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EPR SIGNALS OF NADH : Q OXIDOREDUCTASE SHAPE AND INTENSITY

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SUMMARY

(1) The EPR spectrum of Center 1 of NADH dehydrogenase in isolated Complex I or submitochondrial particles from beef heart consists of two overlapping nearly axial signals of the same intensity. They are defined as Center 1a ($g_{||} = 2.021$, $g_{\perp} = 1.938$) and Center 1b ($g_{||} = 2.021$, $g_{\perp} = 1.928$).

(2) The line shape of the EPR spectrum of the Centers 3+4 can be interpreted as an overlap of two rhombic signals of the same intensity. We define Center 3 by the g-values: $g_z = 2.103$, $g_y = 1.93$-1.94, $g_x = 1.884$, and Center 4 by the values $g_z = 2.04$, $g_y = 1.92$-1.93, $g_x = 1.863$.

(3) Direct quantitation of the individual signals as well as computer simulation suggests that the amount of the Centers 1a and 1b is only 25 % of that of the other individual centers and FMN. As EPR spectra of beef-heart submitochondrial particles at 10–20 K are nearly identical to those of Complex I, the same relative concentrations of the Fe-S centers are also present in the particles.

(4) The signals earlier observed by us in EPR spectra of Complex I and submitochondrial particles at 4.2 K and high microwave powers can now be explained without assuming more than 5 paramagnetic centers in NADH dehydrogenase.

INTRODUCTION

The EPR spectrum of reduced NADH : Q oxidoreductase from beef heart at temperatures below 20 K is composed of at least 4 different signals. These signals were, first reported by Ohnishi et al. [1] in submitochondrial particles from *Candida utilis*, and later observed in material from beef and pigeon heart [2–5]. Orme-Johnson et al. [2, 6] proposed that 4 signals are present with the following apparent g values; Center 1: 2.022, 1.938 and 1.923; Center 2: 2.054, 1.922; Center 3: 2.100, 1.886, 1.862 and Center 4: 2.103, 1.864. Later [7], the possible existence of two more signals, observable at 4.2 K and high microwave powers, was proposed although no relative concentrations were given.

We felt that the overall line shape of the EPR spectra of reduced Complex I both at 10–20 K and 50 K is difficult to understand in terms of the g-value sets proposed by Orme-Johnson et al. [6] especially for the Centers 1, 3 and 4. This paper describes an attempt to determine the individual signal shapes of the Fe-S centra and their relative intensities. A part of this work has been reported at a recent meeting [8].

MATERIALS AND METHODS

EPR samples. Complex I prepared by the method of Hatefi et al. [9, 10] was dialysed overnight against 0.66 M sucrose and 20 mM sodium phosphate buffer (pH 7.5). Additions of reductants were all performed in an optical cuvette at 22 °C. After each addition, the 460 nm absorption was monitored until it had been constant for at least 1 min. Then a sample was transferred into a calibrated EPR tube and frozen and stored in liquid nitrogen.

Acid extractable flavin was determined fluorimetrically, using FMN as a standard. The concentration of the standard was calculated from the difference in absorption at 450 nm before and after addition of excess dithionite using an extinction coefficient of 10.3 $mM^{-1}\,cm^{-1}$ [9].

EPR spectra. Spectra were recorded on a Varian E-9 spectrometer or a Varian E-3 spectrometer extended with a 60 dB microwave attenuator. Samples were cooled with a commercial helium flow cooling system (Air Products) or one constructed according to Lundin and Aasa [11]. In both cases, the sample temperature was monitored by a carbon resistor 1–2 cm below the sample. Extra care was taken when the spectra were used for quantitative purposes. In this case, a tube filled with water and containing 2 carbon resistors at a mutual distance of 2.5 cm was placed at the sample position under exactly the same experimental conditions as the sample tube, except for the microwave power. The two resistors were positioned on both sides of the measuring area of the cavity. The mean of the temperatures given by the resistors was compared with the temperature indication of the resistor placed below the sample and the difference was used to correct the reading during the measurements on the sample. All carbon resistors used were from the Allen-Bradley type. They were calibrated according to Borcherds [12] using liquid helium and liquid nitrogen as reference temperatures. Before calibration they were cooled to 4.2 K and warmed up to room temperature at least 20 times. Even then, recalibration was occasionally necessary.

All spectra were recorded with a field modulation frequency of 100 kHz. The magnetic field strength was measured via a NMR probe, while the microwave frequency was determined with a frequency counter. Microwave attenuators were calibrated using a Fe (III) salt.

EPR conditions are abbreviated in the legends to figures as follows: 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.

Signal quantitation. The intensity of the signal of the centers 1a+1b in 50 K spectra of reduced Complex I was determined by direct double integration. For the Centers 2 and 3 the area of their g_z lines at 2.054 (20 K spectra) and 2.103 (14 K spectra), respectively, was used to compute the intensities. Formulae given by Aasa and Vänngård [13] were used to relate these areas to the double integral values. For

Center 4, the high-field half of the g_x line at 1.863 (14 K spectra) was taken to estimate its intensity. A solution of 1 mM Cu(II) in 2 M $NaClO_4$ and 10 mM HCl [14] was used as a concentration standard. Spectra were not run under identical conditions, but the calculated intensities were corrected for the temperature, gain, microwave power and tube-calibration factor. They were also corrected for the g values using the average intensity factor g_p^{Av} [13]. Conditions were chosen so that the signals were not saturated when spectra were run for quantitation.

Digitizing of spectra. Although the E-9 spectrometer had an on-line connection with a Hewlett-Packard 2100 computer we preferred an off-line procedure to digitize the EPR spectra for practical reasons. A device to do so was set up using a HP 2100 computer together with a scanning digital volt meter (HP 3480 B+HP 3485 A) and a digital plotter (HP 7210 A). The digitizer was constructed as follows: the x-y coordinates were controlled by two potentiometers and read by two channels of the scanning voltmeter. A third channel of the voltmeter was connected to a push button by which manual sampling of an x-y point became possible. The computer continuously monitored the potentiometers and sent their scaled values to the x-y plotter. After determination of some reference points of the curve the computer directed the pointer of the plotter to the desired starting point of the x-axis. From then on, only y values and the push button were monitored by the voltmeter. The pointer then moved along the x-axis in predetermined steps, after a point had been sampled by pushing the button. The x-y points determined in this way were then dumped on either punched papertape or on disc for later retrieval.

The accuracy with which this set-up could digitize curves was dependent on the accuracy with which the operator could place the pointer on the curve and on the specifications of the plotter (0.01 inch). When digitized curves were plotted there was no discernible difference with the originals.

As in practice the microwave frequency at which the EPR spectrometer operates depends on the contents of the cavity, spectra cannot be run at precisely the same frequency. This means that spectral shapes cannot be compared at once as the resonance fields are directly proportional to the frequency. We therefore digitized all curves in such a way that the sampling points had identical g values, i.e. we transformed them to one and the same frequency. As no hyperfine splitting is expected in the EPR spectra of Complex I, this method is allowed. Errors due to the difference in line width are negligible as the experimental frequencies in this paper differ by at most 1 ‰. Having the spectra in a digitized form, where the points match on a g-value scale, addition and subtraction could now be performed without artefacts.

Simulation of EPR spectra. Simulations were carried out on a Cyber 73-26 computer. The simulation program was made for rhombic powder spectra. Instead of the transition probability, the intensity factor g_p was used [13]. Anisotropic line width was used with the same angular dependence as a first-order hyperfine coupling [13, 15]. The powder spectra were assumed to be a convolution of first derivative gaussian lines in all cases.

RESULTS AND DISCUSSION

I. Line shapes and g values

Center I. When Complex I and NADH are reacting during several minutes, the

intensity and shape of the lines at $g = 2.02$ and 1.94 are not stable [10, 16]. In addition to this, Complex I preparations always contain small amounts of the Complexes II, III and sometimes even IV. These are slowly reduced by NADH and as the Fe-S centers present in the Complexes II and III have relaxation rates comparable to Center 1, their EPR spectra will disturb the line shape of Center 1.

In a preliminary experiment, therefore, Complex I was stirred with excess NADH for only 30 s at 0 °C whereafter the mixture was frozen in liquid nitrogen. At 50 K the $g = 2.02$ line and the 1.94 line start saturating at microwave powers greater than 5 mW (see Fig. 1). At the lowest convenient power, where still a reasonable S/N ratio was obtained and no saturation occurred, a spectrum was recorded with a modulation amplitude of 0.63 mT. A scan length greater than 60 mT was chosen on a scale of 1 mT/cm. This was done for two reasons: first, 60 mT scans were needed as these spectra were later compared with the much broader spectra of the Centers 3 and 4; secondly, it minimizes errors in the field position of the steep parts of the spectra, especially in the $g_\perp$ region. As a result of these settings, one spectrum was displayed on two sheets of x-y recorder paper of the Varian spectrometer. The spectrum was then digitized, as described under Materials and Methods, and replotted on a convenient scale. This spectrum is reproduced in Fig. 2, Trace A. Three features can be observed: (a) A line at $g = 2.00$, disturbing the shape of the other lines, that possibly originates from the semiquinone forms of FMN and/or Q-10; (b) the trough of the $g = 1.94$ line is split, whereas the ratio of the positive part and the negative part of this line is approximately 1. This indicates that this line is not a simple $g_\perp$ line; (c) no additional lines on the high-field site of the $g = 1.94$ line can be seen, although such lines show up when dithionite is present as will be shown later on. This means that the contaminating complexes containing Fe-S centra are not reduced under these conditions.

Another way to obtain a good line shape of Center 1 is to make use of the fact that dithionite is not able to reduce Center 1 in beef-heart Complex I. In addition we

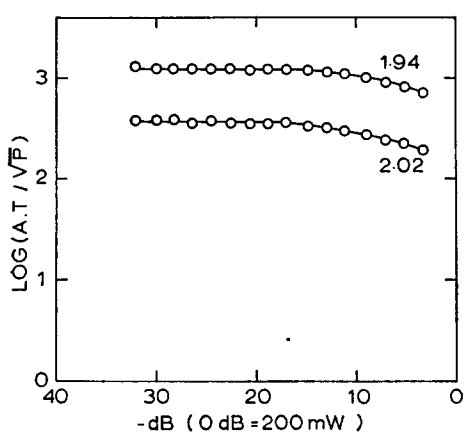

Fig. 1. Microwavepower dependence of the lines of Center 1. Complex I (31 mg/ml) in 0.66 M sucrose and 20 mM sodium phosphate buffer (pH 7.5) was mixed with 6 mM NADH for 30 s at 0 °C before freezing in liquid nitrogen. Spectra at various microwave powers were scanned under the following EPR conditions: F, 9080 MHz; T, 50 K; MA, 0.63 mT; SR, 25 mT/min. The amplitudes (A) of the $g = 2.02$ and 1.94 line were measured. These values were multiplied by the temperature and divided by the experimental gain and the square root of the power and plotted against the power.

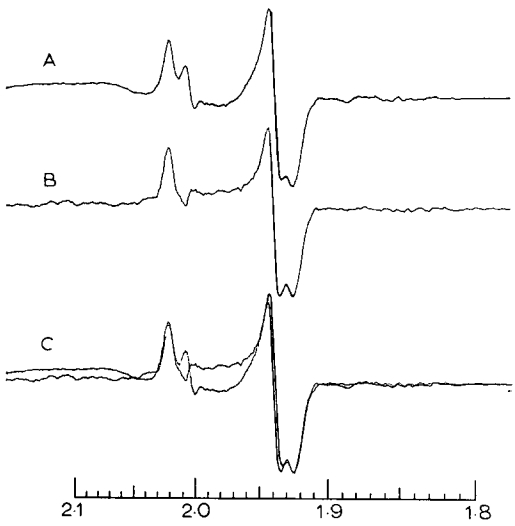

Fig. 2. Comparison of the line shape of Center 1 obtained directly and indirectly. (A) Experimental EPR spectrum of Complex I reduced with NADH as in Fig. 1. (B) The difference of the spectrum of Complex I reduced with dithionite plus 2 mM NADH minus that of Complex I reduced with dithionite (equal to Trace C of Fig. 3). EPR conditions: F, 9080 MHz; T, 50 K; P, 0.5 mW; MA, 0.63 mT; SR, 12.5 mT/min. The y-scaling for B was corrected in order to match the experimental conditions to those of A. (C) Superposition of Traces A and B.

found that dithionite stabilizes the amplitudes and the shapes of the $g = 2.02$ and 1.94 lines during subsequent contact of the Complex I with excess NADH. The reason for this is not known. EPR spectra of the two conditions are shown in Fig. 3, traces B and A, respectively. Fig. 3, Trace B, shows lines originating from Complex II ($g_{z,y,x} =$ 2.02, 1.93, 1.91), Complex III ($g_{z,y,x} = 2.02$, 1.90, 1.86 and even from the Fe-S center of outer membranes [17, 18] ($g_{z,y,x} = 2.01$, 1.94, 1.89), indicating that this particular Complex-I preparation is impure (0.8 nmol FMN/mg protein). This is, however, of no importance for the line-shape analysis of the Fe-S centers of NADH : Q oxidoreductase. No traces of a line at $g = 1.97$ can be detected in Trace A although the NADH was in contact with the Complex-I preparation for more than 7 min at room temperature. When dithionite was omitted, such a line appeared within a few minutes (not shown, see ref. 16) The difference spectrum, shown in Trace C, represents the EPR spectrum of the species that is reduced by NADH but not by dithionite. It is obvious that the shape is very similar to the signal of Center 1 as shown in Fig. 2, Trace A. This is more precisely demonstrated in Fig. 2, Traces B and C. It can be seen that the $g = 2.02$ and 1.94 lines match remarkably well. The radical line is absent in Trace B, since it is already present in the dithionite spectrum. Therefore it is most probably due to Q-10. The facts that the base line of Fig. 2, Trace A on the low-field side of the 2.02 line is higher than in Trace B, and on the high-field side is lower than in Trace B, is explained by the copper of cytochrome oxidase presumably present as a contaminant, which is reducible by dithionite, but not by a short incubation with NADH.

Fig. 2 further demonstrates that the shape and the amplitudes of the lines of Center 1 are the same, whether a short incubation with NADH or long incubation

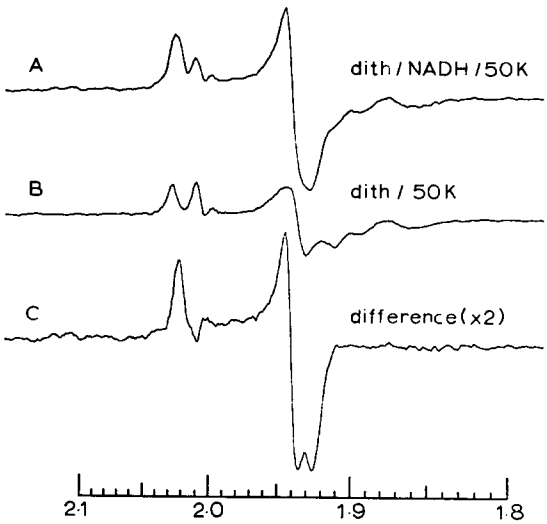

Fig. 3. The line shape of Center 1 obtained indirectly. (A) EPR spectrum of Complex I reduced with dithionite plus NADH. (B) Spectrum of Complex I reduced with dithionite. (C) Difference spectrum, two times enlarged. EPR conditions: F, 9080 MHz; T, 50 K; P, 0.5 mW; MA, 0.63 mT; SR, 12.5 mT/min.

with dithionite followed by NADH is used to reduce Center 1. This is in contrast to what would be expected from the redox potentials in pigeon-heart mitochondria as measured by Ohnishi [19]. We found that the addition of benzyl- and methylviologen (100–200 μM), separate or together, subsequent to dithionite plus NADH has no effect on the redox state of the Fe-S centers. Thus, NADH alone gives maximal reduction of the Fe-S centers in Complex I.

It has been suggested earlier [16, 7] that the split trough of the $g = 1.94$ line indicates the presence of two different lines, especially since on incubation with NADH of Complex I only the low-field trough disappears [16]. However, submitochondrial particles do not show such a time-dependent phenomenon on contact with excess NADH. We found that this instability is introduced in the isolation procedure of Hatefi et al. [9] at the point where Complex I-III is prepared from Complex I-II-III. This made it necessary to investigate whether the line shape of Center 1 in submitochondrial particles is the same as in Complex I. For this purpose, submitochondrial particles freed from outer membranes [18] were used, as NADH, but not succinate, reduces the Fe-S center present in the outer membranes. The result is shown in Fig. 4. Although the spectra were recorded at 84 K and the signal/noise ratio of the difference trace is not very satisfactory, it can be clearly seen that two troughs are present around $g = 1.94$. The conclusion is that the EPR-lines shape of Center 1 in isolated Complex I is very much the same as that in submitochondrial particles.

Whether the particular shape of the $g = 1.94$ line is in fact caused by a superposition of two axial lines or by a possible rhombic line shape can be answered with the help of computer simulation of the spectrum. As the best experimental line shape of Center 1, Trace B of Fig. 2 was taken. Good fits could only be obtained if two independent axial signals with the same $g_{||}$ values, but slightly different $g_{\perp}$ values,

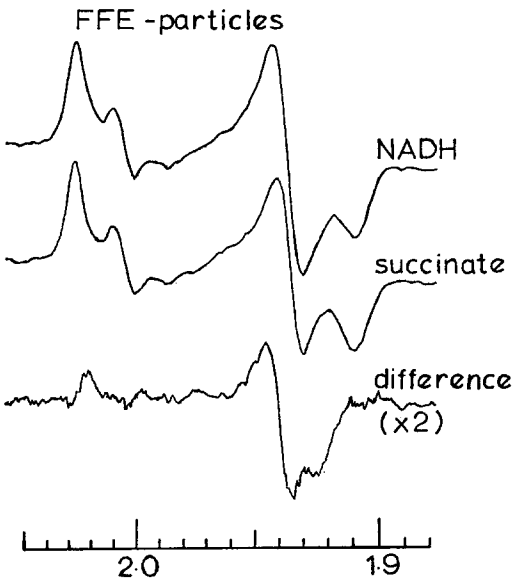

Fig. 4. Line shape of Center 1 in submitochondrial particles. Submitochondrial particles, made by the method of Free Flow Electrophoresis [18], suspended in 0.25 M sucrose and 20 mM Tris · HCl buffer (pH 7.5), were reduced to anaerobiosis with 6 mM NADH or 25 mM succinate. (A) NADH. (B) succinate. (C) Difference spectrum at a two times higher gain. EPR conditions: F, 9014 MHz; T, 84 K; P, 80 mW; MA, 0.63 mT; SR, 12.5 mT/min.

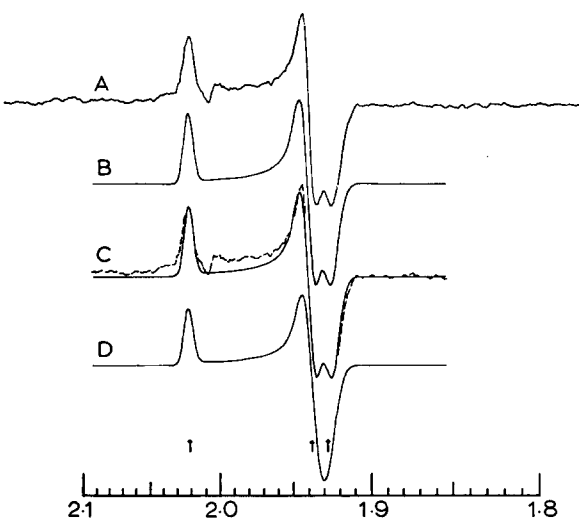

Fig. 5. Simulation of the line shape of Center 1. (A) Trace B of Fig. 2. (B) Simulation as a sum of two axial signals with the same weight. Parameters for 1a: $g_{||} = 2.0213$, $g_{\perp} = 1.9381$; widths: 1.05 mT and 1.55 mT for the z and x-y direction, respectively. Parameters for 1b: $g_{||} = 2.0213$, $g_{\perp} = 1.9278$; widths: 1.05 mT and 1.9 mT for the z and the x-y direction, respectively. (C) Superposition of the traces A and B. (D) Simulation assuming one rhombic signal. Parameters: $g_z = 2.0213$, $g_y = 1.9381$, $g_x = 1.9278$; widths: 1.05 mT, 1.55 mT and 1.9 mT for the z, y and x direction, respectively.

were added in a 1 : 1 ratio. When the three g values used for this simulation were taken to simulate a rhombic spectrum, no fit could be obtained, also when varying the g_y and g_x values, and the ratio of the amplitudes of the top and the trough of the 1.94 line was less than one. This is shown in Fig. 5.

It is concluded that 'the signal of Center 1' as observed in EPR spectra of reduced Complex I or submitochondrial particles in fact consists of two overlapping signals of the same intensity (double-integrated area) having indistinguishable $g_{||}$ values but slightly different $g_{\perp}$ values. From the resolution in X-band EPR spectra, it cannot be determined whether the signals are precisely axial. Measurements at 35 GHz [7] indicated that they probably are slightly rhombic. The two centers are hereafter referred to as Center 1a and 1b for $g_{||} = 2.02$, $g_{\perp} = 1.94$ and $g_{||} = 2.02$, $g_{\perp} = 1.93$, respectively. It is not certain whether the two centers as identified in this way correspond to the Centers 1a and 1b as identified via different ATP response and redox potentials as described by Ohnishi et al. [19]. Although objections can be made to the designation 1a/1b, we retain it in order to indicate that we are very probably dealing with two forms of the same type of center. In this connection it is worth mentioning that no different forms of Center 1 could be detected in EPR spectra of submitochondrial particles of the yeast *Candida utilis* [20].

Center 2. A good line shape of Center 2 can be easily obtained by making use again of the fact that dithionite does not reduce the Centers 1a/1b, but does develop 70–80 % of the $g = 2.05$ line of Center 2 at 18 K and only 15–20 % of the $g = 2.10$ line. However, all species not belonging to NADH dehydrogenase are also reduced and will disturb the line shape. We know by experience, however, that all the paramagnetic centers from the Complexes II and III and from outer membranes as present in completely reduced submitochondrial particles show up in EPR spectra both at 18 K and in the region 50–80 K. A possible exception could be Center 2 of succinate dehydrogenase, about which there is some controversy in the literature [21, 22]. We did not check this in detail as this is of minor importance for our case here, where Complex II or even only parts of Complex II are present as contaminants. Also the line shape of Center 2 of succinate dehydrogenase seems to be very similar to that of Center 1 of that enzyme. Thus a difference of the EPR spectrum of dithionite-reduced Complex I at 17 K minus the spectrum at 50 K, where the gain is corrected for the differences in microwave power, temperature and experimental gain will give a shape of Center 2 which is only slightly disturbed by the presence of lines accompanying the 2.10 line. This is displayed in Fig. 6. The microwave power used for the 17 K spectrum was such that no saturation of the lines of Center 2 occurred. The difference spectrum (Trace C) shows negative peaks around $g = 2.02$ and 2.00. This indicates that these lines slightly saturate at 17 K, so that their amplitude is smaller than expected, i.e. the difference A-B will be negative. Observe, however, that the base line on the high-field side of the $g = 1.92$ line in Trace C is rather flat and shows no positive peaks. This means that the troughs at $g = 1.91$, 1.89 and 1.86 in the Traces A and B are not saturated in the 17 K spectrum. The high-field side base line in Trace C shows two very faint troughs at positions 1.88 and 1.86, which accompany the very weak peak at $g = 2.10$. Trace D shows that if the weighting factor for B in the difference A minus B is decreased so as to eliminate the peaks at $g = 2.02$ and 2.00, the shape of the $g = 1.92$ line is affected and the high-field side base line is no longer flat, but shows troughs at the same positions as in Trace B, indicating that the subtraction was

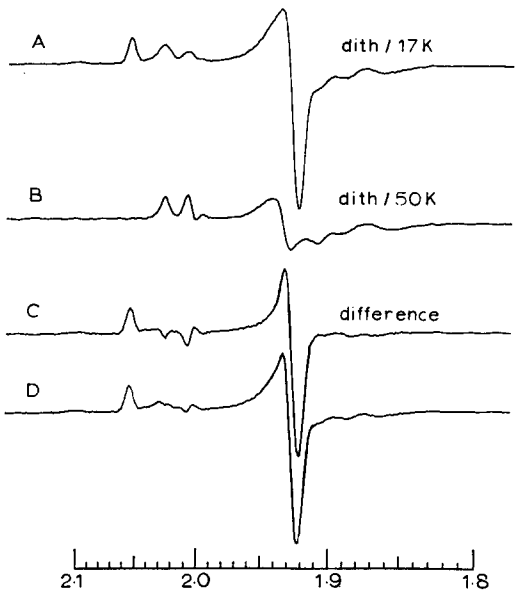

Fig. 6. The line shape of Center 2. A preparation of Complex I reduced with dithionite was used for these traces. (A) EPR spectrum at 17 K. EPR conditions: F, 9080 MHz; T, 17 K; P, 0.2 mW; MA, 0.63 mT; SR, 12.5 mT/min. (B) The spectrum at 50 K. EPR conditions: F, 9080 MHz; T, 50 K; P, 0.5 mW; MA, 0.63 mT; SR, 12.5 mT/min. The y-scaling was corrected to the experimental conditions of Trace A. (C) Difference spectrum A minus B. (D) Difference spectrum A minus $f \times$ B in which the factor f was varied such that the negative peaks around $g = 2.00$–2.02 just disappeared.

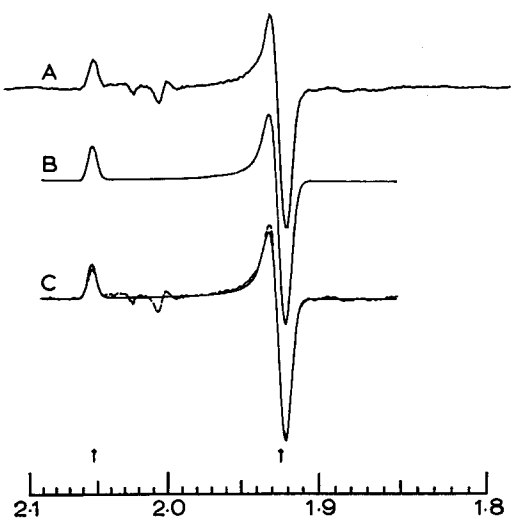

Fig. 7. Simulation of the line shape of Center 2. (A) Trace C of Fig. 6. (B) Simulation as one axial signal. Parameters: $g_{||} = 2.0538$, $g_{\perp} = 1.925$; widths: 1.0 mT for the z and 1.6 mT for the x-y direction. (C) Superposition of A and B. The small arrows at the bottom of the figure indicate the g values.

insufficient for this region. Apparently, the signals seen at 50 K do not saturate homogeneously at 17 K.

If Trace C of Fig. 6 is taken as the best experimental shape of Center 2, simulation as one axial signal is not difficult, as is shown in Fig. 7.

Centers 3 and 4. The strong temperature dependence of the lines at $g = 2.10$, 1.88 and 1.86 can be used to obtain information on the true shape of the complete signals of Centers 3 and 4. This is illustrated in Fig. 8. The difference spectrum, Trace C, should no longer contain signals from the Centers 1a/1b.

A very similar result is obtained when a temperature-difference spectrum is made from Complex I reduced with dithionite and subsequently with NADH. This is shown in Fig. 9.

The two difference spectra of Figs. 8 and 9 are compared in Fig. 10. It can be seen that the overall shape and the amplitudes are nearly identical except for a signal around $g = 2.015$, which is possibly due to a contamination by Complex II which is not quickly reduced by NADH in this Complex-1 preparation. At this point, it should be noted that these temperature-difference spectra at 17 K do not contain any contributions from the signal of Center 2 of succinate dehydrogenase as is shown by the fact that the shape in the $g = 1.93$-1.91 region is the same with NADH alone or dithionite plus NADH as reducing agents. Only dithionite would be able to reduce this Center 2 [21].

The microwave power (0.2 mW) at 17 K used for Figs. 8, 9 and 10 was not saturating for any of the lines at $g = 2.10$, 2.05, 1.92, 1.88 and 1.86. The $g_{\perp}$ lines of the Centers 1a/1b could not be monitored separately at this temperature, but even if they would saturate slightly, this would give rise to negative contributions of these lines in the difference spectra of Figs. 8 and 9. As can be seen in Fig. 10, Trace C, both

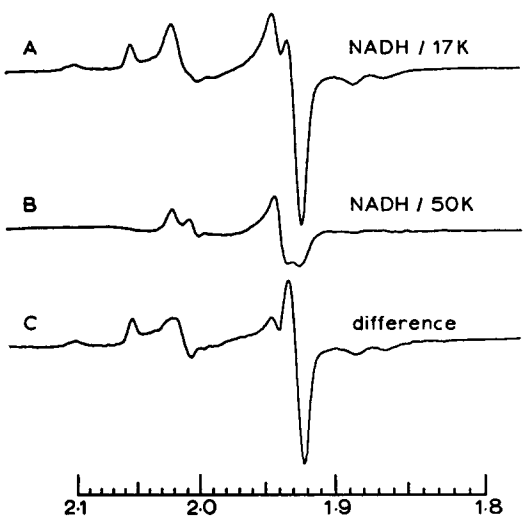

Fig. 8. Information on the line shape of the Centers 3+4. The same EPR tube as used for Fig. 1 was used here. (A) EPR spectrum at 17 K of Complex I reduced with NADH. (B) The spectrum at 50 K. The y-scaling was corrected to the experimental conditions of A. EPR conditions: F, 9080 MHz; T, 17 K for A and 50 K for B; P, 0.2 mW for A and 0.5 mW for B; MA, 0.63 mT; SR, 12.5 mT/min. (C) Difference spectrum A minus B.

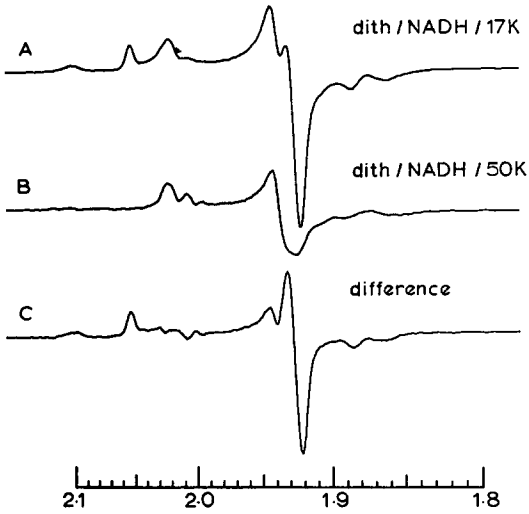

Fig. 9. Information on the line shape of the Centers 3+4. The same EPR tube as used for Fig. 3 was used. (A) EPR spectrum at 17 K of Complex I reduced with dithionite and subsequently with NADH. (B) The spectrum at 50 K. The y-scaling was adjusted to meet the experimental conditions of A. EPR conditions: F, 9080 MHz; T, 17 K for A and 50 K for B; P, 0.2 mW for A and 0.5 mW for B; MA, 0.63 mT; SR, 12.5 mT/min. (C) Difference A minus B.

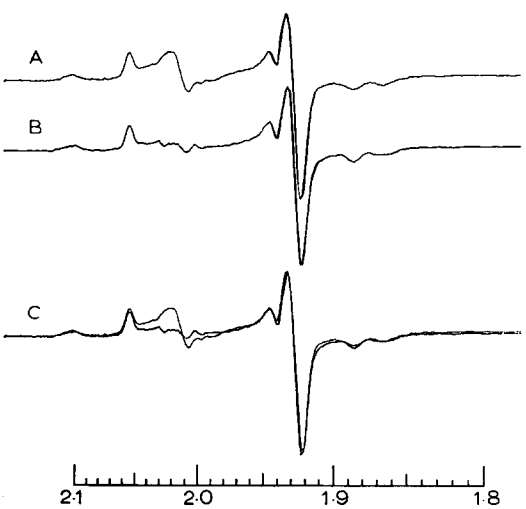

Fig. 10. Comparison of the line shape of the sum spectrum of the Centers 2, 3 and 4 obtained in two ways. (A) Trace C of Fig. 8. (B) Trace C of Fig. 9. The y-scaling was adjusted to correct for the difference in the tube-calibration factors. (C) Superposition of the two spectra.

difference spectra contain a top around $g = 1.945$ of precisely the same amplitude. This top thus belongs to other signals than the Centers 1a/1b or 2 of NADH dehydrogenase. Of course the shape of Center 2 can be subtracted from this difference spectrum, but the resultant spectra had a low signal/noise ratio.

In Fig. 11, an attempt is shown to obtain a spectrum of fully reduced Complex I at 17 K without the line shape of Center 2. Trace C, a difference spectrum, represents all lines from NADH dehydrogenase that develop or increase on adding NADH to dithionite-reduced Complex I. Contributions of contaminants have disappeared by subtraction. Lines at $g = 2.10$, 1.88 and 1.86 increase in amplitude. Centers 1a/1b appear and Center 2 becomes now fully reduced. Trace E contains no contribution from Center 2. It does contain the full amplitude and line shape of Centers 1a/1b and the major part of the amplitudes of the lines of the Centers 3 and 4. Two features attract attention: (a) there is a broad peak around $g = 2.04$ with the same amplitude as the $g = 2.10$ line; (b) the line shape of the $g_\perp$ part of the Centers 1a/1b is rather disturbed. There will be a contribution of the line around $g = 1.94$, which in Fig. 10 we ascribed to Centers 3+4, but this line is expected to overlap pretty well with the $g_\perp$ line of Center 1a. The broad trough around $g = 1.92$ is expected to be the result of an extra 'g_y-type' line, overlapping with the $g_\perp$ lines of Centers 1a/1b.

Summarizing these results, it can be said that 17 K spectra of fully reduced Complex I contain, in addition to the now well-defined signals of the Centers 1a/1b

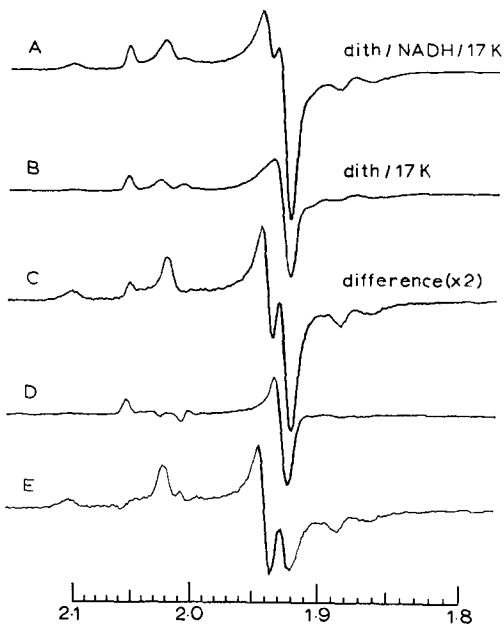

Fig. 11. Information on the line shape of the Centers 3+4. (A) EPR spectrum at 17 K of Complex I reduced with dithionite plus NADH. (B) Spectrum at 17 K of Complex I reduced with dithionite alone. The y-scaling was corrected so as to match the experimental conditions for A. EPR conditions: F, 9080 MHz; T, 17 K; P, 0.2 mW; MA, 0.63 mT; SR, 12.5 mT/min. (C) Two times enlarged difference A minus B. (D) Trace C of Fig. 6 and representing the shape of Center 2. The y-scaling was adjusted to make the $g = 2.05$ line equal to that of Trace C. (E) Difference C minus D. This trace represents the sum spectrum of the centers 1a, 1b, 3 and 4.

and 2 and to the lines at 2.10, 1.88 and 1.86, presumably three other lines: a broad peak around $g = 2.04$ (Fig. 11, Trace E), a line with a top at $g = 1.94$ (Fig. 10) and one with a trough at $g = 1.92$ (Fig. 11, Trace E). These extra lines are not detectable in the normal experimental spectra, where they are hidden under more prominent lines of the Centers 1a/1b and 2. As the lines at $g = 1.88$ and 1.86 as well as the lines at 2.10 and 2.04 have the shape of a normal gaussian peak, the new lines at 1.94 and 1.92 are expected to be the accompanying g_y lines. The conclusion then is that these 6 lines form two signals belonging to Centers 3 and 4. The assignment of a combination of 3 g-values to one signal was solved by computer simulation.

When in a simulation 2 signals were added in a 1 : 1 ratio with a common g_z value at 2.10 (first signal: $g_{z,y,x} = 2.10$, 1.92–1.94, 1.88; second signal: $g_{z,y,x} =$ 2.10, 1.92–1.94, 1.86), then the amplitude of the resulting g_z line was always nearly as great as or greater than that of the 1.88 or 1.86 line. An example is shown in Fig. 12, Trace B. In reality, however, the amplitude of the g_z line at 2.10 is about half that of the 1.88 or 1.86 line as can be seen in Trace A. This trace is an experimental EPR spectrum of NADH-reduced Complex I recorded at 12 K and 2 mW. Under these

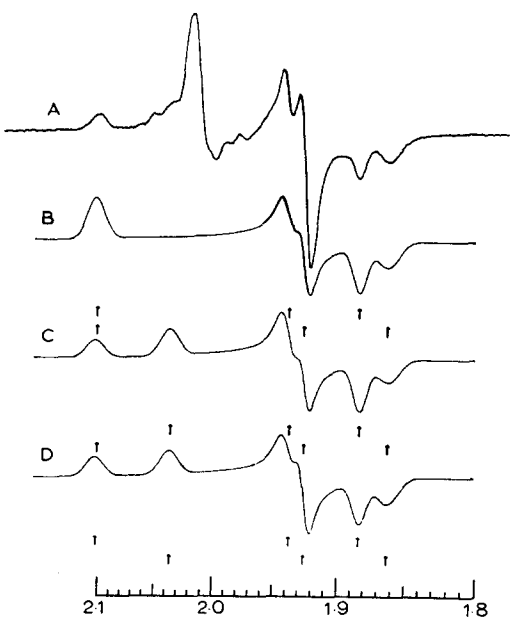

Fig. 12. Determination of the g-values of the Centers 3 and 4 by simulation. (A) Experimental EPR spectrum at 12 K of Complex I reduced with NADH. EPR conditions: F, 9081 MHz; T, 12 K; P, 2 mW; MA, 0.63 mT; SR, 25 mT/min. (B) Simulation of the sum of the centers 3+4 assuming two rhombic signals with a common g_z line at 2.10. Parameters: $g_{x,y,z} = 1.884$, 1.938, 2.103 and widths (x,y,z) 1.9 mT, 1.9 mT and 2.4 mT for the first signal; for the second signal $g_{x,y,z} = 1.863$, 1.9263, 2.103 and widths (x,y,z) 3 mT, 1.4 mT and 2.4 mT. (C) Simulation of the Centers 3+4 assuming two rhombic signals with different g_z values. Parameters: for the first signal $g_{x,y,z} = 1.884$, 1.938, 2.037 and widths (x,y,z) 1.9 mT, 1.9 mT and 2.5 mT, for the second signal $g_{x,y,z} = 1.863$, 1.9263, 2.103 and widths (x,y,z) 3 mT, 1.4 mT and 2.4 mT. (D) as C but the g_z values had been interchanged. Parameters: for Center 3: $g_{x,y,z} = 1.884$, 1.938, 2.103 and widths (x,y,z) 1.9 mT, 1.9 mT and 2.4 mT, for Center 4: $g_{x,y,z} = 1.863$, 1.9263, 2.037 and widths (x,y,z) 3 mT, 1.4 mT and 2.5 mT. The small arrows indicate the g values for the two signals.

conditions, no saturation could be detected for the 2.10, 1.88 and 1.86 lines. Since, however, the lines of the Centers 1a/1b and 2 are partly saturated, their amplitudes are relatively small versus those of the Centers 3 and 4. These simulations indicate that only one of the g_x lines can have an accompanying g_z line at the 2.10 position and that the experimental line around $g = 2.04$ found in difference spectra and also visible in Fig. 12, Trace A, is a second g_z line. In this respect it can be said that, as will be shown later, the area of the 2.103 line corresponds to at most an intensity equal to that of Center 2.

Another result from the simulations was that the ratio of the amplitude of the 1.88 and 1.86 lines could be better compared with experimental spectra if the 1.88 line was coupled to the line at 1.94 and the 1.86 line to the 1.92 line. This was rather independent of the position of the g_z lines (not shown).

A third result was that one g_z line could be coupled only to one particular set of g_y-g_x values. An example is shown in Fig. 12, Traces C and D. The ratio of the 1.88 and 1.86 line in Trace D fits best with the experimental spectrum.

A further argument leading to this choice was that at 4.2 K and high power the so defined lines of center 3 are only partly saturated, whereas the g_z and g_x lines of center 4 (2.04 and 1.86, respectively) are saturated completely (see Fig. 6 of reference 7).

The conclusion is that the line shape of the Centers 3+4 can be interpreted as an overlap of 2 rhombic signals: for the center, which we shall define as Center 3, $g_z = 2.103$, $g_y = 1.93$–1.94 and $g_x = 1.884$ and for the center, which we define as Center 4, $g_z = 2.037$, $g_y = 1.92$–1.93 and $g_x = 1.863$. The g_y values for both centers can not be given more precisely, because no good experimental spectra are available.

II. Concentration of the centers

There are two ways to establish the relative weights of the several signals: quantitation by double integration of the spectra or single integration [13] of parts of the spectra, or simulation of the experimental sum spectrum at 17 K.

Direct double integration of a complete spectrum was only possible for the Centers 1a/1b. The spectra presented in Fig. 2 were used for this purpose. Also two additional Complex-I preparations, which were somewhat purer, were used for quantitation. The double integral value was corrected for the average intensity factor g_p^{Av} [13] using three g-values (2.02, 1.94 and 1.93). The concentration of the individual

TABLE I

CONCENTRATION OF THE SIX PROSTHETIC GROUPS IN ISOLATED COMPLEX I

The concentrations were computed as described under Materials and Methods.

Preparation	FMN content (μmol/g protein)	Concentration of the Fe-S centers in mol (S = ½)/mol FMN			
		1a+1b	2	3	4**
1*	0.80	0.40	0.78	0.56	0.86
2	0.93	0.44	0.77	0.58	1.08
3	0.96	0.41	0.84	0.66	1.07

* This preparation was used throughout this paper.

** The value is overestimated due to overlap of the $g = 1.86$ line by part of the $g = 1.88$ line.

Centers 1a and 1b is only half this value, since the sum of the double integrals of the two individual simulated signals each corrected by using two g values ($g_{||}$ and $g_{\perp}$) is the same as the double integral of the simulated sum signal corrected by using the three above-mentioned g values.

The resulting values for all centers are given in Table I. Orme-Johnson et al. [6] reported concentrations close to that of the FMN for all the centers (in their case no differentiation of Center 1 into 1a and 1b existed).

We felt that simulation of the 17 K EPR spectra of reduced Complex I should also give rather reliable information about the relative concentration of the centers, as the line shape, especially in the $g_{\perp}$ region of the spectra is very sensitive to a change of these concentrations, due to a large amplitude of these lines.

Preliminary simulations were carried out by first mixing the Centers 1a/1b and 2 in different ratios. The best weighting factors were then used to compute a total spectrum including the Centers 3 and 4 and the results were compared with experimental spectra. This is shown in Fig. 13. If one compares the line shapes in the $g =$

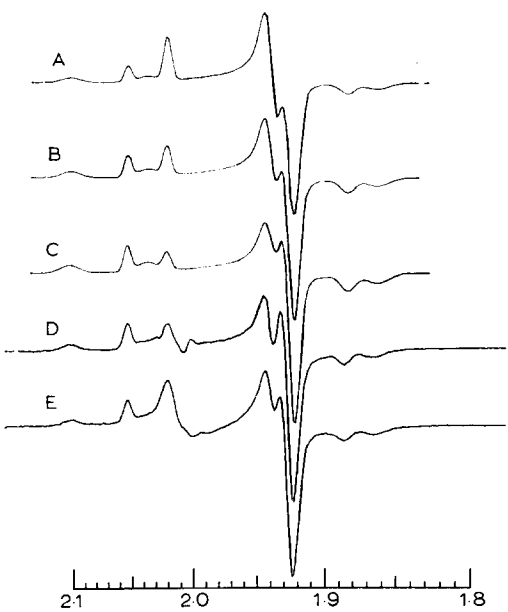

Fig. 13. Simulation of the complete EPR spectrum of reduced Complex I at 17 K. (A) Simulation assuming an equal weight for all the 5 centers (1a, 1b, 2, 3 and 4). (B) Simulation assuming a weighted ratio of the Centers 1a : 1b : 2 : 3 : 4 = 0.5 : 0.5 : 1 : 1 : 1. (C) Simulation as B but with ratio 1a : 1b : 2 : 3 : 4 = 0.25 : 0.25 : 1 : 1 : 1. The maximal amplitude of the three Traces A, B and C was kept the same. Parameters: Center 1a: $g_{x\text{-}y,z}$ = 1.9381, 2.0213, width (x-y,z) = 1.55 mT, 1.05 mT; Center 1b: $g_{x\text{-}y,z}$ = 1.9278, 2.0213, width (x-y,z) = 1.9 mT, 1.05 mT; Center 2: $g_{x\text{-}y,z}$ = 1.925, 2.0538, width (x-y,z) = 1.6 mT, 1 mT; Center 3: $g_{x,y,z}$ = 1.884, 1.938, 2.103, width (x,y,z) = 1.9 mT, 1.9 mT, 2.4 mT; Center 4: $g_{x,y,z}$ = 1.863, 1.9263, 2.037, width (x,y,z) = 3 mT, 1.4 mT, 2.5 mT. (D) Difference of a spectrum of Complex I reduced with dithionite plus NADH taken at 17 K minus one of Complex I reduced with dithionite alone and recorded at 50 K. (E) Experimental EPR spectrum of Complex I reduced with NADH (same tube as used for Fig. 1). EPR conditions for D and E: F, 9080 MHz; T, 17 K; P, for the spectra used for Trace D, 0.2 mW at 17 K and 0.5 mW at 50 K, for Trace E: 0.2 mW; MA, 0.63 mT; SR, 12.5 mT/min.

1.94–1.92 region, it is obvious that the simulation of Trace C fits best to the experimental spectra. The relative concentration of the sum of the Centers 1a/1b is in this case one half that of the other individual centers. Note that the relative amplitudes of the 2.05 and 2.02 lines and the shape of the $g = 1.92$–1.94 region in Trace A resemble very much the experimental spectrum of Fig. 11, Trace C, which contains the full signals of Centers 1a and 1b, but only 20–30 % of the signal of Center 2. This once more points in the direction that the concentration of Center 2 seems to be 4 times that of the individual Centers 1a and 1b. To show the influence of the g_y lines of the Centers 3 and 4 on the line shape in the $g = 1.94$–1.92 region, Fig. 14 is presented. It can be seen that their amplitudes are not negligible, but this depends on the chosen linewidths. As we could not obtain these widths from experimental spectra, the values are rather arbitrary. For the y-direction of Center 3, we used the same linewidth as for the x-direction. For Center 4 this width was chosen about half of the linewidths used for the x-and z-directions. Even if the Centers 3 and 4 were not added to the sum spectrum, as in the preliminary simulations mentioned above, the overall shape of the $g = 1.92$–1.94 region fitted the experimental traces the best if the concentration of Center 2 was taken to be 4 times that of the Centers 1a and 1b. Also the g_z lines at 2.05 and 2.02 fit best to the experimental traces, when this ratio was assumed, as also seen in Fig. 13, Traces C and D. Fitting of the line shape, by varying the relative contributions of the Centers 1a/1b to the total spectrum, thus appears a sensitive way for determining their relative concentrations towards Center 2.

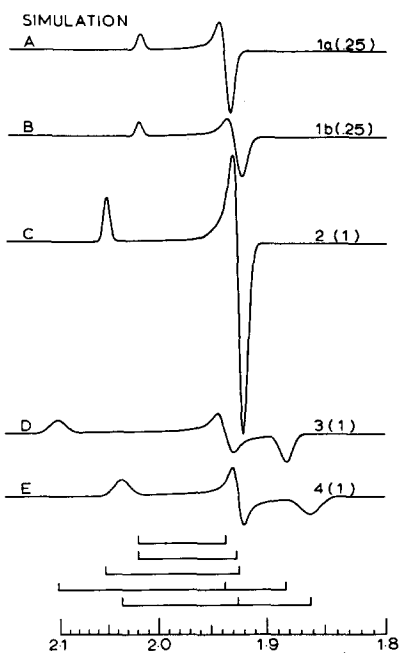

Fig. 14. Plot of the individual simulated signals of the 5 Fe-S centers of Complex I. The weighted ratio was 1a : 1b : 2 : 3 : 4 = 0.25 : 0.25 : 1 : 1 : 1. Parameters as given in Fig. 13. The bars at the bottom of the picture indicate the g-value position of the various signals.

The Centers 3 and 4 have lines with smaller amplitudes of the g_z and g_x lines, since these signals are much broader. Good fits were obtained if they were added in the same concentration as that of Center 2.

Summarizing, these computer simulations as well as direct quantitation (Table I) suggest that, in 17 K spectra of Complex I, the relative ratio ($S = \frac{1}{2}$ system) of the Centers 1a : 1b : 2 : 3 : 4 is 1 : 1 : 4 : 4 : 4, respectively. The concentrations of the Centers 2, 3 and 4 are approaching that of FMN, although the value for Center 3 is rather low (Table I).

EPR studies with ^{57}Fe-containing submitochondrial particles [20] suggest that the Centers 1a/1b, 2, 3 and 4 can account for 13 of the 16 Fe atoms per mol FMN found in isolated NADH dehydrogenase [10, 23], if the FMN concentration is taken to be the same as that of the Centers 2, 3 and 4,

Although these results complicate the picture of Complex I considerably, we feel that we should communicate them at this stage.

III. Additional lines at 4.2 K

We have reported earlier [7] that at 4.2 K, new lines appeared at powers varying between 2 and 200 mW. One of these lines was a clearly separated g_z peak at $g = 2.06$, another one was a separated peak at 2.10 and thus at the same position as the g_z line of Center 3. As to this last peak, we concluded that if the g value interpretation of Orme-Johnson et al. [6] for the Centers 3 and 4 was correct (Center 3, $g_{x,y,z} = 1.86$, 1.88 and 2.10; Center 4, $g_{x-y,z} = 1.86$ and 2.10) and if we assumed no inhomogeneous saturation behaviour of these centers, the line remaining at $g = 2.10$ at high powers at 4.2 K could not belong to either Center 3 or Center 4 because their line at 1.86 could be completely saturated under these conditions. After the above evaluation of the line shape and the g values of the Centers 3 and 4, however, this conclusion may no longer be drawn. In fact, the position of three of the lines which cannot be saturated completely at 4.2 K (X-band) coincides with the g values of Center 3.

As we communicated earlier [8], quantitation of the species responsible for the 2.06 line, assuming $g_y = 1.92$ and $g_x = 1.89$ as the other g values, resulted in a concentration of much less than that of Center 2 ($\leqslant 6\,\%$). Beinert and Ruzicka [24] came to a similar conclusion. In addition, they have isolated a soluble Fe-S protein from beef-heart mitochondria, which exhibits very similar g values. We found no trace of this $g = 2.06$ line in 4.2 K spectra of submitochondrial particles from the yeast *Candida utilis*. We feel safe to conclude, therefore, that it is not an essential component of NADH dehydrogenase.

Summarizing, it can be said that no additional centers of NADH dehydrogenase can be observed at 4.2 K.

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