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CHARACTERIZATION OF IRON-SULPHUR CENTRES OF PLANT MITOCHONDRIA BY MICROWAVE POWER SATURATION

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Summary

The electron spin relaxation of iron-sulphur centres and ubisemiquinones of plant mitochondria was studied by microwave power saturation of the respective EPR signals. In the microwave power saturation technique, the experimental saturation data were fitted by a least-squares procedure to a saturation function which is characterized by the power for half-saturation ($P_{1/2}$) and the inhomogeneity parameter (b). Since the theoretical saturation curves were based on a one-electron spin system, it became possible to differentiate between EPR signals of iron-sulphur centres which have similar g values but different $P_{1/2}$ values. If the difference in the $P_{1/2}$ values of the overlapped components was small, no significant deviation from these theoretical saturation curves was observed, as shown for the overlapped signals of centre S-3 and the Ruzicka centre of mung bean mitochondria. By contrast, the microwave power saturation data for the $g = 1.93$ signal (17—26 K) of *Arum maculatum* submitochondrial particles reduced by succinate could not be fitted using one-electron saturation curves. Reduction by NADH resulted in a stronger deviation. Since the iron-sulphur centres of Complex I were present only in an unusually low concentration in *A. maculatum* mitochondria, it was proposed that an iron-sulphur centre of the external NADH dehydrogenase contributes to the spectrum of centre S-1. For mung bean mitochondria, the $g = 1.93$ signal below 20 K could be attributed mainly to centre N-2. The microwave power saturation technique was also suitable for detecting magnetic interactions between paramagnetic centres. From the saturation data of the complex spectrum attributable to centre S-3 and an interacting ubisemiquinone pair in mung

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bean mitochondria (oxidized state) followed that centre S-3 has a faster electron spin relaxation than the ubisemiquinone molecules. It is noteworthy that the differences in the relaxation rates were maintained despite the interaction between centre S-3 and the ubisemiquinones. Furthermore, a relaxation enhancement was observed for centre S-1 of *A. maculatum* submitochondrial particles upon reduction of centre S-2 by dithionite. This indicated a magnetic interaction between centres S-1 and S-2.

Introduction

Plant mitochondria show a number of EPR signals at cryogenic temperatures due to iron-sulphur centres, flavin and ubiquinone radicals [1—5]. Most of the iron-sulphur centres are located in the low-potential region of the respiratory chain. Iron-sulphur centres have been detected in the NADH-ubiquinone reductase region (Complex I) and assigned to centres N-1b, N-2 and N-3 or N-4. These centres behave like ferredoxins and show a signal upon reduction. In the succinate-ubiquinone reductase region (Complex II) three centres have been characterized. Centres S-1 and S-2 belong to the ferredoxin type, whereas centre S-3 exhibits a signal in the oxidized state. A HiPIP-type signal was found also for the Ruzicka centre. This soluble high-potential-type iron-sulphur protein isolated from beef heart mitochondria has recently been found to be identical with aconitase [6]. In contrast to plant mitochondria, a greater number of iron-sulphur centres have been observed in mammalian mitochondria and their physicochemical properties are well established [7].

A way of resolving these different iron-sulphur centres is by oxidation-reduction potential titration [8]. However, overlapped signals are difficult to distinguish by this potentiometric technique, unless the oxidation-reduction potentials are separated by at least 40 mV [2]. Overlapping signals can also be distinguished on the basis of the electron spin relaxation of the iron-sulphur centre. In principal, either the temperature at which the EPR signals are observed can be varied or different microwave magnetic fields can be used [9]. From the temperature- and microwave power-dependent EPR signals, conclusions can be drawn with regard to the rate of electron spin relaxation of the electron carrier. In a comprehensive study on a wide range of soluble iron-sulphur proteins the rate of electron spin relaxation of an iron-sulphur centre was found to be a characteristic feature for a particular iron-sulphur centre [10]. The relaxation rate was generally higher for a [4Fe-4S] centre compared to a [2Fe-2S] centre. Within each group the relaxation behaviour was variable, apparently depending on the spin-orbit coupling of the spin system. A convenient way for probing electron spin relaxation proved to be microwave power saturation of the EPR signals. Based on the equation for homogeneously or inhomogeneously broadened lines given by Beinert and Orme-Johnson [11], it was possible to interpret experimental saturation data in terms of the power for half-saturation and a so-called inhomogeneity parameter. This promising method was applied to membrane-bound iron-sulphur centres of chloroplasts (Rupp, H., de la Torre, A. and Hall, D.O., unpublished results) and mitochondria [12]. In this report the electron spin relaxation of iron-sulphur centres of

mitochondria from mung beans and *Arum maculatum* spadix was studied in terms of the degree of saturation and the line-broadening mechanism. Although mitochondria from both species exhibit a cyanide-resistant respiration, *A. maculatum* mitochondria are exceptional in oxidizing exogenous NADH at very high rates by an externally located non-phosphorylating NADH dehydrogenase [13]. Furthermore, in *A. maculatum* mitochondria, the internal NADH-ubiquinone reductase (Complex I) is present only in a low amount as judged from the low concentrations of iron-sulphur centres in Complex I [2] and from low rates of malate oxidation in comparison to that of succinate and exogenous NADH. It is, therefore, possible to relate EPR signals of mitochondria having either a normal or a low concentration of internal NADH dehydrogenase. In the case of *A. maculatum* mitochondria, a possible contribution from the external NADH dehydrogenase to the EPR properties might be expected.

Materials and Methods

Materials. All chemicals used were of the highest grade commercially available. Mung bean (*Phaseolus aureus*) hypocotyls were grown for 5—7 days in a dark room maintained at 28°C. *A. maculatum* inflorescences were collected at the stage just prior to the opening of the spathe and sterile spadices were used. The epidermal anthocyanins were removed from the spadices by wiping them with a damp cotton.

Mitochondria and submitochondrial particles. Mitochondria were prepared as described by Bonner [14]. Submitochondrial particles were prepared by sonic disruption of hypotonic mitochondria. In this technique, mitochondria were initially diluted to ten times their original volume with ice-cold distilled water adjusted to pH 7.2 with KOH and containing 0.3 mM ATP, 1 mM $MgCl_2$ and 0.1% (w/v) bovine serum albumin as described by Rich and Bonner [5]. The suspension was subjected to three times 20 s sonication by using a Dawe Soniprobe (Type 1130A - Dawe Instruments Ltd., London) tuned to an output of 3—4 A at setting 8. The sample was immersed in ice during sonication. The suspension was centrifuged at 10 000 × *g* (10 min) to remove unbroken mitochondria and the supernatant was centrifuged at 100 000 × *g* for 1 h to yield a pellet of submitochondrial particles. The pellet was suspended in a small volume of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.2) and 0.1% (w/v) bovine serum albumin to a final concentration of approximately 15—50 mg protein/ml. Submitochondrial particles prepared in this manner retained full succinate and NADH respiratory activities. Typical rates of oxygen consumption for *A. maculatum* submitochondrial particles were 566 and 2355 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein for succinate and NADH, respectively. Protein was measured by the method of Lowry et al. [15] using bovine serum albumin as standard.

EPR measurements. Samples for EPR measurements of mitochondria in various steady states were prepared as described previously [16]. EPR spectra were recorded on a Varian E4 spectrometer (Varian Associates, Palo Alto, CA, U.S.A.). This instrument permits a 1000-fold range of microwave power attenuation, from a nominal value of 200—0.2 mW. EPR spectra were recorded digitally on a Nicolet 1020A digital oscilloscope (Nicolet Instrument Corp.,

Madison, WI, U.S.A.) interfaced to a HP 9830 calculator (Hewlett Packard Inc., Palo Alto, CA, U.S.A.). Low-temperature studies were performed using an ESR-9 continuous flow cryostat (Oxford Instruments, Oxford, U.K.). The temperature was monitored with a gold/iron chromel thermocouple below the sample position and was checked periodically with a carbon resistor thermometer placed at the sample position. Samples for EPR measurements were frozen in an isopentane/methylcyclohexane (5 : 1, v/v) freezing mixture (81 K) and stored in liquid nitrogen.

Microwave power saturation curves. The experimental saturation data (EPR signal amplitude as a function of incident microwave power) were fitted by a weighted least-squares procedure to the equation given by Beinert and Orme-Johnson [11]. The computer programme was devised by Dr. R. Cammack. Further details are given in Ref. 10. The calculations were carried out on a Hewlett Packard 9830 calculator and the saturation data were plotted on a Hewlett Packard 7202A graphic plotter.

Results and Discussion

Simulation of microwave power saturation curves

The interpretation of the saturation data is straightforward when only a homogeneous electron spin system contributes to the EPR properties. As pointed out by Beinert and Orme-Johnson [11], macroheterogeneity of the samples results in a rather complex saturation behaviour. In the past, no theoretical curves were used for fitting of the experimental data. However, this approach is necessary for characterizing EPR signals which are heterogeneous with respect to electron spin relaxation.

The signal amplitude (S) depends on the microwave power (P) as given by Eqn. 1 (Ref. 10, see also Ref. 11).

$$S \propto \sqrt{P}/(1 + P/P_{1/2})^{b/2} \tag{1}$$

$P_{1/2}$ is the power for half-saturation and b is the 'inhomogeneity parameter' which is 1.0 for an inhomogeneously and 4.0 for a homogeneously broadened line [10]. For representing power saturation data, $\log(S/\sqrt{P})$ is plotted against $\log P$ (Refs. 9—13). A line parallel to the abscissa indicates no saturation within the range of microwave power used. Relief from saturation with microwave power is expressed by a higher value of $P_{1/2}$. It was found that the inhomogeneity parameter generally decreases from 1.34 for soluble [2Fe-2S] proteins to 1.22 for soluble [4Fe-4S] proteins, reflecting an increased width of the spectral envelope compared to the width of the spin packet [10]. Theoretical saturation curves were reported [12] for a typical [4Fe-4S] centre.

A complex situation arises, if the spectrum consists of components of different saturation behaviour but identical g values. In order to illustrate this point, it was assumed that two species, A ($P_{1/2}$ = 1 mW) and B ($P_{1/2}$ = 100 mW), overlap in a varying ratio giving a composite spectrum. The saturation points were derived from Eqn. 1 (Fig. 1). The ratio of species A and B contributing to the total signal amplitude was varied from 80% A, 20% B (Fig. 1e) to 20% A, 80%

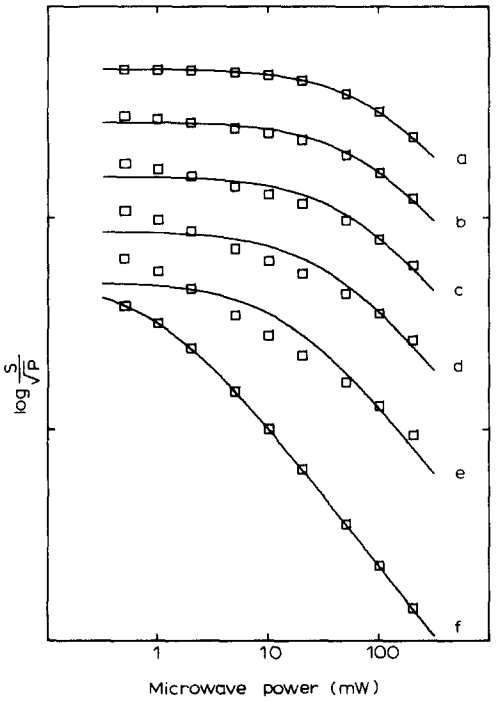

Fig. 1. Theoretical saturation data for two components A ($P_{1/2}$ = 1 mW) and B ($P_{1/2}$ = 100 mW) which overlap in a varying ratio, namely (b) 20% A, 80% B, (c) 40% A, 60% B, (d) 60% A, 40% B, (e) 80% A, 20% B. The saturation curves of A and B are given in (f) and (a), respectively. The data points were calculated from Eqn. 1 (b = 1.34) and were fitted using a weighted least-squares procedure (b = 1.34, $P_{1/2}$ variable, one-electron spin system). (Note, the numbers along the abscissa refer to the microwave powers used and do not represent the log of these powers.)

B (Fig. 1b). The saturation curves of the constituting components A and B are given in Fig. 1f and Fig. 1a, respectively. Since two components of different $P_{1/2}$ values contribute to the signal (Fig. 1b—e), the saturation points could not any more be fitted using a least-squares procedure to Eqn. 1 and assuming a one-electron spin system.

HiPIP-type iron-sulphur centres in mung bean mitochondria

Centre S-3 of succinate dehydrogenase and the Ruzicka centre (aconitase) have overlapped EPR signals with derivative maxima at g = 2.02 and g = 2.03. respectively. S-3 is markedly broadened above approximately 10 K, whereas the Ruzicka centre exhibits a signal which can be detected best around 13 K [17,18]. The overlapping signals of S-3 and of the Ruzicka centre provide a good opportunity for determining the 'resolution' of the microwave power saturation technique. A convenient way for observing the HiPIP signals without a greater interference from the split ubisemiquinone components is provided by the addition of ethanol [4,16]. Fig. 2 shows saturation curves for the derivative peak near g = 2.02 for mung bean mitochondria in the presence of 0.1% (v/v) ethanol.

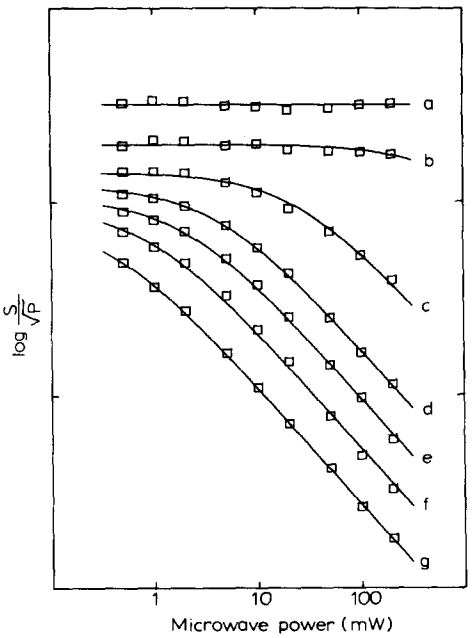

Fig. 2. Microwave power saturation curves for EPR signals (g = 2.02—2.03) of mung bean mitochondria (aerobic state) in the presence of 0.1% (v/v) ethanol at (a) 18.7 K, (b) 15.7 K, (c) 12.7 K, (d) 10.9 K, (e) 9.9 K, (f) 8.9 K, (g) 7.9 K. The data points were fitted using a weighted least-squares procedure for a one-electron spin system assuming b = 1.22 and treating $P_{1/2}$ as a free parameter. EPR conditions: modulation amplitude 1 mT, microwave frequency 9.26 GHz.

The experimental points can apparently be fitted within experimental uncertainty all over the temperature range studied, although two centres contribute to the spectra in a varying ratio depending on the temperature [17]. The least-squares fit for one component was carried out assuming an inhomogeneity parameter of 1.22 and treating $P_{1/2}$ as a variable. By comparing these saturation curves with theoretical curves, an upper limit can be given for the ratio of $P_{1/2}$ (S-3) over $P_{1/2}$ (Ruzicka centre), namely approximately 10 at 10 K. This example demonstrates that the absence of a significant deviation from theoretical saturation behaviour (one-electron system) does not necessarily imply a homogeneous electron spin system.

Based on the assumption that centre S-3 and the Ruzicka centre do not interact with other paramagnetic components of higher relaxation rate, we can deduce [4Fe-4S] centres in both cases. [2Fe-2S] centres are saturated strongly at 15 K using a microwave power ranging from 0.5 to 200 mW [10].

In this respect, it is worthwhile considering the saturation data presented by Case et al. [19]. They resolved the composite saturation curve of centre S-3 and of the Ruzicka centre in pigeon heart mitochondria into two components by adding their predicted absorption contributions in a procedure termed 'linear combination'. A saturation curve for the Ruzicka centre was derived which shows a strong saturation at 18.0 K using a microwave power setting of 100 mW. Although the microwave magnetic field at the sample obviously depends on the Q-factor of the cavity used, it is apparent from Fig. 2 that the

Ruzicka centre is not expected to be saturated at 18.0 K using 100 mW microwave power. This conclusion is supported by the known temperature dependence [17]. Furthermore, both centres were reported to be saturated in a manner characteristic for Lorentzian EPR lines. As already noted, the saturation behaviour of homogeneously broadened EPR lines (i.e. Lorentzian line shape) is characterized by an inhomogeneity parameter $b = 4.0$. The data points at 7.9 K were, therefore, also fitted using $b = 4.0$ and treating $P_{1/2}$ as free parameter (not shown). It was readily apparent that the EPR signal of centre S-3 is not homogeneously broadened. (Note, the Ruzicka centre is not contributing greatly to the spectrum at 7.8 K [17].) On the contrary, the saturation data could be treated as for a bacterial [4Fe-4S] centre.

Ferredoxin-type iron-sulphur centres in A. maculatum mitochondria

Mitochondria from *A. maculatum* oxidize exogenous NADH at very high rates via a piericidin A-insensitive NADH dehydrogenase, located on the outer surface of the inner membrane. The iron-sulphur centres of Complex I are present only in an unusually low concentration [2]. Succinate dehydrogenase (Complex II), however, is present in *A. maculatum* mitochondria in high amounts. This can be inferred from the oxidation of succinate and from the presence of an EPR signal attributable to centre S-3 [2]. Information from soluble succinate dehydrogenase preparations [20—23], indicates that two ferredoxin-type centres are present in Complex II. Centre S-1 can be reduced by succinate, whereas centre S-2 is reducible only by dithionite. The oxidation-reduction potentials of centre S-1 (—7 mV) and centre S-2 (—240 mV) in *A. maculatum* submitochondrial particles [2] suggest that centre S-2 in *A. maculatum* is also reducible only by dithionite. In Fig. 3A saturation curves are shown for the $g = 1.93$ signal of submitochondrial particles from *A. maculatum*, reduced by succinate. In the temperature range 12—47 K the signal is strongly saturated reflecting a slow electron spin relaxation of the iron-sulphur centre. The saturation behaviour is characteristic for [2Fe-2S] centres [10]. Indeed, for mammalian soluble succinate dehydrogenase [22] and centre S-1 from yeast *Candida utilis* [24] there is strong evidence that centre S-1 is a [2Fe-2S] protein. On this basis, the succinate reducible signals at $g = 1.93$ and $g = 2.02$ are attributed mainly to centre S-1 of succinate dehydrogenase. As in the case of centre S-3 and the Ruzicka centre no indication was found for a saturation behaviour characteristic for a homogeneously broadened line. From Fig. 3A it may be noted that between 17 and 26 K a deviation from theoretical saturation behaviour (one-electron spin system) is observed. In order to account for such a deviation, we have to conclude that at least one additional centre of higher relaxation rate contributes to the $g = 1.93$ signal in this temperature range. At low temperature (12.2 K) the data points can be fitted. Presumably, if the additional component is involved in the oxidation of NADH, then its contribution to the $g = 1.93$ signal would be expected to be more pronounced following reduction by NADH. This is indeed the case (Fig. 3B).

A stronger deviation from ideal saturation behaviour (one-electron spin system) is apparent in the range 17—26 K (Fig. 3B). The saturation behaviour at 47 K was identical, within experimental error, for the NADH and succinate-reduced particles. In both cases, the power for half-saturation was 6 mW. Intact

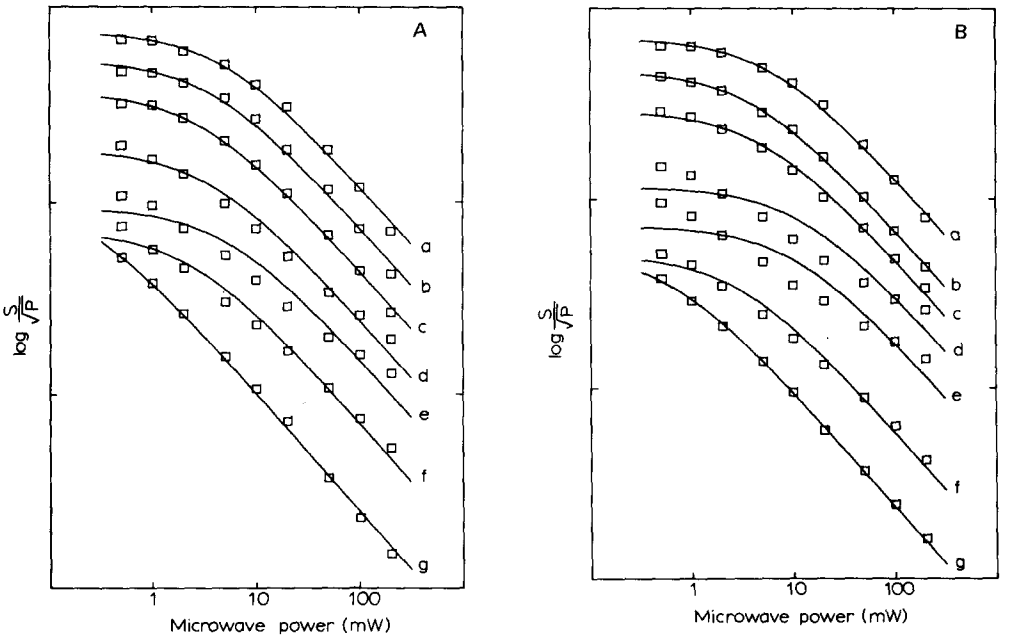

Fig. 3. (A) Microwave power saturation curves for the $g = 1.93$ feature of *A. maculatum* submitochondrial particles reduced by succinate at (a) 47.0 K, (b) 36.6 K, (c) 32.2 K, (d) 26.2 K, (e) 23.2 K, (f) 17.2 K, (g) 12.2 K. Experimental data points were fitted assuming a one-electron spin system and using $b = 1.22$, $P_{1/2}$ was treated as free parameter. EPR conditions: modulation amplitude 1 mT, microwave frequency 9.26 GHz. (B) Microwave power saturation curves for the $g = 1.93$ feature of *A. maculatum* submitochondrial particles reduced by NADH at (a) 47.0 K, (b) 36.6 K, (c) 32.2 K, (d) 26.2 K, (e) 23.2 K, (f) 17.2 K, (g) 12.2 K. Treatment of data points and EPR conditions as in (A).

mitochondria from *A. maculatum* reduced by succinate showed a similar saturation behaviour at 23 K as particulate preparations.

At present, the iron and labile sulphide content of external NADH dehydrogenase is not known. A low iron and sulphur content might be inferred from the loss of Site I energy coupling in *Torulopsis utilis* cells upon limitation of iron or sulphur in the growth medium [25]. However, it might be assumed that the external NADH dehydrogenase contains comparable iron-sulphur centres to those observed in Complex I. This should be borne in mind, when relating a NADH-reducible signal in plant mitochondria solely with Complex I by analogy to mammalian mitochondria. Obviously, it would not be justified to classify the iron-sulphur centres of the external NADH dehydrogenase using the nomenclature for Complex I [26–28].

Ferredoxin-type iron-sulphur centres in mung bean mitochondria

Mung bean mitochondria show EPR signals arising from iron-sulphur centres of internal NADH-ubiquinone reductase and succinate-ubiquinone reductase. The activity of external NADH dehydrogenase is low compared to that of *A. maculatum*. Representative spectra are given in Fig. 4c and d.

In mitochondria (aerobic state) from mung beans a signal was detectable at $g = 1.93$ beside the signals due to centre S-3, the Ruzicka centre and the ubi-

semiquinone species (Fig. 4c). Following reduction by NADH, the $g = 2.05$ and $g = 1.93$ features were more pronounced (Fig. 4d). Additional signals were observed at $g = 1.94$ and $g = 1.87$ attributable to centres N-1b and N-3 or N-4 of Complex I. Less unequivocal is the assignment of the $g = 1.93$ feature. Both centres S-1 and N-2 can contribute to the overlapped signal. A way of resolving this complex signal is provided by the microwave power saturation technique. The $g = 1.93$ signal of mung bean submitochondrial particles reduced by NADH is only slightly saturated at 23.2 K ($P_{1/2} = 378$ mW) (Fig. 5).

No significant deviation from theoretical saturation behaviour (calculated for one component) was detected. By contrast, the $g = 1.93$ signal of *A. maculatum* mitochondria due to centre S-1 is strongly saturated under these conditions (Fig. 3A). Thus, the $g = 1.93$ signal of mung bean mitochondria below 20 K can be attributed mainly to centre N-2. Centre S-1 does not contribute greatly to the $g = 1.93$ feature under these conditions. The saturation behaviour of centre N-2 provides a valuable clue for the characterization of the unknown component in the saturation curves of *A. maculatum* mitochondria (see Fig. 3): from the marked deviation from theoretical saturation behaviour for one

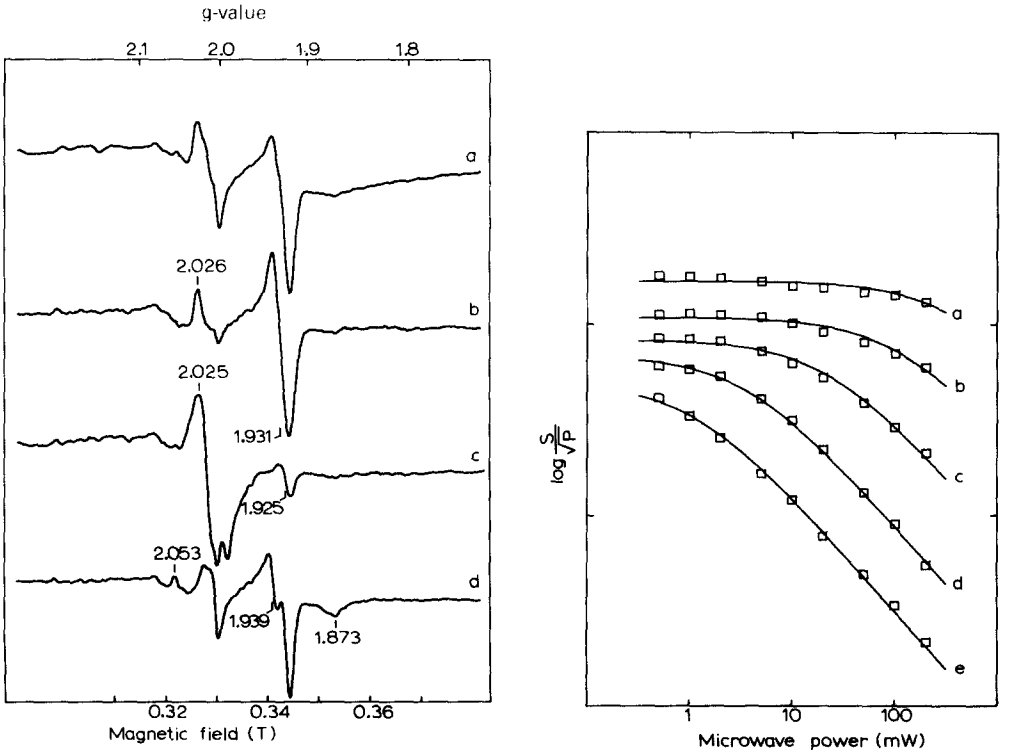

Fig. 4. EPR spectra of *A. maculatum* (a) mitochondria reduced by succinate, (b) submitochondrial particles reduced by dithionite and spectra of mung bean (c) mitochondria in the aerobic state, (d) submitochondrial particles reduced by NADH. EPR conditions: temperature 23.2 K, modulation amplitude 1 mT, microwave frequency 9.26 GHz, microwave power 20 mW.

Fig. 5. Microwave power saturation curves of the $g = 1.93$ signal of mung bean submitochondrial particles reduced by NADH at (a) 23.2 K, (b) 20.2 K, (c) 17.2 K, (d) 14.2 K, (e) 12.1 K. EPR conditions: modulation amplitude 1 mT, microwave frequency 9.26 GHz. Treatment of data points as in Fig. 2.

homogeneous spin system follows that the unknown component must have a much higher relaxation rate than centre S-1. This can be inferred from Fig. 1. A possible candidate would be an iron-sulphur centre with saturation properties like centre N-2. However, we are inclined not to relate the unknown centre in *A. maculatum* solely with centre N-2 of Complex I. If it were due to centre N-2, one would expect to detect also the $g = 1.94$ signal of centre N-1b. There is no evidence for the presence of N-1b in *A. maculatum* mitochondria reduced by dithionite (Fig. 4b). At higher temperatures the spectra would account also only for a very low concentration of N-1b. Since the deviation was more apparent in the presence of NADH than succinate, it is tempting to assign this additional centre to the external NADH dehydrogenase although the possibility that it may be due to centre N-2 of Complex I does exist (i.e. high concentration of centre N-2 and low concentration of centre N-1b). However, the very low concentrations of centre N-1b observed in this report (and see Cammack and Palmer [2]) and the extremely high rates of NADH oxidation in *A. maculatum* would tend to discount this possibility. In a recent examination of the major ferredoxin-type centres in mitochondria of the poky mutant of *Neurospora crassa* [29] it was concluded that the system only consisted of centres S-1 and S-2 of succinate dehydrogenase and that there was no unique iron-sulphur centre associated with the external NADH oxidation pathway. However, since no power saturation data were presented and furthermore, since centres S-1 and S-2 have similar g values to that of the unknown component the possibility of a component other than centre S-1 or S-2 cannot be discounted. Whether the $g = 1.87$ signal seen in *A. maculatum* mitochondria is only due to centre N-3 or N-4 or whether it is also partially due to a centre of the external NADH dehydrogenase requires further investigation.

A further iron-sulphur centre of mung bean mitochondria is centre N-1b ($g = 1.94$) which is strongly saturated below 20 K. At 35 K centre N-1b exhibits a saturation behaviour similar to [2Fe-2S] proteins. The $P_{1/2}$ values for centre N-1b and spinach [2Fe-2S] ferredoxin are 24 mW and 59 mW, respectively. [4Fe-4S] proteins are not saturated at 35 K using microwave powers up to 200 mW ($P_{1/2} \geqslant 1000$ mW). Thus, in the absence of magnetic interactions, the microwave power saturation of centre N-1b would support the presence of a [2Fe-2S] centre for centre N-1b.

Magnetic interaction between ubisemiquinones and iron-sulphur centre S-3 in mung bean mitochondria

At low temperature (less than 20 K) additional signals beside the HiPIP-type and ferredoxin-type centres have been observed in mammalian and plant mitochondria. The most distinctive features occur at $g = 2.04$, $g = 1.99$ and $g = 1.96$ [4,16,30—32]. These signals have been attributed to an interaction between two ubisemiquinone molecules. In addition, an interaction with a fast relaxing paramagnetic component, most probably centre S-3, has to be considered in order to explain the low temperatures necessary to observe these signals. The split signal without the presence of centre S-3 was observed as the difference EPR spectrum of mung bean mitochondria in the presence and absence of salicylhydroxamic acid [16,33]. It has been reported that centre S-3 and the ubisemiquinones in beef heart submitochondrial particles have not greatly dif-

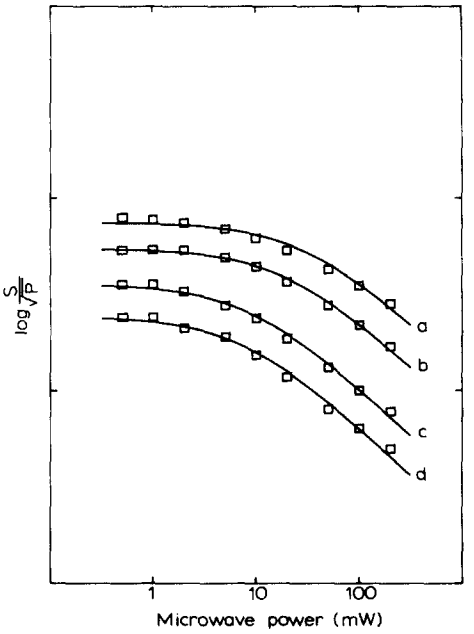

Fig. 6. Microwave power saturation curves for the (a) $g = 2.02$, (b) $g = 2.04$, (c) $g = 1.99$ and (d) $g = 1.97$ features of mung bean mitochondria in the aerobic state. EPR conditions: temperature 12.7 K, modulation amplitude 1 mT, microwave frequency 9.26 GHz. Treatment of data points as in Fig. 2 beside $b = 1.0$.

ferent relaxation rates [31]. Furthermore, a similar behaviour was inferred for mung bean mitochondria [4]. Since in both cases the saturation data were not fitted using theoretical saturation curves, the microwave power saturation of these signals was reexamined. In Fig. 6 saturation data are shown for the features at $g = 2.02$ (Fig. 6a), $g = 2.04$ (Fig. 6b), $g = 1.99$ (Fig. 6c) and $g = 1.97$ (Fig. 6d).

The saturation of the signals increases in the above order. Most desaturated is the $g = 2.02$ signal which is mostly due to centre S-3 and the Ruzicka centre, whereas the most saturated one is the $g = 1.97$ feature of the split ubisemiquinone spectrum. Centre S-3 is expected not to contribute greatly to the $g = 1.97$ signal [32]. From the significant difference in the saturation parameters ($P_{1/2} = 30.0$ mW for $g = 2.02$, $P_{1/2} = 7.4$ mW for $g = 1.97$) it follows that the ubisemiquinone species and centre S-3 do not have identical relaxation rates. (Note, the absence of the Ruzicka centre would result in a still greater difference in the half-saturation powers.) Thus, centre S-3 has a higher relaxation rate than the ubisemiquinone species. Such a behaviour is expected for a dipolar interaction between the HiPIP-type centre S-3 and the ubisemiquinone pair.

Magnetic interaction between iron-sulphur centres

As already noted, centre S-2 of succinate dehydrogenase in *A. maculatum* mitochondria is only reducible by dithionite. In soluble succinate dehydrogen-

ase preparations the electron spin relaxation of centre S-1 is enhanced upon reduction of centre S-2 [23]. It has been suggested that both centres S-1 and S-2 are located on the larger subunit of soluble succinate dehydrogenase (molecular weight about 70 000) both being in close contact [18]. A striking feature of the saturation curves (Fig. 7) of the $g = 1.93$ signal of *A. maculatum* submitochondrial particles reduced by dithionite are markedly higher $P_{1/2}$ values over the temperature range 12.2—47.0 K compared to particles reduced by succinate (Fig. 3A).

Furthermore, at least two components can be distinguished from the microwave power saturation curves. Thus, $P_{1/2}$ increases from 6.4 mW (12.2 K) up to 36.9 mW (23.2 K) and decreases then again to 16.0 mW (47.0 K). We attribute this change in $P_{1/2}$ to two overlapped signals arising from a faster relaxing component (centre S-2) and a slower relaxing species (centre S-1). It is noteworthy that $P_{1/2}$ for $g = 1.93$ (47.0 K) in the case of dithionite reduction (16.0 mW) is greater than that of the succinate-reducible component (6.0 mW). This clearly indicates a relaxation enhancement for centre S-1, most probably due to magnetic interaction with centre S-2. Since the saturation data can be fitted reasonably well in the range 20—25 K, we have to assume similar saturation characteristics for centre S-2 and the centre we have associated with the external NADH dehydrogenase. A significant deviation from theoretical saturation curves (one-electron spin system) would not be expected for overlapping components with similar $P_{1/2}$ values (Fig. 1).

It has been shown that the equilibration between the various iron-sulphur centres of the internal NADH dehydrogenase system is very fast, when an elec-

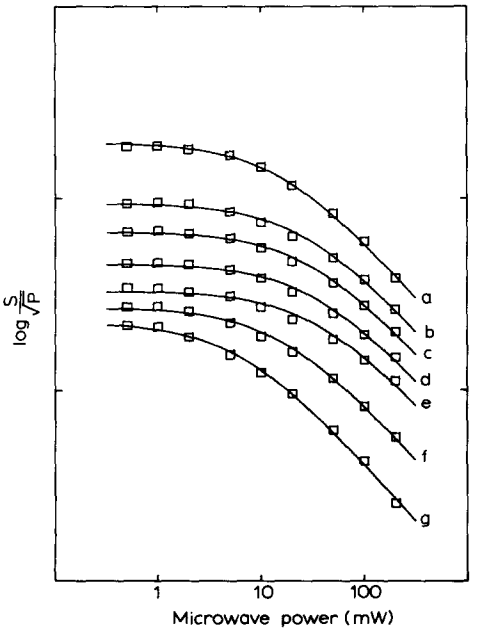

Fig. 7. Microwave power saturation curves for the $g = 1.93$ feature of *A. maculatum* submitochondrial particles reduced by dithionite at (a) 47.0 K, (b) 36.6 K, (c) 32.2 K, (d) 26.2 K, (e) 23.2 K, (f) 17.2 K, (g) 12.2 K. Treatment of data points and EPR conditions as in Fig. 3A.

tron has entered the molecule [7]. This indicates that a special pathway exists between the iron-sulphur centres. Magnetic interactions between the iron-sulphur centres of the internal NADH dehydrogenase are, therefore, highly probable. Furthermore, centre N-2 has the highest oxidation-reduction midpoint potential of all iron-sulphur centres of Complex I and is expected to be oxidized last in the functional electron transport chain [7]. Beside interactions between the iron-sulphur centres, an interaction of N-2 with the ubiquinone system might also be envisaged. It might be inferred from the different temperatures which are necessary for observing the N-centres that a strong magnetic interaction, such as cross-relaxation, does not exist. The saturation properties of centre N-1b ($g_{\parallel} = 2.025$, $g_{\perp} = 1.935$) are similar to those of adrenodoxin ($g_{\parallel} = 2.025$, $g_{\perp} = 1.932$). [2Fe-2S] centres are present in both cases. This would indicate that centre N-1b does not interact with a faster relaxing one. By contrast, centre N-2 which also exhibits an axial signal ($g_{\parallel} = 2.05, g_{\perp} = 1.93$) is much less saturated than centre N-1b. This observation could be rationalized by assuming a [4Fe-4S] centre for centre N-2 or alternatively, by assuming magnetic interactions of a [2Fe-2S] centre with faster relaxing species. Centre N-3 has a rhombic spectrum ($g_z = 2.11$, $g_y = 1.93$, $g_x = 1.87$) [3] which can be observed best around 8 K. Rhombic spectra are generally less saturated than axial spectra due to a strong spin-orbit coupling [10]. (The spin-orbit interaction couples the electron spin to orbital angular momentum, thus providing a way for the spin system to pass its energy to the lattice vibrations via the orbit lattice interaction.) The fast relaxation of centre N-3 or N-4 would suggest a [4Fe-4S] centre. By contrast, [2Fe-2S] centres of a high spectral anisotropy, such as Rieske's iron-sulphur protein, are readily detectable up to 80 K [7].

Centre N-2 provides a convenient example for detecting any possible magnetic interactions. In mitochondria, in the aerobic state, the $g = 1.93$ signal can be assigned to centre N-2, no other N-centres are expected to be reduced under these conditions [7]. Following reduction by NADH, centres N-1b and N-3 or N-4 are reduced. It is notworthy that the saturation behaviour of centre N-2 is indeed changed in the NADH-reduced particulate preparation. The power for half-saturation at 15.4 K is increased from 0.8 mW (mitochondria, aerobic state) to 3.0 mW (submitochondrial particles, reduced by NADH). (Note, the reduction of centre S-1 ($g = 1.93$) cannot be responsible for this relaxation enhancement.) The most probably centre responsible for the relaxation enhancement is centre N-3 or N-4. However, it might well be that centre N-2 in mitochondria in the aerobic state is also interacting with fast relaxing ubisemiquinone species. These magnetic interactions would fit well within the hypothesis that centre N-2 is involved in the energy transduction reaction of Site I [34]. The saturation data, thus, indicate that magnetic interactions do occur both in Complex I and Complex II.

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