STUDIES ON SUCCINIC AND DPNH DEHYDROGENASE PREPARATIONS BY PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY*

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We have previously shown by EPR spectroscopy that at least part of the iron contained in a DPNH cytochrome c reductase preparation is reducible by substrate (Beinert and Sands, 1959). It was our aim to extend these studies to more integrated preparations of DPNH dehydrogenase and to other electron transport enzymes containing non-heme iron (NHI) in order to assess the possible significance of the earlier observations and to detect changes in these preparations on oxidation-reduction, which can be studied by EPR.

Data on the preparations used are given in Table I. They were tested aerobically and anaerobically at -100°. Fig. 1 shows the anaerobic titration of (I) with DPNH. First a free radical appears which increases while the ferric iron (signal at g=4.3) is reduced. This iron represents about 1% of the total NHI and occurs at a molar NHI:flavin ratio of 1:4. At the point of maximal radical formation (Fig. 1c,d) a new asymmetric signal appears at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94, which increases on further addition of DPNH. Dithionite leads to a small additional rise after excess DPNH has been added. Reoxidation by ferricyanide reverses these changes completely. DPN at a 5-fold excess over the DPNH present is unable to reoxidize the component indicated by the new signal.

Fig. 2 shows the anaerobic titration of two separate samples of (II) with DPNH and succinate, respectively. DPNH promptly reduces the iron (g=4.3) which

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Table I.

Analytical Data on Enzyme Preparations

Preparation No.	Function	Ref.	Quantity used in 0.3 ml	flavin	NHI	Cyto-chrome b	Cyto-chrome c + c_1
			mg	mμmoles/mg			
I	DPNH cyt. c reductase (particulate)	Hatefi et al., 1960	27	0.9	16	1.1	0.8
II	DPNH and succinic cyt. c reductase (particulate)	Rabinowitz and DeBernard, 1957	25	0.6	7	1.1	0.65
III	Succinic CoQ reductase	Ziegler and Doeg, 1959	24.5	3.4	32	4	----
IV	Succinic dehydrogenase	Singer et al., 1956	36.0	1.0	4.7	---	----
V	Succinic dehydrogenase	Basford et al., 1957	30	4	8	---	----

in this preparation represents about 10% of the total NHI present. There are two non-equivalent iron signals visible which show by different width. A free radical appears and fades as reduction proceeds. A weak signal similar to that seen with (I) at $g_{\perp}$ =1.94 is also visible. It is only on addition of succinate after DPNH, however, that this signal develops significantly (Fig. 2f). The free radical signal at g=2.003 can be differentiated by changing the incident microwave energy. The new signal contains structure not seen in the signal given by reduced (I) (Fig. 1). In contrast to DPNH, succinate is unable to reduce the iron represented by the signal at g=4.3. Only partial reduction is apparent after an hour's incubation with excess succinate at 0° (Fig. 2j). Succinate, when added to (II) in the absence of DPNH also produces the new signal just described (Fig. 2g-i). It may be noted that on reduction of (I) with

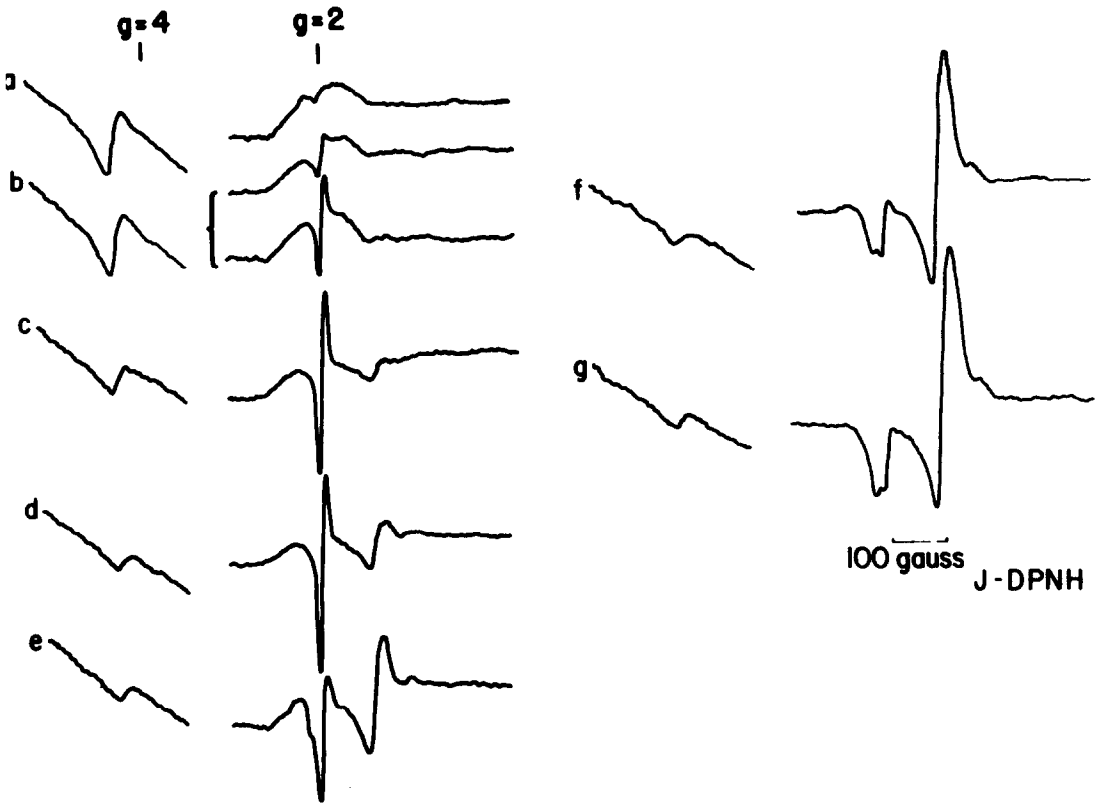

Fig. 1 27 mg of (I) in 0.3 ml of 15% sucrose, 0.05 M in Tris chloride of pH 7.8. Total mμmoles of enzymatically reduced DPNH added: a,0; b,57; c,114; d,171; e,228; f,2000; g, excess of $Na_2S_2O_4$. Tracing of a and upper tracing of b at g=2 recorded at -9db, others at -15 db. Frozen 20 seconds after additions.

DPNH the new signal appears when the free radical concentration has reached a maximum and starts to decline, while during the reduction of (II) by succinate the radical signal and the new signal develop almost simultaneously until the radical starts to decline on further reduction.

Fig. 3 shows the anaerobic titration of (III) with succinate. The sequence of development of signals is similar to that in Fig. 2g-k. The new signal observed when (II) is reduced by succinate appears also with (III). Again this signal and a free radical signal initially grow almost simultaneously. The iron indicated by the signal at g=4.3 is only partly reduced by excess of succinate. This iron does not represent more than 1% of the total NHI present and occurs in this enzyme at a molar ratio to flavin of only about 1:10.

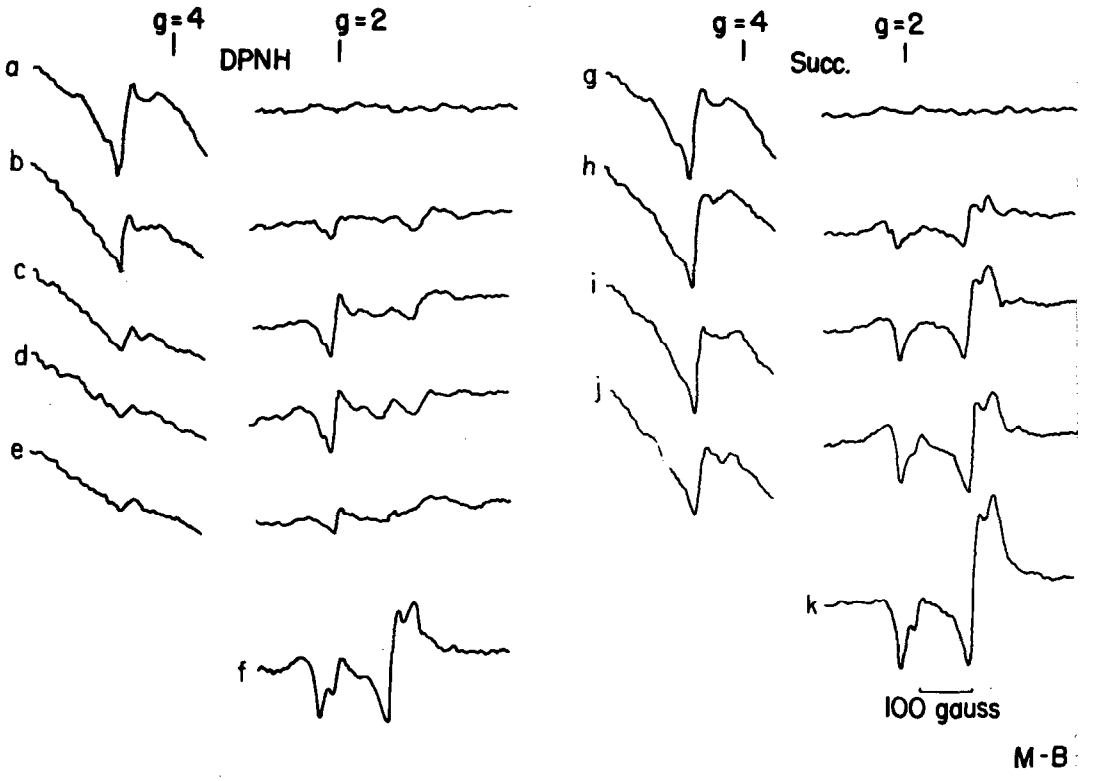

Fig. 2 25 mg of (II) in 0.3 ml of 5% sucrose, 0.025 M in phosphate of pH 7.4. Total mμmoles of DPNH added: a,0; b,69; c,345; d and e,3480; f,3480 plus 2.5 μmoles of succinate. Total mμmoles of succinate added: g,0; h-j,3; k, excess of $Na_2S_2O_4$. b,c, and h, frozen 20 seconds; e,f, and i, 15 minutes, and j, 60 minutes after additions. Incubations at 0°.

We would therefore not like to attribute any significance to the partial reduction of this iron. It is of interest that fumarate added at a 7.5-fold excess over the succinate present is able to reoxidize the material represented by the new signal partly (Fig. 3e). This material is immediately and completely reoxidized by Coenzyme Q_2 and ferricyanide. The signal of Fig. 3g was obtained in the liquid state at 25°C. It was not possible in preliminary experiments to ascertain whether this signal represented both the free radical and the new signal observed in the frozen state or only the free radical signal.

The succinic dehydrogenase preparations (IV) and (V) showed essentially the EPR pattern of preparation (III), when they were reduced by succinate. In each case the new signal at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94 appeared. There was some variation, however, as to the quantity of this material which was reducible by succin-

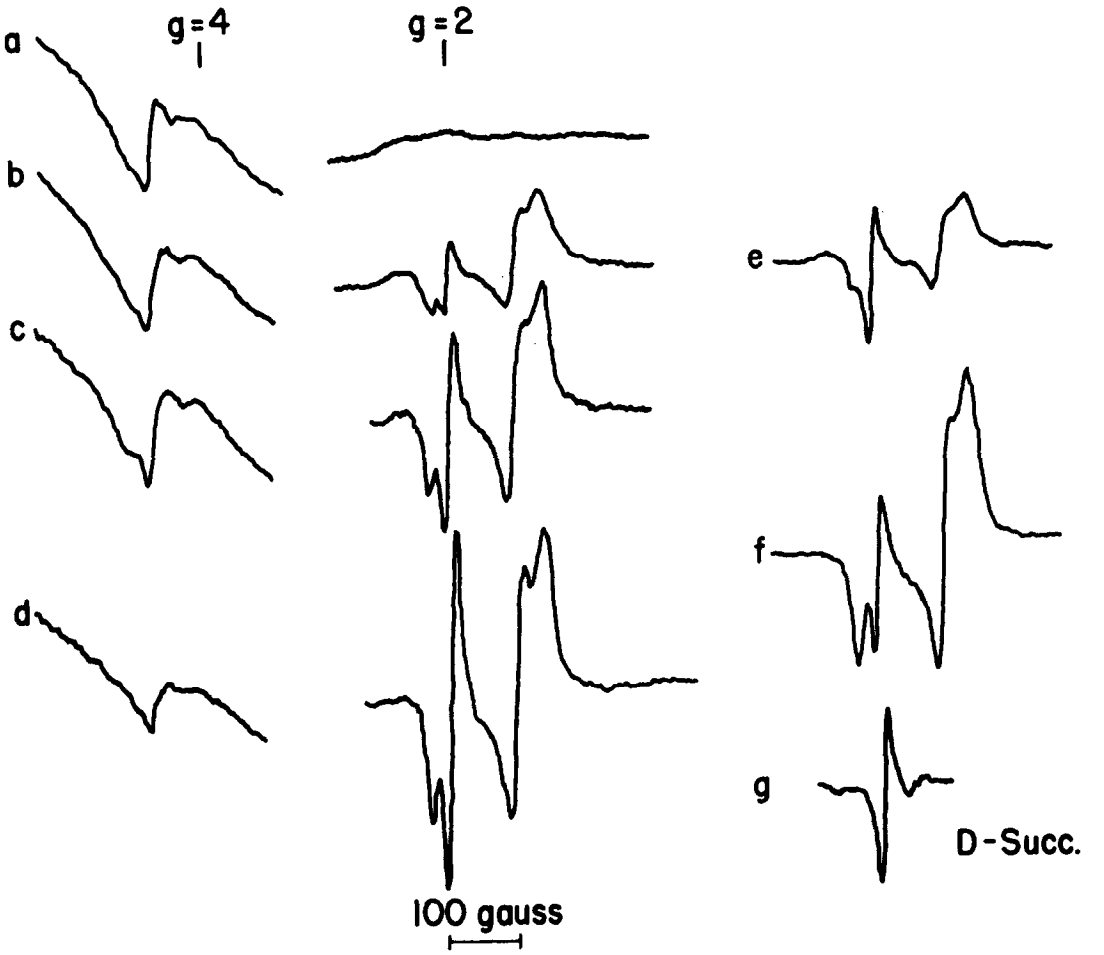

Fig. 3 24.5 mg of (III) in 10% sucrose, 0.1 M in phosphate of pH 7.4. Total μmoles of succinate added: a,0; b,0.4; c,0.8; d,2; e,2 plus 15 of fumarate; f, excess of $Na_2S_2O_4$; Frozen 20 seconds after additions; g,16.5 mg of (III) dissolved in 0.2 ml of sucrose-phosphate plus 10 μmoles of succinate at 25°C.

ate compared to that which was reducible by dithionite. In every case (I-V) this material was present at a molar concentration of the order of the flavin concentration.

We conclude from these experiments that DPNH specifically reduces the ferric iron of g=4.3. The molar concentration of this iron in the DPNH dehydrogenase (II) is about equivalent to the flavin concentration, while it is less in (I). Free radicals, probably flavin semiquinones, are readily formed on reduction or reoxidation of all DPNH or succinic dehydrogenases studied. Reduction by succinate and by DPNH of unknown components in these enzymes is indicated by the new

type of signal observed at $g_{\parallel}$ =2.00, $g_{\perp}$ =1.94. The appearance of this signal on extensive reduction, its position in the EPR spectrum, its structure and its behavior, when the incident microwave energy is varied, indicate that it is probably not due to a free radical but more likely to a paramagnetic ion. According to chemical analysis iron is the only transition metal occurring in significant amounts in the preparations studied. It appears therefore possible that the appearance of the new signal indicates reduction of iron in sites other than those yielding the signal at g=4.3. To our knowledge no analogies from simple model complexes are available**. If iron should indeed be the material indicated by the new signal, EPR spectroscopy can furnish information on structurally and functionally different iron in these preparations.

We would like to emphasize that the titration technique which we have used does not permit us to state whether the intermediates or components which are indicated by EPR are true intermediates on the main pathway of the reactions catalyzed by the enzymes studied. Our work does, however, establish that reducing or oxidizing equivalents are taken up by these components and that they must be considered as branching points for side reactions if not as true intermediates on the main path.

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** R. C. Bray, B. G. Malmström and T. Vänngård [Biochem. J. 73, 193 (1959)] found a somewhat similar signal at g=1.97 with xanthine oxidase, which they ascribe to Mo^{V}. This signal, however, persists at room temperature, in contrast to our signal, cf. Fig. 3g. We could not detect significant amounts of Mo in our preparations by chemical analysis. There were traces of copper present.