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SOME NEW PARAMAGNETIC CENTERS IN SUBMITOCHONDRIAL PARTICLES DETECTABLE BY EPR SPECTROSCOPY

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SUMMARY

1. In EPR spectra at 77 °K from NADH-reduced submitochondrial particles, routinely prepared by sonication of beef-heart mitochondria, a new signal was recognized due to a paramagnetic center not in contact with the respiratory chain.

2. Evidence is presented for the existence of two more paramagnetic centers in NADH-dehydrogenase as observed in EPR spectra of NADH-reduced submitochondrial particles or isolated Complex I at 4.2 °K and high microwave powers.

3. The $g = 1.94$ line of NADH dehydrogenase as normally seen in 9 GHz-EPR spectra at 77 °K of isolated Complex I reduced with NADH is composed of at least two slightly rhombic signals one of which disappears on prolonged incubation with NADH.

INTRODUCTION

Since Ohnishi et al. [1] observed that EPR spectra of reduced submitochondrial particles from *Candida utilis* showed more lines at temperatures below 25 °K, than seen at 77 °K, similar observations were reported for submitochondrial particles from beef and pigeon heart [2–5]. All the species responsible for the new resonances in the reduced particles were situated in the NADH–cytochrome *c* reductase region of the respiratory chain and most of them could be located before the rotenone-sensitive site [3–5] and were present in isolated Complex I [2]. In this report evidence is brought forward for the presence of several additional paramagnetic centers in reduced submitochondrial particles which were prepared by sonication of beef-heart mitochondria. Furthermore, it is suggested that the '$g = 1.94$ EPR line' of NADH dehydrogenase as present in isolated Complex I receives contributions from at least two different species. EPR absorption lines for some of these new centers have been reported recently by Ohnishi [15] and by Albracht [16].

Abbreviations: PMS, phenazine methosulphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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MATERIALS AND METHODS

Beef-heart submitochondrial particles were prepared as described by Löw and Vallin [6] (Mg–ATP particles) or Fessenden and Racker [7] (A-particles). For EPR measurements samples containing about 40–60 mg protein/ml were used. Isolated Complex I was a gift of Dr G. Dooijewaard from the Laboratory of Biochemistry, University of Amsterdam. EPR spectra at 9 GHz with a field-modulation frequency of 100 kHz were recorded on a Varian E-3 spectrometer adapted for use at low microwave powers. Low temperatures were obtained by a helium cooling system described by Lundin and Aasa [8]. EPR spectra at 35 GHz with a field-modulation frequency of 100 kHz were recorded with a Varian V-4503 spectrometer. Low temperatures were in this case obtained by a helium cooling system that will be described elsewhere [9]. The magnetic field strength was measured by means of a NMR probe, while the microwave frequency from the E-3 spectrometer was determined with a frequency counter. EPR conditions are specified in the legends to the figures using the following abbreviations: *F*, microwave frequency; *T*, temperature; *P*, microwave power; *MA*, modulation amplitude; *SR*, scanning rate. The scale at the bottom of each figure is a *g*-value scale.

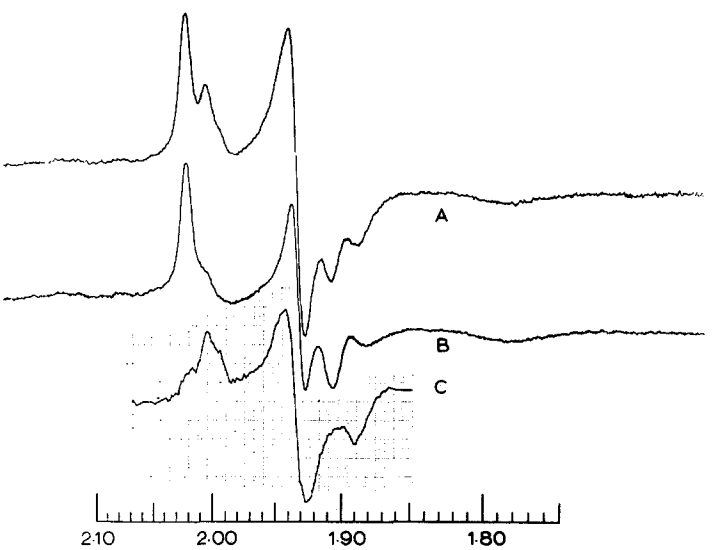

Fig. 1. Comparison of EPR spectra of beef-heart submitochondrial particles reduced with NADH or succinate. Mg–ATP particles in 0.25 M sucrose, 10 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer (pH 7.5), 10 mM ATP (pH 7.5) and 1 mM dinitrophenol were incubated with 10 mM NADH (A) or 25 mM succinate (B) for 3 min at 20 °C and the suspension was then frozen in liquid nitrogen. EPR conditions for A and B: *F*, 9.1 GHz; *T*, 77 °K; *P*, 82 mW; *MA*, 12.5 G; *SR*, 250 G/min. Trace C is a difference spectrum (NADH—succinate) computed from spectra of the same samples recorded on a 5 times expanded horizontal scale at 130 mW and an *SR* of 50 G/min and plotted on the same horizontal scale as the experimental Traces A and B. The sample tubes used were calibrated and the frequency was measured with a frequency meter so the spectra could be normalized to the same conditions before subtracting them. The higher power used for C causes the line amplitudes to be 1.3 times the real difference of Traces A and B.

RESULTS AND DISCUSSION

EPR spectra of substrate-reduced anaerobic submitochondrial particles at 77 °K

In Fig. 1 EPR spectra of submitochondrial particles reduced with NADH or succinate are compared. In the spectrum of particles reduced with succinate (Fig. 1, Trace B) two signals are present. The first one has three lines at $g = 2.02$, 1.93 and 1.91 and is considered to be a summation of signals from succinate dehydrogenase and two other iron–sulphur proteins [10]. The second signal contains also three lines with g values at 2.03, 1.90 and 1.78. This signal is much better resolved at 35 °K and high microwave powers due to saturation of the first signal (not shown). The second signal has been ascribed to an iron–sulphur protein in the vicinity of cytochrome c_1 by Rieske et al. [11]. The shoulder visible around $g = 2.00$ is a highly saturated radical signal due to flavin and Q-10 [12].

When NADH is used as substrate (Fig. 1, Trace A) increases in amplitude are observed around $g = 2.01$, 1.94, 1.91 and 1.89, whereas nearly no increase is seen at $g = 2.02$. This last observation seems somewhat surprising because the increase in amplitude around 1.94 suggests reduction of Center 1 of NADH dehydrogenase which also has an absorption maximum at 2.02. The line at $g = 1.78$ has the same amplitude as with succinate as substrate.

A difference spectrum (NADH reduced—succinate reduced) is shown in Trace C. From the amplitude of the shoulder at $g = 2.02$ and from the overall shape of this difference spectrum it is clear that center 1 of NADH-dehydrogenase is not the only species contributing to this spectrum.

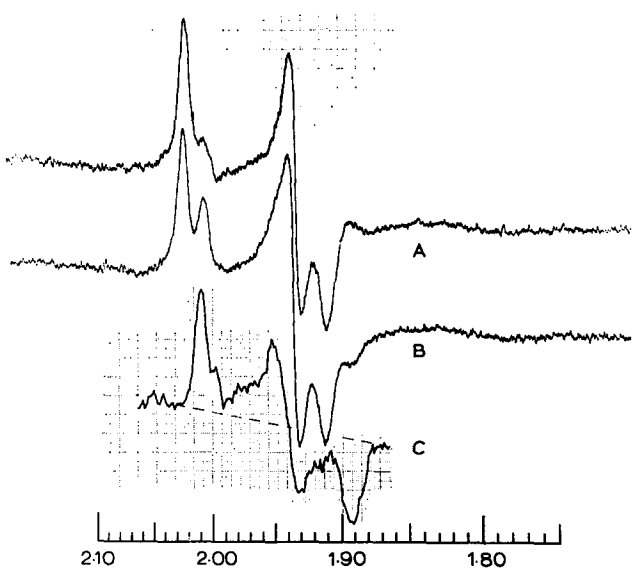

Fig. 2. Effect of PMS on the EPR spectrum of succinate-reduced beef-heart submitochondrial particles. A particles in 0.25 M sucrose were mixed with 25 mM succinate (A) or 0.15 mM PMS+ 25 mM succinate (B) and frozen in liquid nitrogen after 3 min at 20 °C. EPR conditions for A and B: F, 9.1 GHz; *T*, 77 °K; *P*, 82 mW; *MA*, 12.5 G; *SR*, 250 G/min. Trace C is a 2.4 times enlarged difference spectrum (B−A) computed from spectra run on an extended horizontal scale at 82 mW as described in Fig. 1.

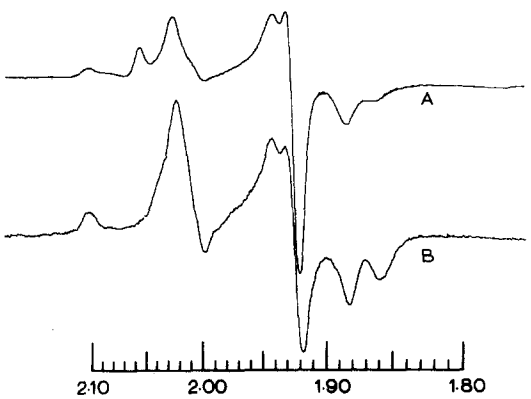

Fig. 3. EPR spectra of NADH reduced submitochondrial particles below 20 °K. The sample used here was the same as that used for Fig. 1, Trace A. EPR conditions: *F*, 9.1 GHz; *P*, 2 mW; *MA*, 12.5 G; *SR*, 500 G/min. Trace A: *T*, 16 °K; gain×1. Trace B: *T*, 11.5 °K; gain×2.5.

It was found that when phenazine methosulphate (PMS) was added to submitochondrial particles reduced with succinate increase of line amplitudes around $g = 2.01$, 1.93, 1.91 and 1.89 could also be seen. This is demonstrated in Fig. 2, Traces A and B. The amplitude of the 1.89 line is the same when NADH alone or succinate plus PMS are used as substrates. Because succinate, in the absence or presence of PMS, is not able to reduce Center 1 of NADH-dehydrogenase the difference, (succinate plus PMS reduced)—(succinate reduced), will now give a spectrum free from contribution of Center 1 as shown in Fig. 3, Trace C. The shape of this difference spectrum suggests that it is due to a rhombic paramagnetic center. The *g*-values for this signal are 2.009, 1.938 and 1.891. Two observations indicate that the center responsible for this signal is probably not in contact with the normal electron transport chain: (1) The signal from this center can be observed in beef heart submitochondrial particles reduced with NADH, but it is not detectable in reduced isolated Complex I. (2) Succinate is not able to reduce this center but needs the mediator PMS to do so.

The shape as well as the *g*-values of the signal in Fig. 2, Trace C are very similar to a signal recently described by Bäckström et al. [13] originating from an iron–sulphur protein of the mitochondrial outer membrane.

These properties make it likely, that the above described signal, found in submitochondrial particles routinely prepared by sonication of beef-heart mitochondria, is due to contaminating outer membrane fragments. Note the fact that the increase in amplitude of the line around $g = 1.93$–1.94 when NADH is added to succinate reduced submitochondrial particles is thus not simply a qualitative measure of the amount of reduced Center 1 of NADH-dehydrogenase.

EPR spectra of reduced submitochondrial particles and isolated Complex I below 20 °K

In Fig. 3 spectra of submitochondrial particles reduced by NADH are shown. In Trace A, recorded at 16 °K, contributions from Centers 1, 2, 3 and 4 of NADH dehydrogenase as defined by Orme-Johnson et al. [2] can be seen, whereas the more easy saturating signals from Center 5 as defined by Ohnishi et al. [5] and from the iron-

sulphur center described by Rieske et al. [11] are also still detectable. The new iron–sulphur center discussed in the previous section is probably still contributing to the spectrum as judged from the shoulder around $g = 2.01$. On decreasing the temperature to 11.5 °K, using the same power, the lines due to Centers 3 and 4 intensify relative to the other lines as is seen in Trace B. Center 2 is highly saturated as the 2.05 line is no longer distinct.

At 4.2 °K and low power lines due to Center 1, 2, 3 and 4 are still visible as seen in Fig. 4, Trace A, although the shape of the lines from Center 3 and 4 in the region 1.88–1.86 is different from that at 11 °K. When the power was increased a factor 100, Trace B, the lines at 2.10 and 1.88 became relatively more intense. The $g_{\|}$ of Center 2 at $g = 2.05$ is barely noticeable and a line at $g = 2.06$ appears. Still a first derivative maximum is seen at 1.94 and a minimum at 1.92. Another increase in microwave power of a factor 100, Trace C, causes a relative intensification of the lines around 2.06, 1.92 and 1.88. No resonance is left at $g = 1.86$, whereas still a clear peak is seen at 2.10. Both lines around $g = 1.92$–1.94 are better resolved and line shape changes can be noticed around 2.00 and 1.90.

From Fig. 5 information can be obtained about the possible localization in the respiratory chain of the paramagnetic species giving rise to these spectra at high power. Traces A and B show spectra at 4.2 °K of submitochondrial particles reduced with succinate and NADH, respectively. The only similarity is found in the $g = 2.00$ region. From these results several conclusions can be drawn: (a) The species responsible for all the lines in Fig. 5, Trace B, cannot be reduced by succinate, which makes it likely that they belong to the NADH–Q-10 segment of the respiratory chain or to NADH-reducible contaminations. The species connected to the signal

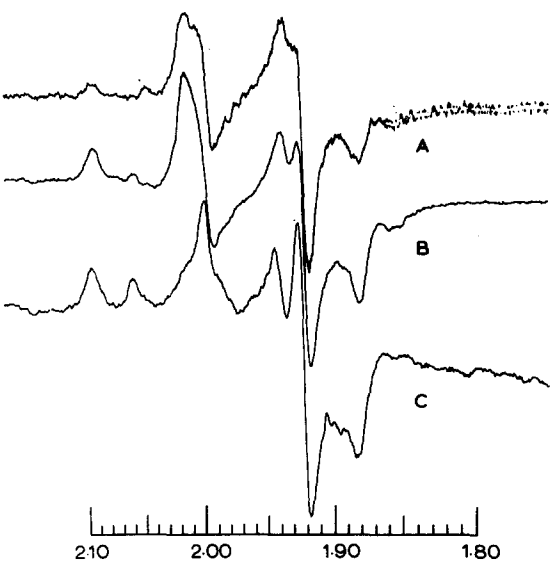

Fig. 4. Effect of microwave power on EPR spectra at 4.2 °K of NADH reduced submitochondrial particles. The sample used was the same as that used for Fig. 1, Trace A. EPR conditions: *F*, 9.1 GHz; *T*, 4.2 °K; *MA*, 12.5 G; *SR*, 250 G/min. Trace A: *P*, 0.02 mW; gain ×2.6. Trace B: *P*, 2 mW; gain × 1.6. Trace C: *P*, 207 mW; gain × 1.

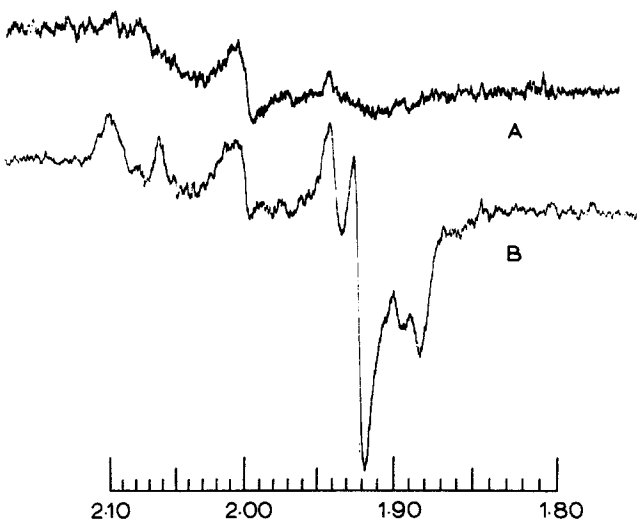

Fig. 5. Comparison of 4.2 °K EPR spectra of submitochondrial particles reduced with succinate or NADH. Mg–ATP particles in 0.25 M sucrose, 10 mM $MgCl_2$ and 60 mM Tris buffer (pH 7.5) were incubated with 12 mM succinate for 5 min (Trace A) or with 10 mM NADH for 3 min (Trace B), both at 20 °C and then frozen in liquid nitrogen. EPR conditions: *F*, 9.1 GHz; *T*, 4.2 °K; *P*, 10 mW; *MA*, 6.3 G; *SR*, 250 G/min.

around $g = 2$ is affected by both substrates in the same way. (b) The fact that no line is seen at $g = 1.92$ in the succinate spectrum, Fig. 5, Trace A, indicates that Center 2 is completely saturated under the conditions used. This would mean that the line around $g = 1.92$ seen in the NADH spectrum, Fig. 5, Trace B, is not due to Center 2, which is in agreement with the absence of a line at 2.05. At 20 °K one can clearly observe, as shown previously [3], that about one quarter of Center 2 can be reduced by succinate. (c) The fact that a top at 1.94 is seen relatively sharp at 4.2 °K and high powers makes it less likely to further assign it to Center 1 under these conditions. (d) The absence of a line at $g = 1.86$ under extreme conditions, Fig. 4, Trace C, suggest the complete saturation of Centers 3 and 4. Summarizing these conclusions, it is questionable whether any of the Centers 1–4 contribute to the spectrum of Fig. 4, Trace C. It must be stated here that these as well as all other conclusions in this report are based on the assumption that the various paragmagnetic centers are homogeneous, behave independent and have a homogeneous saturation behaviour.

Because Orme-Johnson et al. [2] reported that in isolated Complex I no other signals than those from the Centers 1–4 could be detected at temperatures equal to or above 4.2 °K the possibility exists that the resonances reported here are originating from species not belonging to the respiratory chain or that Complex I is modified during the isolation procedure. To clarify this, spectra of isolated Complex I reduced with NADH were run at 4.2 °K and different powers. The results, presented in Fig. 6, can be directly compared with those in Fig. 4, except for the absolute gain. Apart from the $g = 2$ region, the shape of the spectra at the highest power, Traces C in Figs 4 and 6, are very similar, showing that all the resonances are also present in isolated Complex I and thus very likely belong to paramagnetic centers, presumably

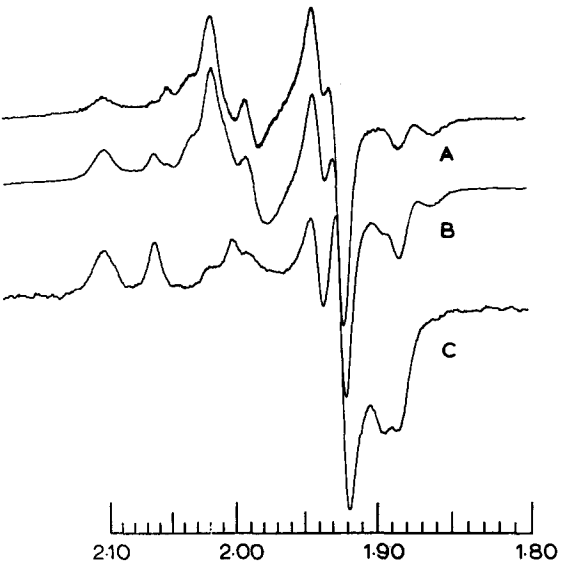

Fig. 6. Effect of microwave power on EPR spectra at 4.2 °K of isolated Complex I reduced with NADH. Isolated Complex I (0.6 nmole FMN/mg protein) in 0.66 M sucrose, 50 mM Tris buffer (pH 8.0) and 1 mM histidine was mixed with 7 mM NADH for 45 s at 0 °C and then frozen in liquid nitrogen. EPR conditions: *F*, 9.1 GHz; *T*, 4.2 °K; *MA*, 12.5 G; *SR*, 250 G/min; Trace A: *P*, 0.02 mW; gain × 2.5. Trace B: *P*, 2 mW; gain × 1.6. Trace C: *P*, 207 mW; gain × 1.

iron–sulphur centers, of NADH-dehydrogenase. As judged from the well separated low-field lines at $g = 2.103$ and 2.064, seen in Traces C of Figs 4 and 6, it is concluded that at least two more iron–sulphur centers with very short relaxation times are present in NADH-dehydrogenase.

Under the conditions used here, 4.2 °K and 207 mW, all the signals still observable are partly saturated and the used field modulation frequency of 100 kHz might distort the true signal shape. Although preliminary experiments with lower modulation frequencies showed no gross changes in line shape, the results were not conclusive enough to be presented here.

EPR spectra at 35 GHz of isolated Complex I

In Fig. 7 a 35 GHz EPR spectrum of isolated Complex I reduced with NADH is shown. The trough of the $g = 1.94$ line is split, indicating possible rhombicity.

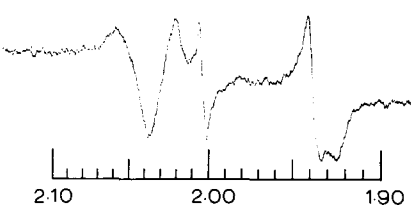

Fig. 7. 35 GHz EPR spectrum at 60 °K of isolated Complex I reduced with NADH. The sample used was prepared in the same way as specified in Fig. 6. EPR conditions: *F*, 35 GHz; *T*, 60 °K; *P*, 3 mW; *MA*, 10 G; *SR*, 1000 G/min.

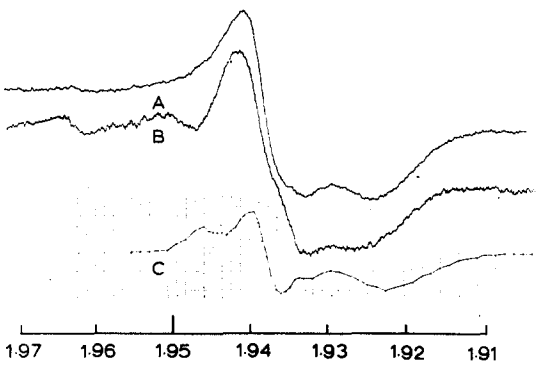

Fig. 8. Effect of incubation of isolated Complex I with NADH on the line shape around $g = 1.94$ in 35 GHz EPR spectra at 60 °K. The sample used was the same as in Fig. 8. Trace A was recorded after mixing for 45 s at 0 °C. The sample was then thawed and kept at 20 °C for 10 min after which Trace B was recorded at a 1.6 times higher gain. Trace C is the difference A−B and is plotted at the same gain as Trace A. EPR conditions: *F*, 35 GHz; *T*, 60 °K; *P*, 4.5 mW; *MA*, 22 G; *SR*, 200 G/min.

The resonance at the low field side around $g = 2.04$–2.06 is from an unknown source in the sample. The slightly rhombic line shape obtained here is in agreement with earlier observations at 9 GHz, where this was also visible [14], though less clearly. It was shown by Albracht and Slater [14] that when isolated Complex I was incubated with NADH at room temperature, the amplitude of the 1.94 line observed at 9 GHz halved, whereas that of the 2.02 line decreased even more. During this process the trough of the 1.94 line sharpened and at the same time a new rhombic type of signal appeared.

This experiment was now repeated looking only at the shape of the signal in the $g = 1.94$ region as shown in Fig. 8. Trace A is a magnification of the 1.94 region of Fig. 7. Trace B is the shape obtained after 10 min of incubation at 20 °C and recorded with a 1.6 times larger gain. Trace C is the corrected difference spectrum of A—B, plotted at the same gain as A, representing the shape of the disappearing signal. The top around $g = 1.946$ in Trace C does not belong to this signal because this is caused by the appearance of a new signal with a g_x value around 1.95 [14]. It can be seen that the shape of the three traces is different in the region 1.92–1.94. The shape of the signal in Trace B suggests that this is not due to one single center either. The conclusion is that the 1.94 line of NADH dehydrogenase as normally seen at 60–80 °K in 9 GHz spectra of isolated Complex I is composed of at least two slightly rhombic signals with different shapes, one of which disappears on prolonged incubation with NADH. The resolution seen in Figs 7 and 8 could not be obtained in NADH-reduced submitochondrial particles due to interference with the succinate dehydrogenase type of signals and the new iron–sulphur center not attached to the respiratory chain, mentioned above.

In Fig. 9 a 35 GHz EPR spectrum of isolated Complex I reduced with NADH is shown, taken at 19 °K. The resolution is nearly the same as that from the 9 GHz spectrum shown in Fig. 6, Trace A, and no additional lines can be detected. Spectra of NADH-reduced submitochondrial particles are nearly identical to the one in Fig. 9 at the same temperature. More work has to be done to characterize the individual signal shapes of the new signals appearing at 4.2 °K Quantitation can then be carried

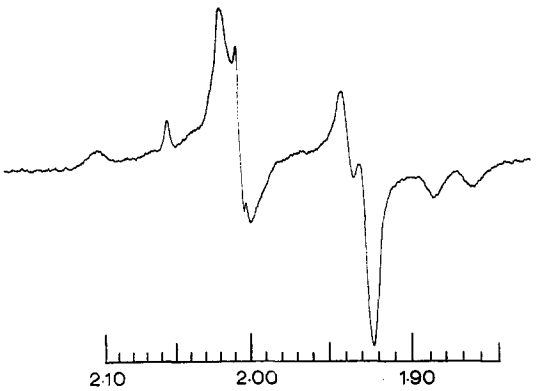

Fig. 9. 35 GHz EPR spectrum at 19 °K of isolated Complex I reduced with NADH. The sample used was prepared as specified in Fig. 6. EPR conditions: *F*, 35 GHz, *T*, 19 °K; *P*, 1 mW, *MA*, 10 G; *SR*, 1000 G/min.

out to determine whether the amounts are significant enough with relation to the FMN content of Complex I.

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