STUDIES ON MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES BY PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY.*

R. H. Sands and Helmut Beinert

Randall Laboratory of Physics, University of Michigan, Ann Arbor, Michigan and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin

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We have shown in preceding communications (Beinert and Sands, 1959, 1960) that certain features of the oxidation-reduction of purified mitochondrial electron transport enzymes may be studied by EPR spectroscopy. Four principal types of reactions, indicated by specific EPR signals, could be followed:

1. Symmetrical signals of g=2.00 were seen on partial reduction of all preparations which contained flavoprotein components. These signals are attributable to free radicals, mainly flavin semiquinones, presumably.

2. A signal at g=2.05 was observed principally in cytochrome oxidase preparations. This signal decreased in intensity in the presence of substrates of the oxidase. It is characteristic of Cu^{II}.

3. A signal at g= 4.3 was found with most electron transport enzymes, whether they contained cytochrome components or not. In all preparations which have DPNH dehydrogenase activity this signal readily disappeared on addition of DPNH. By analogy with signals given by certain Fe^{III} complexes and Fe^{III} containing glasses this signal is attributed to Fe^{III}. It has so far not been observed with pure intact heme compounds which contain Fe^{III} and is therefore thought to be due to non-heme iron.

4. A new kind of asymmetric signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 was described in the accompanying communication (Beinert and Sands, 1960). This signal appeared on

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reduction of a variety of DPNH or succinic dehydrogenase preparations by their respective substrates. The signal showed somewhat different shape, depending on the substrate used. Its origin is unknown.

All the described signals can also be produced or abolished, respectively, when chemical reducing agents take the place of substrates. These changes can be reversed by oxidizing agents.

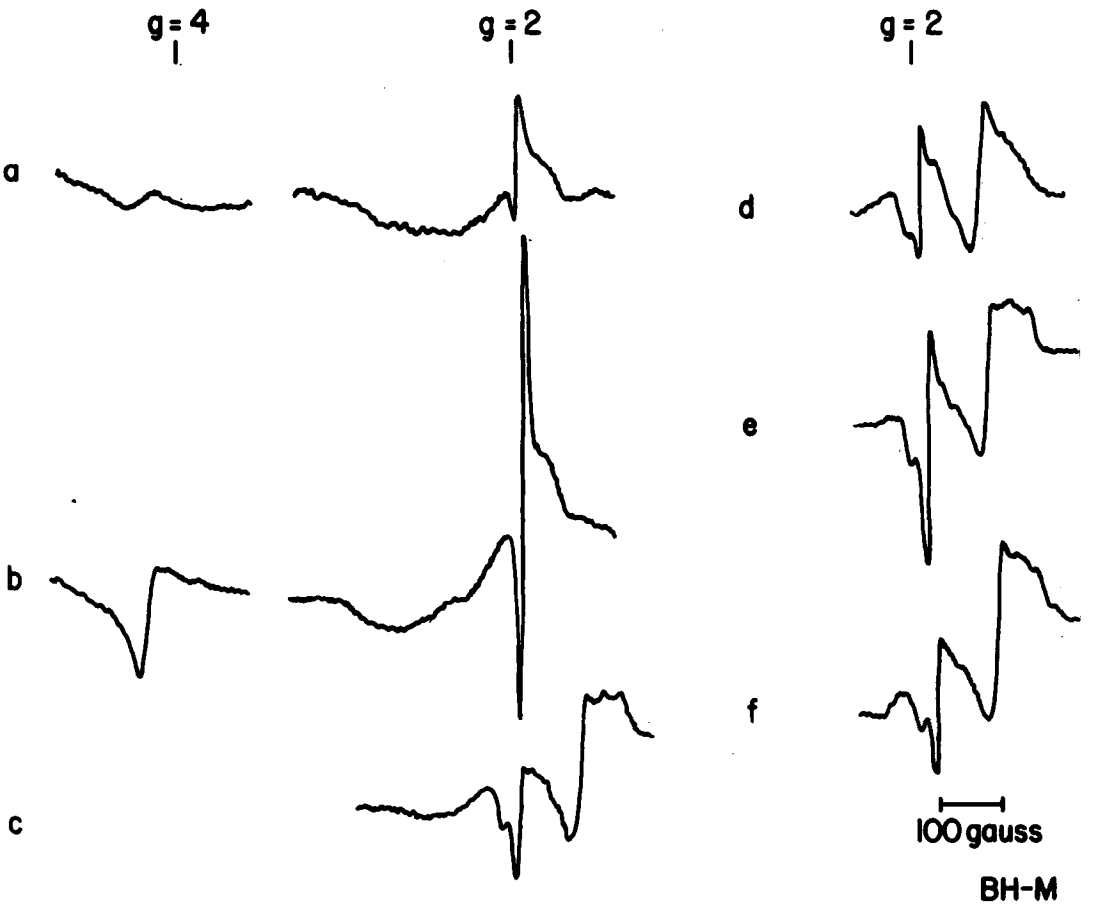

Fig. 1 EPR signals of 24 mg of "heavy" beef heart mitochondria suspended in 0.25 M sucrose containing 10^{-4} M versene and about 10^{-2} M Tris chloride of pH 7.5, aerobically at -100°: (a) untreated; (b) after addition of 10 μmoles of ferricyanide; (c) new sample after addition of 10 μmoles of succinate; (d) new sample after addition of 2.85 μmoles of DPNH; (e) excess dithionite added to (c); (f) excess dithionite added to (d). All samples frozen after 30 seconds except (c) which was frozen 30 minutes after addition.

We wish to report here that all the described types of signals can also be observed with mitochondria and with particles, which are derived from mitochondria by sonic oscillation and which contain most of the electron transport components of mitochondria.

"Heavy" beef heart mitochondria (Hatefi and Lester, 1958) and particles derived from beef heart mitochondria (Ziegler and Linnane, 1958, Beinert and Lee, 1959) were used. In the presence of versene these preparations are very stable and retain most of their activities on freezing and storage. According to optical spectroscopy the electron transport components of mitochondria are partly reduced even under aerobic conditions, because of the presence of endogenous substrate. In the submitochondrial particles used the electron carriers are in the oxidized state unless cyanide is added. In agreement with this, EPR spectroscopy showed that in aerobic mitochondria only a weak Cu^{II} signal and a trace of a Fe^{III} signal were present, but free radicals were observed, indicating partial reduction (Fig. 1a). A signal at g=4.3 (Fe^{III}) was, however, readily produced, when ferricyanide was added (Fig. 1b). When succinate (Fig. 1c) or DPNH (Fig. 1d) was added to mitochondria**, the free radical signal was slightly increased and the signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 appeared, which had previously been seen with purified DPNH and succinic dehydrogenases. When dithionite was added after one of the substrates only a 10 to 20% increase of the signal was observed (Fig. 1e,f). This suggests that the material represented by the signal is basically the same whether succinate or DPNH is the reductant, although in purified preparations a difference in shape was apparent (Beinert and Sands, 1960).

Experiments with the submitochondrial particles confirmed the conclusion that the material indicated by the signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 is largely the same whether DPNH or succinate is the reductant. In contrast to mitochondria these particles showed a strong Fe^{III} and Cu^{II} signal under aerobic conditions (Fig. 2a). A small free radical signal was also present. Addition of DPNH reduced the Fe^{III} signal considerably, increased the free radical signal and

** DPNH will react with these mitochondria after freezing.

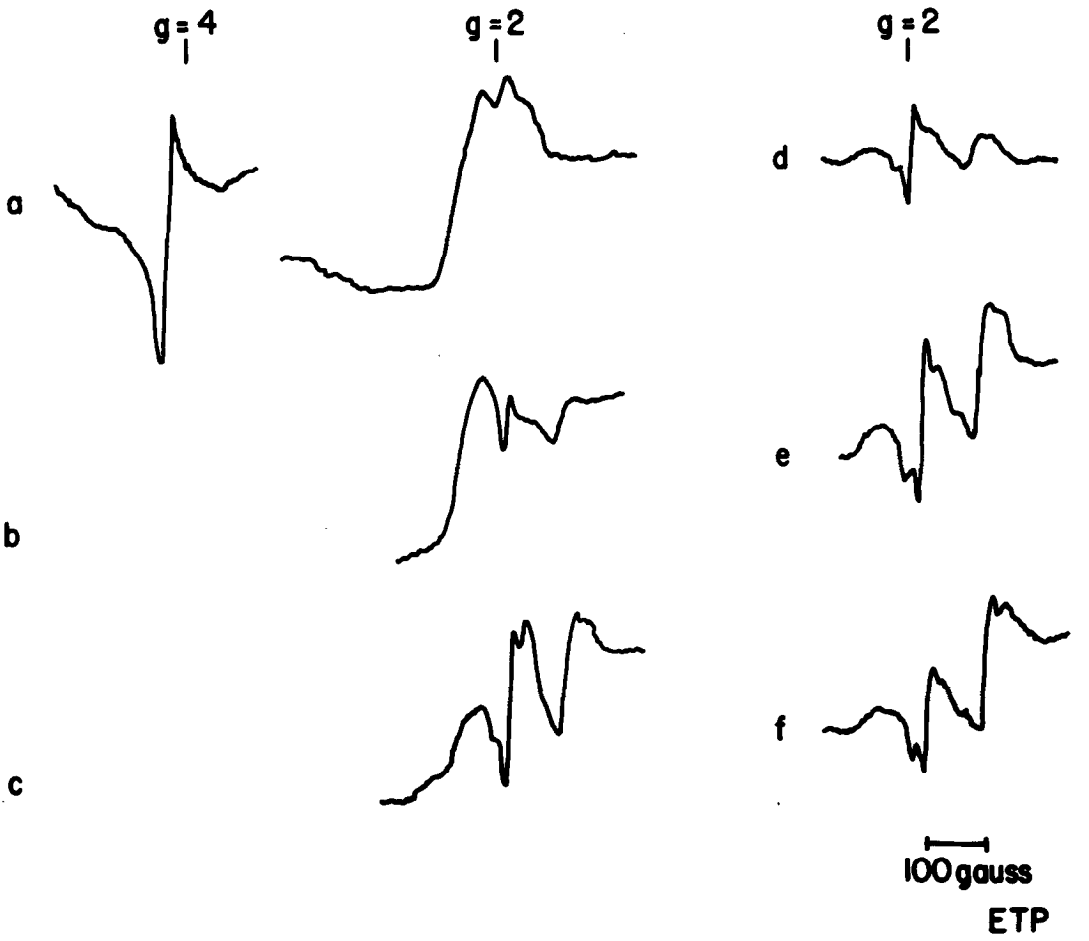

Fig. 2 EPR signals of 25 mg of submitochondrial particles suspended in 0.5 M sucrose containing 10^{-3} M versene and 0.02 M Tris acetate of pH 7.4, aerobically at -100°: (a) untreated; (b) after addition of 2.85 μmoles of DPNH; (c) after addition of 1.42 μmoles of DPNH and 6 μmoles of KCN to (b); (d) same as (c) but at -15db instead of -9db as used in all other experiments of Fig. 1 and Fig. 2; (e) after addition of 10 μmoles of succinate to (c); (f) after addition of an excess of dithionite to (e). All samples frozen 30 seconds after additions except for (c) which was frozen after 10 minutes.

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produced the new signal at $g_{\perp}$ =1.94 (Fig. 2b). On further addition of DPNH and incubation at 0° this signal increased and the Fe^{III} signal disappeared. When cyanide was then added the Cu^{II} signal appeared to be somewhat reduced, whereas the signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 and the free radical signal increased (Fig. 2c). The free radical signal can be differentiated from the $g_{\parallel}$ =2.00 portion of the new asymmetric signal by changing the incident microwave energy

(Fig. 2d). Little further change was produced when succinate was added after DPNH (Fig. 2e). Dithionite led to an additional increase of the new signal of about 15% (Fig. 2f). Succinate, when added to a fresh sample of particles, produced changes similar to those described for DPNH except that the Fe^{III} signal at g=4.3 was not changed. This agrees with observations made on purified succinic and DPNH dehydrogenases. In DPNH dehydrogenase preparations the non-heme iron indicated by the signal at g=4.3 was always promptly reduced by DPNH but the iron of g=4.3 in succinic dehydrogenase preparations was slowly, if at all, reduced by succinate.

The fact that the same EPR signals are observed with mitochondria as they are under similar conditions with purified preparations derived from mitochondria gives some assurance that the signals are not due to artifacts. However, the true significance of the reported observations for the electron transport process in mitochondria can only be evaluated on the basis of kinetic studies. Such work is at present beset with great technical difficulties.

As discussed previously, the material represented by the asymmetric signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 is not identified. It may be a known or an unknown component of the electron transport system. The properties of this EPR signal, among these its maximal development at full reduction, make it likely that it is a paramagnetic ion, which becomes reduced by substrate. A semiquinoid intermediate should tend to disappear as reduction proceeds. It is also unlikely that the unidentified signal belongs to one of the cytochromes. Preparations free of cytochromes show the signal, and in mitochondria, which have partly reduced cytochrome components, as discussed above, the signal is absent. The kinetics of appearance and disappearance of the new signal and the Fe^{III} signal at g=4.3 preclude the possibility that the material indicated by the new signal is the reduced form of the material represented by the signal at g=4.3.

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