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EPR studies at 20° K on the mitochondrial respiratory chain

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SUMMARY

EPR spectrometry at 20°K of oxidized phosphorylating submitochondrial particles has revealed new paramagnetic species, with lines at g = 2.014 (centre) and 1.990 (trough), respectively. The reduction by NADH of the iron-sulphur centre 2 (N.R. Orme-Johnson, W.H. Orme-Johnson, R.E. Hansen, H. Beinert and Y. Hatefi, *Proc. Second International Symp. on Oxidases and Related Oxidation–Reduction Systems, Memphis, Tennessee, 1971*, in the press) of NADH dehydrogenase, with lines at g = 2.052 and 1.922, is unaffected by rotenone. Succinate also partially reduces this species in phosphorylating sub-mitochondrial particles. An additional species with lines at g = 2.027 (top) and 1.886 is also seen in reduced particles.

Ohnishi *et al.*[1] have recently reported new lines in the EPR spectrum at 20°K of sub-mitochondrial particles from *Candida utilis* reduced with NADH in the presence of Piericidin A, and have allocated them to a new signal with g_x = 1.89, g_y = 1.93–1.94 and g_z = 2.05. This report prompted us to extend our studies[2-4] on the EPR spectrum at 83°K of submitochondrial particles and isolated complexes prepared from beef-heart mitochondria to lower temperatures. The new lines described by Ohnishi *et al.*[1] are also clearly visible in these preparations, as well as additional lines derived from unknown components[5]. Orme-Johnson *et al.*[6] have also reported the presence of these and additional lines in the EPR spectra below 25°K of rat-heart tissue and sub-mitochondrial particles and complexes isolated from beef-heart mitochondria. The present report deals with additional information on the spectrum in non-energized phosphorylating sub-mitochondrial particles isolated from beef-heart. The effect of ATP is examined in the accompanying paper[7]. Some of these results were reported at a recent symposium[8].

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

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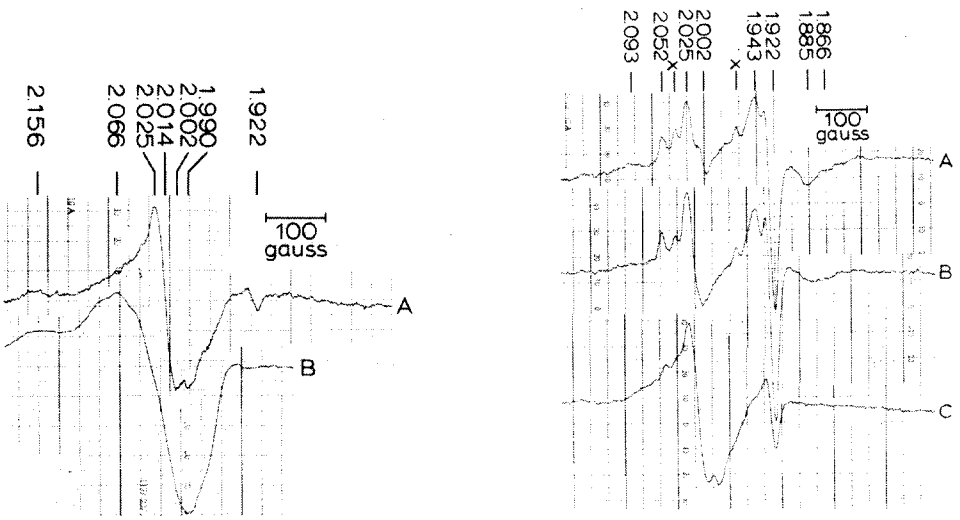

Fig. 1. Comparison of the EPR spectra at 20°K of Mg-ATP particles and isolated cytochrome oxidase in the absence of electron donor. A. Mg-ATP particles[10] (40 mg/ml) in 0.25 M sucrose and 10 mM $MgCl_2$. B. Cytochrome oxidase (100 mg/ml), prepared by the method of Fowler *et al.*[11] as modified by MacLennon and Tzagoloff[12], in 50 mM Tris–H_2SO_4 buffer (pH 8.0). The EPR spectra were obtained with a Varian V-4500-10 A EPR spectrometer equipped with a Hewlett–Packard counter 5245 L, a Hewlett Packard plug-in unit 5255 A and a AEG nuclear-resonance magnetic field meter. The gain for trace A was 5 times higher than for trace B. Modulation amplitude 5 gauss, microwave power 16 mW.

Fig. 2. Effect of rotenone and of reoxidation on the appearance of the EPR signals at 20°K of 'A' particl reduced with NADH. A and B. 'A' particles (45 mg/ml) in 0.25 M sucrose were mixed with 2 mM ascorbate, 4 mM KCN, 4 mM Tris–HCl buffer (pH 8.0) and 4 mM NADH for 10 sec at 0°C and then rapidly frozen in liquid nitrogen. A, no rotenone. B, particles pretreated with rotenone (2 nmoles/mg) for 1 min at 0°C. The EPR spectra were measured at 20°K as in Fig. 1. The lines marked with X are due to the insert Dewar flask used. C. Mg-ATP particles (40 mg/ml) in 0.25 mM sucrose and 10 mM MgCl were made anaerobic by adding 0.9 mM NADH. After 2 min at 15°C oxygen was bubbled through the suspension and 4 min later, when polarographic measurements showed that the suspension was aerobic, a sample was taken and frozen in liquid nitrogen. The EPR spectrum was measured at 20°K as in Fig. 1.

At 83°K the EPR spectra of sub-mitochondrial particles and of cytochrome *c* oxidase, both measured in the absence of electron donor, appear similar, being dominated by a line at $g = 1.990$, ascribed in cytochrome *c* oxidase to Cu(II) (see, *e.g.*, ref. 9). At first sight, this also appears to be the case at 20°K (Fig. 1). When, however, the relative intensities of the lines at $g = 2.156$ (top) and $g = 1.990$ (trough) at the two temperatures are compared (Table I), it is clear that, in Mg-ATP particles, lowering the temperature from 83°K to 20°K has a relatively smaller effect on the $g = 1.990$ line than on the $g = 2.156$ line, whereas in isolated cytochrome *c* oxidase there is no difference in the effect of temperature on the two lines. If it may be assumed that the effect of temperature on the EPR spectrum of cytochrome oxidase in the particles is the same as that with isolated oxidase, it follows that oxidized particles contain an additional component contributing to the $g = 1.990$ line at 20°K. A line at $g = 2.014$ (peak at $g = 2.025$, trough at $g = 2.002$) is also clearly visible in the spectrum taken at 20°K. The only other significant difference

TABLE I

COMPARISON OF THE RATIO'S OF THE INTENSITIES OF THE $g = 1.990$ (TROUGH) AND THE $g = 2.156$ (TOP) LINES AT 83°K AND 20°K OF Mg-ATP PARTICLES AND ISOLATED CYTOCHROME OXIDASE

Intensities (arbitrary units) were measured against a base line recorded with water. Spectra at 83°K and 20°K were recorded with different instruments and under different conditions. Thus, the relative line intensity at the two temperatures (b/a) has no absolute significance but the different values of b/a may be compared with one another. At one temperature, however, the conditions were the same for all preparations, except for the gain, for which the values in the table are corrected. The EPR spectra at 20°K were measured as in Fig. 1. The spectra at 83°K were measured in a Varian E-3 EPR spectrometer with modulation amplitude 12.5 gauss and microwave power 80 mW.

	Line intensity					
	g = 1.990 (trough)			*g = 2.156 (top)*		
	83°K (a)	*20°K (b)*	*b/a*	*83°K (a)*	*20°K (b)*	*b/a*
Cytochrome oxidase	832	240	0.29	240	70	0.29
Cytochrome oxidase	928	273	0.29	208	64	0.31
Particles	45	32	0.72	16	6	0.36
Particles	70	45	0.64	20	5	0.25

seen between the spectra of particles and oxidase at 20°K, between $g = 1.3$ and 7.6, are small lines at $g = 1.922$ (see Fig. 1) and 6.05 in the particles.

Fig.2. shows the EPR spectra at 20°K of 'A' particles[13] reduced with NADH in the absence (A) and presence (B) of rotenone. In the absence of rotenone lines at $g = 2.052$ and 1.922 are clearly visible as well as less intense lines at $g = 2.093$ and 1.885 and a shoulder at 1.866 (*cf.* ref. 6). Rotenone affects the spectrum only in the region of the lines at $g = 1.885$ and 2.025. The increase of the height of the top at $g = 2.025$ is probably due to oxidation of the species responsible for the line at $g = 2.014$ (see above). The decrease of the depth of the $g = 1.885$ trough is probably due to oxidation of the species reducible by ascorbate + tetramethyl-p-phenylenediamine (TMPD) (see Fig.3, Trace B of accompanying paper[7]). In contrast to the findings at 83°K (ref. 3), rotenone has no effect on the line at $g = 1.943$ (top), indicating that iron–sulphur proteins associated with succinate dehydrogenase, with $g_Y = 1.93$ and $g_X = 1.91$, whose reduction by NADH is inhibited by rotenone[3], make a negligible contribution to the line at $g = 1.943$ at 20°K. Fig. 3 shows that rotenone has no effect on the EPR signals given by Complex I [14], indicating that all four iron–sulphur centres identified by Orme-Johnson *et al.* [6] lie before the rotenone-sensitive site. Thus the rotenone-sensitive decline of $A_{460\text{-}510\ nm}$ on addition of NADH to Complex I, shown by Hatefi[15], cannot be correlated with components detectable by EPR spectroscopy at either 83°K (ref. 3) or 20°K.

Oxidation of NADH-reduced phosphorylating particles by aeration sufficient to oxidize all the NADH resulted in the disappearance of all lines at 83°K and all lines at 20°K characteristic of reduced preparations, except those at $g = 2.052$ and $g = 1.922$ (Fig. 2C). These belong to iron–sulphur centre 2 of NADH dehydrogenase, the one with the highest potential of the four centres identified by Orme-Johnson *et al.*[6].

The EPR spectra of isolated succinate dehydrogenase and Mg-ATP particles, both reduced by succinate, are quite different at 20°K (Fig. 4) although they are very similar

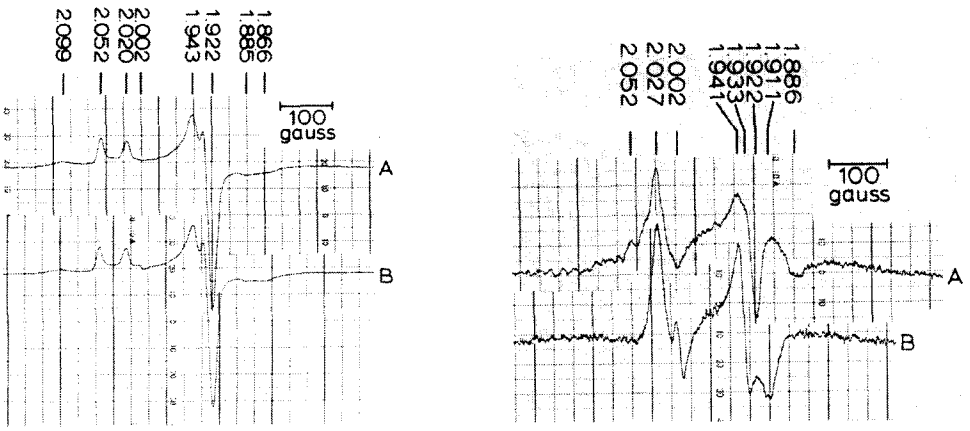

Fig. 3. Effect of rotenone on the appearance of the EPR signals at 20°K of isolated Complex I reduced with NADH. Complex I (37 mg/ml) in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine was treated with rotenone (2.5 nmoles/mg) or an equivalent amount of ethanol for 2 min at 0°C. The suspension was then mixed with 5 mM NADH for 15 sec and rapidly frozen in liquid nitrogen. A, no inhibitor. B, with rotenone. The EPR spectra were measured at 20°K as in Fig. 1.

Fig. 4. Comparison of the EPR spectra at 20°K of Mg-ATP particles and isolated succinate dehydrogenase both reduced with succinate. A. Mg-ATP particles (40 mg/ml) in 0.25 M sucrose and 10 mM $MgCl_2$ incubated 2 min at 22°C with 20 mM succinate and then frozen in liquid nitrogen. B. Isolated succinate dehydrogenase[16] (26 mg/ml) in 0.1 M sodium phosphate buffer (pH 7.6), 1 mM EDTA and 20 mM succinate. The EPR spectra were measured at 20°K as in Fig. 1. The gain for the upper trace was 1.6 time larger than for the lower.

at 83°K. From the intensity of the $g = 1.933$ line in the particles measured at 83°K, and the effect of temperature on this line in isolated succinate dehydrogenase, it can be calculated that it would be barely visible at 20°K. The line at $g = 1.922$ in the EPR spectrum at 20°K of particles is not, then, derived from iron–sulphur proteins associated with succinate dehydrogenase, but presumably from iron–sulphur centre 2 (ref. 6) associated wit NADH dehydrogenase. Its intensity was about one-quarter that obtained with NADH. It is concluded, then, that, under the conditions of this experiment, succinate is able to reduce some of this species, indicating that its potential is much higher than the −305 mV found by Wilson *et al.*[17] for the low-potential Fe–S species (presumably iron–sulphur centr 1 of NADH dehydrogenase[6]). The potential gap between centres 1 and 2 might be involved in Site-I phosphorylation.

The top at $g = 2.027$ seen in succinate-reduced particles is also much too intense to be accounted for by the iron–sulphur proteins associated with succinate dehydrogenase. Since there is no trough at $g = 2.002$, it is not derived from the species with top at $g = 2.025$ seen in oxidized particles. The line at $g = 2.027$ as well as the trough at $g = 1.886$ probably belong to a species reducible by ascorbate + TMPD (see Fig. 3, Trace B of accompanying paper[7]). In any case, it is unlikely that the iron–sulphur protein of Rieske *et al.*[18] is responsible for the trough at $g = 1.886$ in Fig. 4, because this protein does not seem to be reduced by succinate[3,4] (see also Peak C in Fig. 3 of ref. 19).

The nature of the new species with signals at $g = 2.014$ and $g = 1.990$ seen in

oxidized particles is not known. In the accompanying paper[7], it will be shown that both can undergo energy-dependent redox reactions in phosphorylating sub-mitochondrial particles. Orme-Johnson *et al.*[6] have reported a line with $g = 2.019$ in particles and Complex III, and ascribe it to high-spin ferric iron in a 'cubic' environment. The $g = 1.990$ trough is probably not derived from the 'non-visible' Cu(II), associated with cytochrome a_3 [20], since after treatment with azide followed by NADH, the trough was not visible. Under these conditions, this copper atom is oxidized[21]. The $g = 1.990$ trough was not present in succinate dehydrogenase, Complex III, Complex I+II+III or cytochrome *c* oxidase, although it is reducible by ascorbate–TMPD in non-energized phosphorylating particles[7]. Thus, the presence of the species responsible for this line seems to be correlated with the ability of the preparation to catalyse oxidative phosphorylation.

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REFERENCES

1 T. Ohnishi, T. Asakura, H. Wohlrab, T. Yonetani and B. Chance, *J. Biol. Chem.*, 245 (1970) 901.
2 S.P.J. Albracht and E.C. Slater, *Biochim. Biophys. Acta*, 223 (1970) 457.
3 S.P.J. Albracht, H. van Heerikhuizen and E.C. Slater, *Biochim. Biophys. Acta*, submitted for publication.
4 E.C. Slater, I.Y. Lee, B.F. van Gelder, S.P.J. Albracht and J.A. Berden, *Biochim. Biophys. Acta*, submitted for publication.
5 S.P.J. Albracht, *Abstr. 7th Meeting Federation European Biochemical Societies, Varna, 1971.*
6 N.R. Orme-Johnson, W.H. Orme-Johnson, R.E. Hansen, H. Beinert and Y. Hatefi, *Proc. Second International Symp. on Oxidases and Related Oxidation–Reduction Systems, Memphis, Tennessee, 1971*, in the press.
7 S.P.J. Albracht and E.C. Slater, *Biochim. Biophys. Acta*, 245 (1971) 508.
8 S.P.J. Albracht, E.C. Slater, I.Y. Lee and B.F. van Gelder, *Proc. Second International Symp. on Oxidases and Related Oxidation–Reduction Systems, Memphis, Tennessee, 1971*, in the press.
9 H. Beinert, D.E. Griffiths, D.C. Wharton and R.H. Sands, *J. Biol. Chem.*, 237 (1962) 2337.
10 H. Löw and I. Vallin, *Biochim. Biophys. Acta*, 69 (1963) 361.
11 L.R. Fowler, S.H. Richardson and Y. Hatefi, *Biochim. Biophys. Acta*, 64 (1962) 170.
12 D.H. MacLennan and A. Tzagoloff, *Biochim. Biophys. Acta*, 96 (1965) 166.
13 J.M. Fessenden and E. Racker, *J. Biol. Chem.*, 241 (1966) 2483.
14 Y. Hatefi, A.G. Haavik and D.E. Griffiths, *J. Biol. Chem.*, 237 (1962) 1676.
15 Y. Hatefi, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 733.
16 D.V. DerVartanian, *On the mechanism of action of succinate dehydrogenase*, Ph.D. Thesis, Amsterdam, Hofman, Alkmaar, 1965.
17 D.F. Wilson, M. Erecinska, P.L. Dutton and T. Tsudzuki, *Biochem. Biophys. Res. Commun.*, 41 (1970) 1273.
18 J.S. Rieske, R.E. Hansen and W.S. Zaugg, *J. Biol. Chem.*, 239 (1964) 3017.
19 H. Beinert and W. Lee, *Biochem. Biophys. Res. Commun.*, 5 (1961) 40.
20 B.F. van Gelder and H. Beinert, *Biochim. Biophys. Acta*, 189 (1969) 1.
21 B.F. van Gelder and A.O. Muijsers, *Biochim. Biophys. Acta*, 118 (1966) 47.