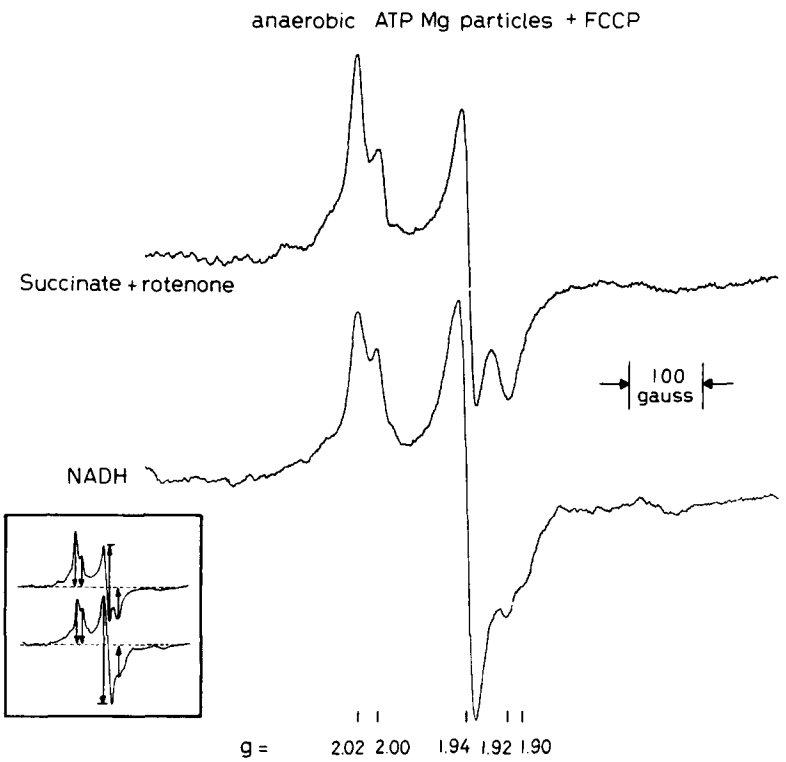

Fig. 1. EPR spectrum of succinate- and NADH-reduced anaerobic ATP–Mg particles in the presence of 50 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The particles were suspended in 200 mM sucrose, 50 mM Tris–acetate buffer (pH 7.4) and 7.5 mM $MgCl_2$ at a final concentration of 60 mg/ml. Total volume, 0.2 ml. The suspension was frozen 2 min after incubation with substrate (15 mM NADH or 50 mM succinate) and the EPR spectra were measured at 83 °K with micropower 100 mW, modulation amplitude 12.5 gauss. The inset shows how the intensities of the different lines were calculated.

brings about a decline in the intensity of the EPR spectra of one of the three iron–sulphur proteins with a line at $g = 1.92$ in non-phosphorylating particles. Fig. 3 (see also Fig. 2) shows that in phosphorylating particles also it causes a decrease of

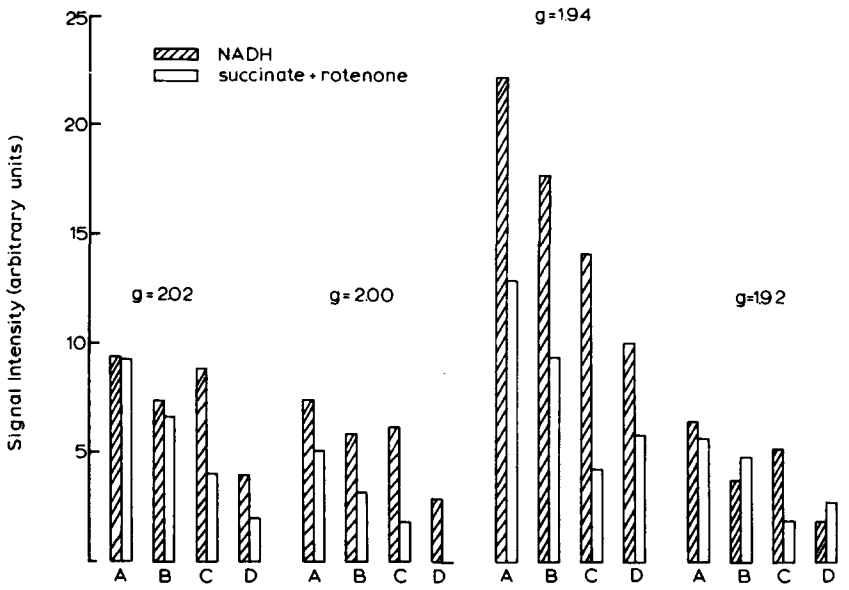

Fig. 2. Comparison of effect of ATP and antimycin on intensity of signals at various g values in NADH- and succinate-reduced ATP–Mg particles. A, in the presence of FCCP (control); B, in the presence of FCCP and antimycin; C, in the presence of ATP; and D, in the presence of ATP and antimycin. The data refer to the spectra shown in Figs. 1 and 3–5.

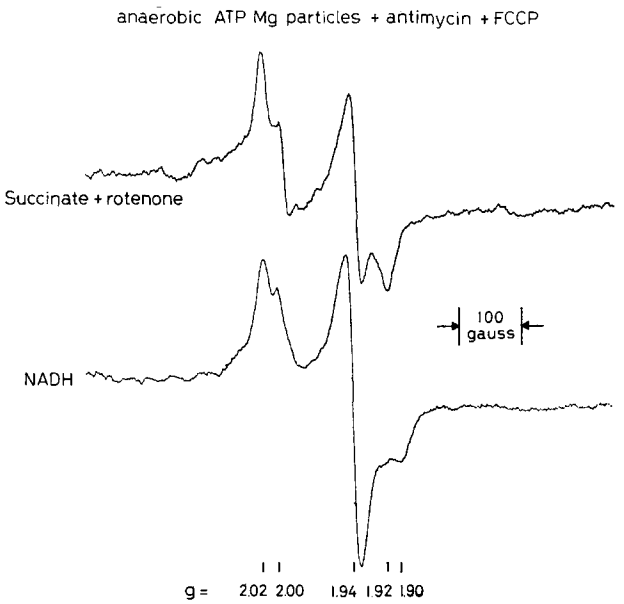

Fig. 3. EPR spectrum of succinate- and NADH-reduced anaerobic ATP–Mg particles in the presence of antimycin (2.08 μg/mg protein). Experimental conditions are identical to those described in Fig. 1.

the intensity of all three lines characteristic of these proteins, at $g = 2.02$ (by 21 % with NADH, 28 % with succinate), $g = 1.94$ (20 and 27 %) and $g = 1.92$ (41 % and 14 %). It is strange that the effect of antimycin on the $g = 1.92$ line is greater with NADH than with succinate as substrate. This was confirmed in a second experiment (34 % and 9 %, respectively).

Antimycin also caused a substantial decline in the intensity of the free-radical signal with $g = 2.00$ (21 % with NADH and 37 % with succinate). With NADH, this decline was confirmed in two other experiments, but it was not found in an additional experiment with succinate. Errors in measuring the height of this signal are, however, large, because the high power used to reveal the iron–sulphur signals is not the most suitable for measuring the intensity of the free-radical signals.

Effect of ATP on EPR spectrum

ATP added to substrate-reduced phosphorylating sub-mitochondrial particles causes the reduction of both long-wavelength and short-wavelength species of *b* (refs. 15–17). ATP caused a considerable decline in all signals with succinate as substrate (in the presence of rotenone) and a smaller decline with NADH, except for the $g = 1.94$ signal, the decline of which was about the same with both substrates (see Figs. 4 and 2). It may be concluded that, with succinate as substrate in the presence of rotenone, an Fe–S species with $g_z = 2.02$, $g_y = 1.94$ and $g_x = 1.92$ is affected by ATP. The effect of ATP on this species is less with NADH as substrate. The fact that the decline in intensity of the $g = 1.94$ line is the same with both succinate and NADH suggests that a species contributing to the $g = 1.94$ line, but relatively little to the $g = 2.02$ line, also declines in intensity when ATP is added with NADH as substrate.

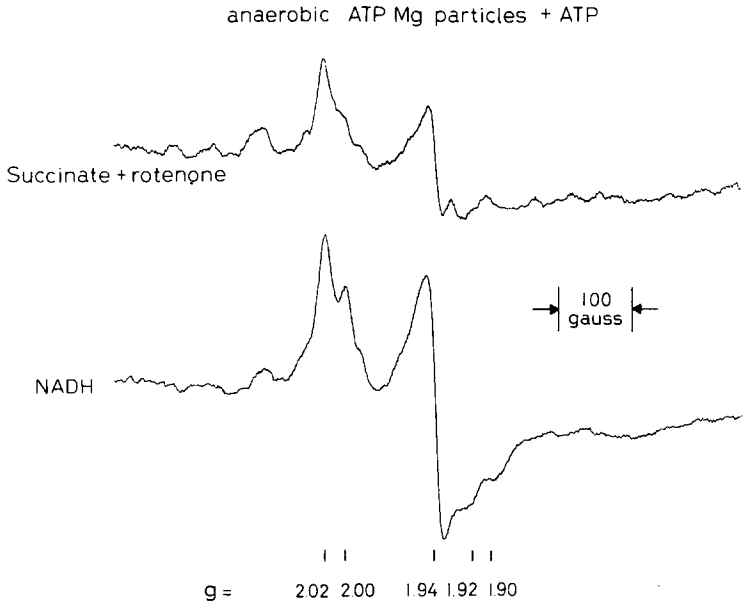

Fig. 4. EPR spectrum of succinate- and NADH-reduced anaerobic ATP–Mg particles in the presence of 25 mM ATP. Experimental conditions are identical to those described in Fig. 1.

Effect of ATP in the presence of antimycin on EPR spectrum

In the presence of antimycin, ATP added to substrate-reduced phosphorylating sub-mitochondrial particles causes the oxidation of cytochrome *b* (refs. 15–17). Fig. 5 (see also Fig. 2) shows that it causes a decline of all signals of the free radicals and the

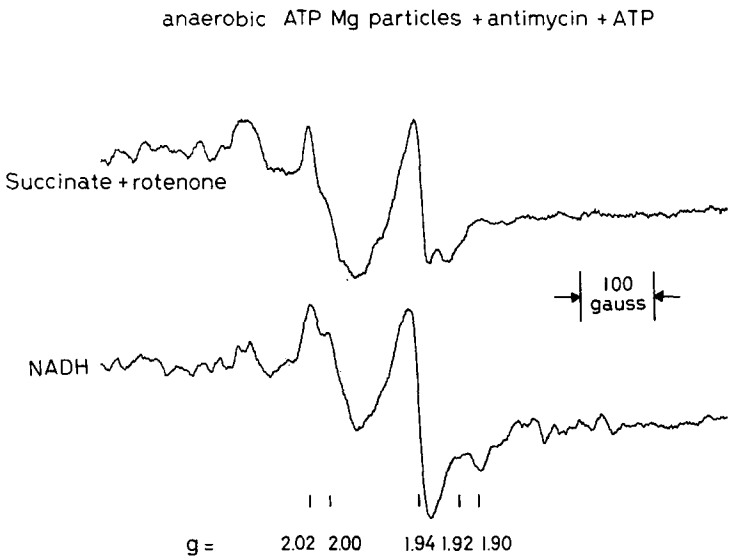

Fig. 5. EPR spectrum of succinate- and NADH-reduced anaerobic ATP–Mg particles in the presence of 25 mM ATP and antimycin (2.08 μg/mg protein). Experimental conditions are identical to those described in Fig. 1.

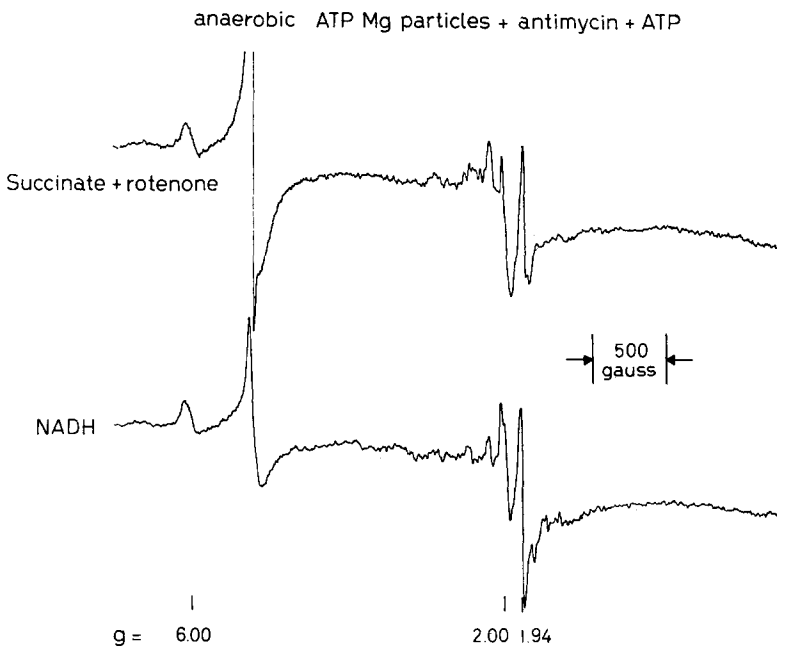

Fig. 6. EPR spectrum of Fig. 5 recorded with a wider scan range.

iron–sulphur proteins. The lines at $g = 2.02$, 2.00 and 1.92 declined by about the same amount with both NADH and succinate, but a greater decline of the $g = 1.94$ line was seen with NADH.

The EPR spectrum measured with a wider scanning range reveals a signal at $g = 6$ and the $g_\perp$ line of Cu(II), not seen in the absence of either ATP or antimycin (Fig. 6). Control experiments showed that the $g = 6$ signal did not appear when cyanide was added.

DISCUSSION

The addition of either ATP or antimycin causes a decline in the intensity of the EPR signals derived from free radicals and the iron–sulphur paramagnetic centres. The decline in the intensity of the free-radical signal could be due to oxidation or reduction of ubisemiquinone, to oxidation of the flavoprotein semiquinone or to a conformation change in the flavoprotein–semiquinone–iron–sulphur complex leading to displacement of an electron to the flavin. In the light of these many alternatives and in view of the difficulty of quantitating the free-radical signal, further discussion of the effects of ATP and antimycin on this signal is profitless.

Similarly, a decline in the intensity of the iron–sulphur signal may be caused by either oxidation of the iron–sulphur protein or a conformation change in the iron–sulphur paramagnetic centre. The concentration of the particle suspension was so high that anaerobiosis and complete reduction of components of the respiratory chain beyond the antimycin block occur within a few seconds of adding substrate in the presence of uncoupler. This is confirmed by the absence of an EPR signal of Cu(II) in the suspensions to which antimycin was added. As already suggested by ALBRACHT *et al.*[7], it is likely that antimycin brings about a decline in the intensity of the EPR signals derived from the iron–sulphur proteins by inducing a conformation change. BRYŁA *et al.*[20] and BERDEN[21] have proposed that antimycin brings about a conformation change in cytochrome b_1 that enables it to receive electrons from substrate. The same conformation change of the membrane in which cytochrome b_1 and the Fe–S proteins are situated could conceivably result in a redistribution of electrons within the paramagnetic centre in such a way that a less intense signal results.

The addition of ATP to phosphorylating sub-mitochondrial particles also brings about the reduction of cytochrome b_1 (ref. 17), but ATP is much less effective than antimycin in this respect. Since it has a greater effect on the EPR spectrum, it seems likely that this is due to oxidation of iron–sulphur proteins. Two species appear to be involved, one with $g_z = 2.02$, $g_y = 1.94$ and $g_x = 1.92$, and one with $g_\perp = 1.94$ and a $g_\parallel$ of low intensity. With NADH as substrate, the main effect of ATP is to cause the oxidation of the latter species; with succinate (in the presence of rotenone) the main effect is on the former species.

In the presence of antimycin with NADH as substrate, ATP causes the oxidation of both species. With succinate as substrate, in the presence of rotenone, ATP has a much smaller effect on the $g = 1.94$ line in the presence of antimycin than in its absence. This may indicate that the species with $g_\perp = 1.94$ is reduced under these conditions. Ubiquinone is also reduced under these conditions[17], whereas cytochrome b is oxidized [14–17].

The species with $g_x = 1.92$ is one of the Fe–S proteins proposed by ALBRACHT

et al.[7] to be operating between the 2-thenoyltrifluoroacetone block and the cytochromes in the succinate oxidation pathway. The species with $g_{\perp} = 1.94$ is presumably one or both of the Fe–S components of the NADH dehydrogenase complex. If we may place the rotenone block between these two species, the simplified version shown in Fig. 7 of the respiratory chain proposed by ALBRACHT *et al.*[7] suffices as a basis for a discussion of the effects of ATP described in this paper. Fe–S_P represents the two species with $g_x = 1.92$ described by ALBRACHT *et al.*[7] while Mo and 'P' have been omitted.

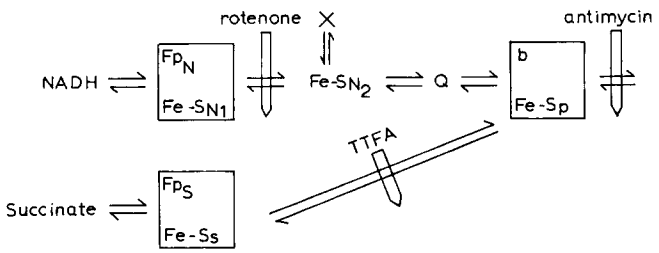

Fig. 7. Simplified version of respiratory chain proposed by ALBRACHT *et al.*[7]. The upper arrow indicates the direction of electron transfer in the presence of uncoupler and the lower arrow in the presence of ATP. TTFA, thenoyltrifluoroacetone.

The experiments carried out in the presence of antimycin, which inhibits the supply of electrons from the oxygen end of the chain, are easier to interpret. With NADH as substrate, reversal of the respiratory chain induced by ATP leads to oxidation of the iron–sulphur proteins (Fe–S_P, Fe–S_{N_2} and/or Fe–S_{N_1}), b^{2+} and possibly flavin semiquinone, while, as we have shown elsewhere[17], Q becomes more reduced. The addition of an uncoupler to a system containing NADH, rotenone, antimycin and ATP leads to reduction of ubiquinone and cytochrome *b* as well as of the iron–sulphur proteins[17]. This can only be explained on the basis of the chain proposed in Fig. 7 if ATP in the presence of rotenone induces electrons to flow not only into Q and Fe–S_{N_2} but into an unidentified electron sink, in equilibrium with the latter, and identified as X in Fig. 7.

Fe–S_{N_2} is not reduced by succinate in the absence of ATP. In the presence of both antimycin and rotenone, ATP causes the oxidation of Fe–S_P, b^{2+} and perhaps succinate dehydrogenase flavin, and both Q and possibly Fe–S_{N_2} become more reduced.

In the presence or absence of antimycin, and absence of ATP, cytochrome oxidase was completely reduced by the time the EPR tube was frozen in liquid nitrogen. The addition of ATP leads to some oxidation of cytochrome aa_3, as shown by spectrophotometric measurements at 605–630 nm and 445–455 nm, but no EPR signal derived from cytochrome aa_3 was visible. When antimycin was present, however, ATP induced the formation of the $g = 6$ signal, characteristic of a_3 in partially reduced cytochrome aa_3 (ref. 21), as well as the $g_{\perp}$ line of Cu(II). The sensitivity of the signal to cyanide confirms that it is derived from cytochrome a_3. Spectrophotometric observations showed that, under these circumstances, a_3 was 22 % oxidized and *a* 8 % oxidized, in comparison with 24 % and 18 %, respectively, in the absence of antimycin. WIKSTRÖM AND SARIS[23] have also reported data that can be interpreted as indicating that a_3 is more oxidized than *a* in the presence of ascorbate, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine, ATP, antimycin, and absence of oxygen.

The following conclusion may be tentatively drawn from these observations. During ATP-induced reversal of the respiratory chain cytochrome *c* oxidase becomes oxidized as a unit, without the redistribution of electrons required for an EPR signal. When, however, reversal of the chain is inhibited by antimycin, ATP induces a displacement of electrons within the cytochrome *c* oxidase molecule that results in a_3 being more oxidized than *a*, and the appearance of an EPR signal characteristic of partially oxidized cytochrome aa_3. The destination of the electrons is obscure. The EPR-detectable Cu(II) also becomes more oxidized.

Indeed, the characteristic result of adding ATP to NADH-reduced phosphorylating sub-mitochondrial particles in the presence of antimycin and absence of oxygen is that cytochromes *b* and aa_3, the EPR-detectable Cu(II), the iron–sulphur proteins detectable by EPR spectroscopy at 80°K and perhaps the flavins all become more oxidized. Ubiquinone is the only component that becomes more reduced, but on the subsequent addition of uncoupler all components become more reduced, including the ubiquinone. GUTMAN *et al.*[18] have shown too that ATP causes the oxidation of a pigment absorbing at 470 nm, previously reduced by NADH in the presence of rotenone. The destination of the electrons in this case also is unknown.

Thus, despite the fact that 15 or 16 electron carriers have been indentified in the respiratory chain[7], there still remain unidentified electron sinks where electrons disappear on adding ATP and reappear on adding uncoupler. Maybe the large amount of iron[24,4] or the one copper atom[25] not visible in the EPR spectrum at 80°K play a role.

METHODS

ATP–Mg sub-mitochondrial particles were prepared from heavy beef-heart mitochondria according to the method of LÖW AND VALLIN[26].

EPR measurements were made in a Varian E-3 EPR spectrometer. The sample tubes were calibrated with Cu(II)-EDTA and the signal intensities corrected to a constant sample size.

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