2. Practical Aspects

2.1 The EPR Spectrometer

EPR spectrometers come in different sizes and models, dependent on the year they were produced and on the frequency and field they operate. The first EPR spectrometers functioned at very low frequencies. As with NMR spectroscopy, over the years the frequency was increased to improve resolution and to obtain higher sensitivity. Unlike NMR, however, an optimum sensitivity in EPR is reached in the 8-12 GHz range. It is not possible to produce frequency sources in this microwave frequency region that cover a wide range of frequencies in a continuous mode. Microwave frequencies can only be produced at particular small frequency windows called bands. The names of the bands originate in military communication partially dating back to World War II. The so-called X-band with a frequency window around 9.6 GHz, is right in the middle of the 8-12 GHz range. This is the reason that X-band EPR spectroscopy is the most common EPR technique and is therefore also the one available in our Department.

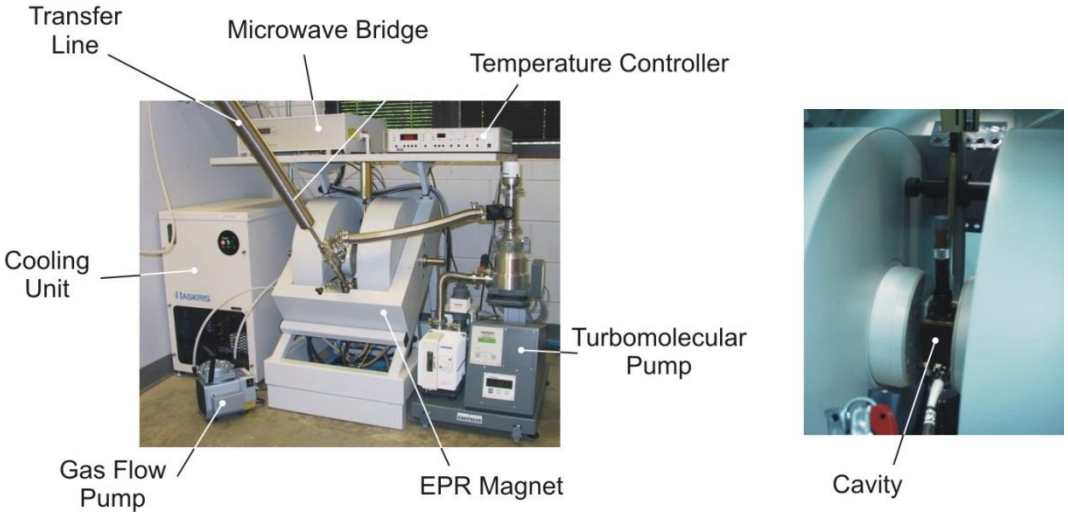

Fig. 1: Different parts of the EPR spectrometer in the Department of Chemistry and Biochemistry.

The important parts of the spectrometer are the ***microwave bridge*** and the ***EPR magnet***. The ***microwave bridge*** is where the X-band waves are generated. These are directed through a wave guide towards the ***cavity*** or ***resonator*** where the sample under study is situated. The ***cavity*** and the sample are subjected to a magnetic field during the EPR measurements generated by the ***EPR magnet***. In this case the coils of the magnet are water cooled, with the cold water coming from the ***cooling unit***. Samples can be measured at room temperature, but in most cases lower temperatures are needed, sometimes as low as 4.2 K. To be able to do that, the spectrometer can be fitted with a cryostat. Liquid and gaseous helium will flow through this system in response to the working of the ***gas flow pump***. The liquid helium comes from a large storage Dewar via a ***transfer line***. The cold helium is protected from the 'hot' environment by a high vacuum generated by the ***turbomolecular pump***.

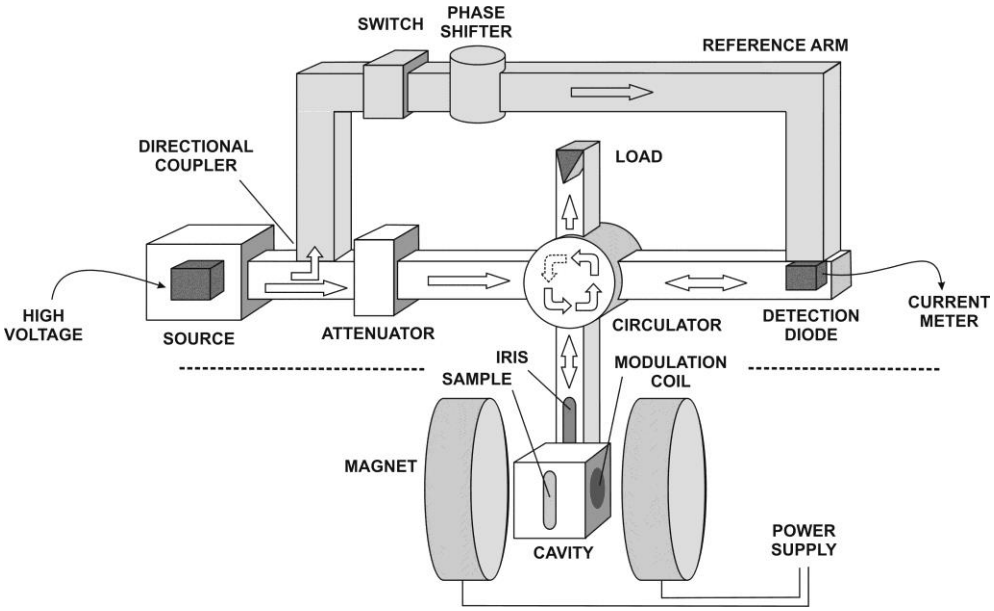

Fig. 2: Flow of the microwaves in the EPR spectrometer (Adapted from Hagen, W.R. (2009) *Biomolecular EPR Spectroscopy*).

Figure 2, shows the flow of the microwaves in the EPR spectrometer. The parts above the dashed lines are all hidden inside the Microwave Bridge box. On the left is a monochromatic source of microwaves of constant output (200 mW) and slightly (10%) tunable frequency. The produced radiation is transferred by means of a rectangular, hollow wave guide to an attenuator where the 200 mW can be reduced by a factor between 1 and 10^6. The output of the attenuator is transferred with a waveguide to a circulator that forces the wave into the resonator/cavity. The entrance of the cavity is marked by the iris, a device to tune the amount of radiation reflected back out of the cavity. The reflected radiation returns to the circulator and is directed to the diode for the detection of microwave intensity. Any remaining radiation that reflects back from the detector is forced by the circulator into the upward waveguide that ends in a wedge to convert the radiation into heat. A small amount of the 200 mW source output is directed through the reference arm directly to the detector to produce a constant working current. The reference arm contains a port that can be closed and a device to shift the phase of the wave. These parts are important for the tuning of the cavity. This will be explained in the following section.

2.2 Important EPR Spectrometer Parameters

More detailed, step-by-step, instructions on how to operate the EPR spectrometer can be found in the following chapter. Here more background information is provided that explain the idiosyncrasies related to this measuring technique that the reader needs to understand to be able to tune the spectrometer and obtain useful data.

Most EPR spectrometers are reflection spectrometers. This means that they measure the changes (due to spectroscopic transitions) in the amount of radiation reflected back from the microwave cavity containing the sample. The detector should only detect the microwave radiation coming back from the cavity.

Simply put, a microwave cavity is a metal box with a rectangular or cylindrical shape which resonates with microwaves much as an organ pipe resonates with sound waves. The cavity or resonator is designed to set up a pattern of standing microwaves in its interior (Fig. 3). Standing electromagnetic waves have their electric and magnetic field components exactly out of phase - where the magnetic field is maximum, the electric field is minimum and vice versa. The place where the sample is situated has a minimum electric field and maximum magnetic field.

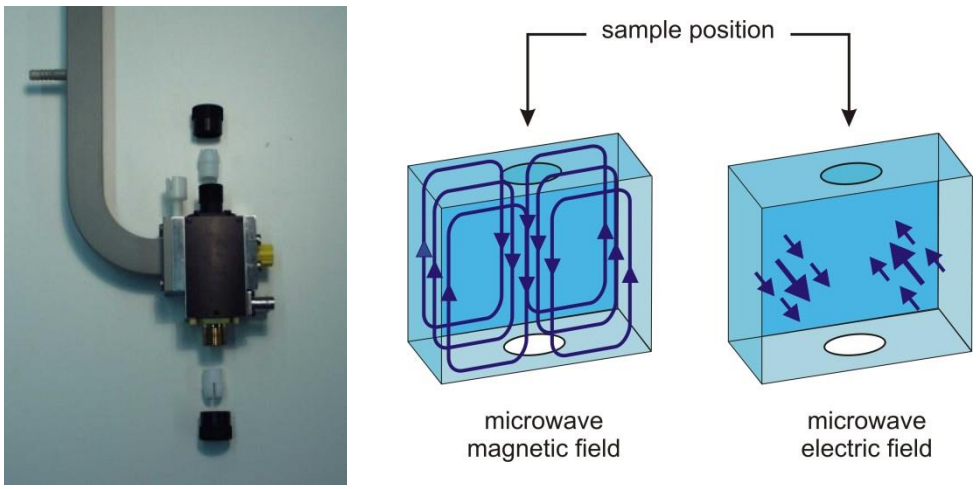

Fig. 3: Detail of the waveguide and the cavity. The diagrams on the right show the directions of the microwave magnetic field and the microwave electric field when the cavity is critically coupled.

Resonance means that the cavity stores the microwave energy; therefore, at the resonance frequency of the cavity, no microwaves will be reflected back, but will remain inside the cavity. Energy can be lost to the side walls of the cavity because the microwaves generate electrical currents in the side walls of the cavity which in turn generates heat.

Cavities are characterized by their Q or quality factor, which indicates how efficiently the cavity stores microwave energy. We can measure *Q* factors easily:

$$Q = (\nu_{res})/(\Delta\nu) \quad (1)$$

where ν_{res} is the resonant frequency of the cavity and $\Delta\nu$ is the width at half height of the resonance.

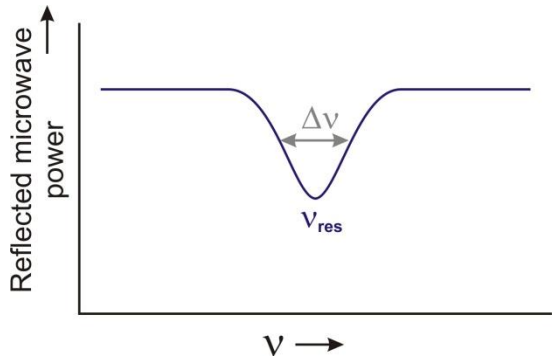

Fig. 4: Plot of the reflected microwave power vs. the frequency produced by the microwave source. The 'dip' is the result of the loss of reflected waves at the resonance frequency of the cavity.

In order for the microwaves to enter the cavity one of its end walls must have an opening: the iris. The size of the iris controls the amount of microwaves which will be reflected back from the cavity and how much will enter the cavity. Just before the iris is a small metal plate (attached to the iris screw). Moving this plate up or down changes the amount of coupling. Only for one unique position is the cavity critically coupled: all powers enter the cavity, and no radiation is reflected out.

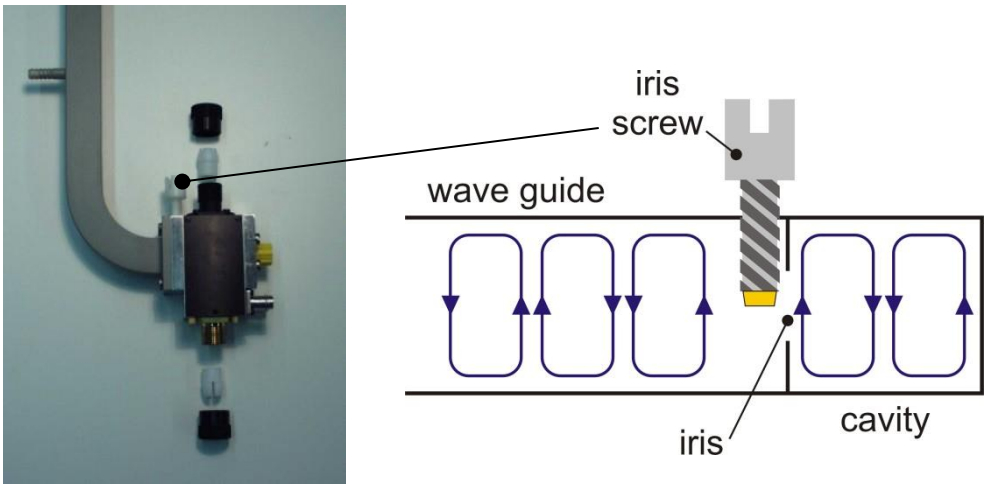

Fig. 5: Detail of the waveguide and the Cavity. The diagrams on the right show the positions of the iris and the iris screw. Turning the iris screw causes the iris to become larger and smaller. Only at one position is the cavity critically coupled. This position will be different, however, with different samples and different temperatures.

The actual EPR signal is the result of the EPR sample absorbing the microwave energy. At the beginning of a measurement, the system is tuned resulting in the cavity being critically coupled. During the measurement, when the sample absorbs the microwave energy, the Q is lowered because of the increased losses and the coupling changes. The cavity is therefore no longer critically coupled and microwaves will be reflected back to the bridge, resulting in an EPR signal.

Tuning the Microwave Cavity and Bridge

Figure 6, shows what the computer screen will look like when you are in the process of tuning the bridge. The window with the yellow curve on the black background is a display of the microwave power reflected from the cavity and the reference arm power as a function of the microwave frequency. The dip corresponds to the microwave power absorbed by the cavity and thus is not reflected back to the detector diode. By centering the dip on the display monitor, using the frequency slider, the microwave source is set to oscillate at the same frequency as the cavity resonant frequency.

Additional steps will be needed and include the adjustment of the signal reference phase. On the display this will result in the dip looking symmetric and having maximal depth. Next the bias levels are adjusted, keeping the diode meter needle in the center. This is followed by the critical coupling of the cavity, by increasing the power step wise and adjusting the iris position, again trying to keep the diode meter in the center. A more detailed description can be found in the next chapter.

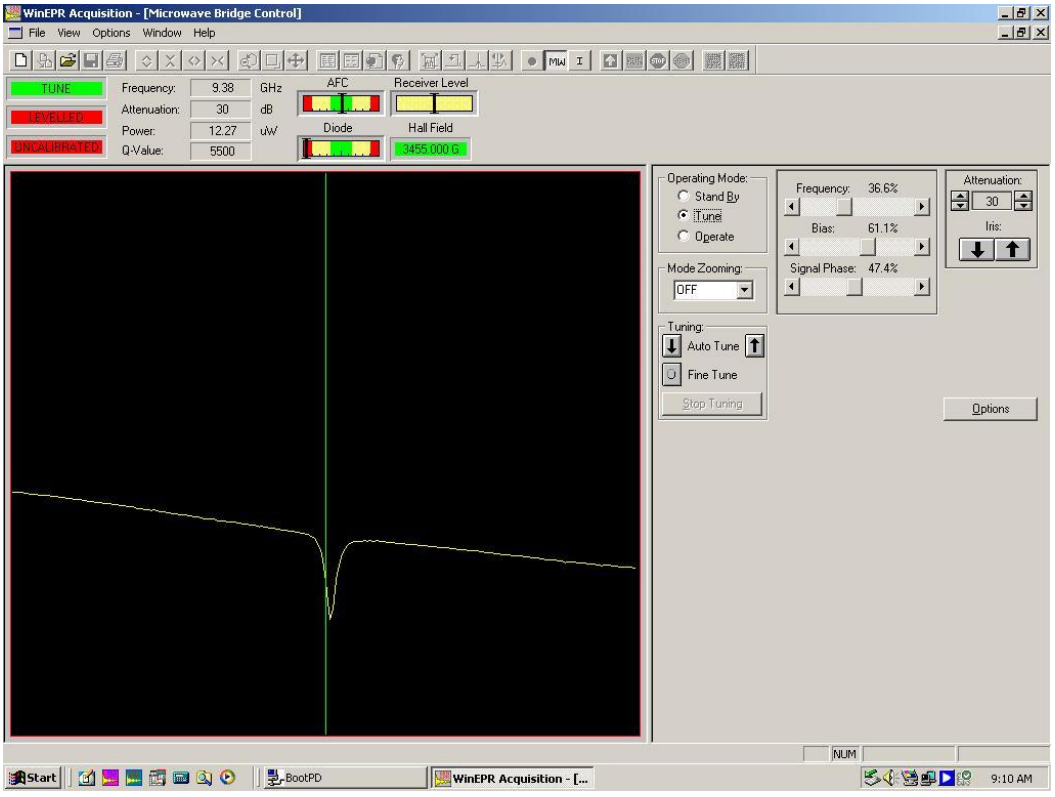

Fig. 6: Screen shot of the EPR computer during the tuning process.

Microwave Bridge Parameters

Most of the parameters are just important for getting a signal somewhere in the middle of your computer screen during the measurement. The ***center field*** and ***sweep width*** determine what region of the field is measured. Our magnet covers the field region from 100 Gauss all the way up to 6000 Gauss. Ideally, you chose the center field value right in the middle of your EPR signal and the sweep width as wide as the width of the signal. These values will be different for each type of sample and will have to be determined for each new type of sample you run.

Another parameters related to the display of the spectrum is the ***gain***. The gain is adjusted to give the desired size of display and where possible should be increased to use the full range of the digitizer. The receiver gain must be high enough to show all details in the spectrum. Figure 7 shows the results of insufficient or excessive receiver gain. If the receiver gain is too low the effect of digitization will be evident in the spectrum (Fig. 7, trace B), whereas at high gain the signals will be clipped due to an overload in the signal channel (Fig. 7, trace C).

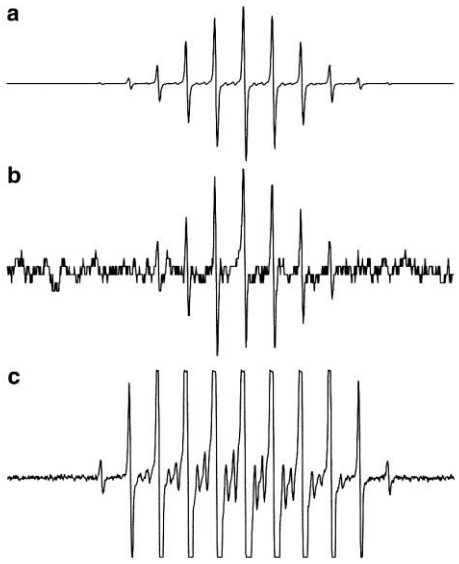

Fig. 7: Effect of using gain setting that are either (**a**) optimal, (**b**) too low, or (**c**) too high on an EPR spectrum.

Some of the other parameters, however, need a better understanding since the wrong setting might result in deformation and/or complete loss of the signal. Several of these are related to how the EPR signal is detected. The EPR spectrometer makes use of phase sensitive detection. This enhances the sensitivity of the spectrometer significantly and diminishes the noise from the detection diode and baseline instabilities due to the drift in DC electronics. This is achieved by modulating the magnetic field at the site of the sample. The field is modulated (varied) sinusoidally at a set modulation frequency. If there is an EPR signal, the field modulation quickly sweeps through part of the signal and the microwaves reflected from the cavity are amplitude modulated at the same frequency (Fig. 8). Only the amplitude modulated signals are detected. Any signals which do not fulfill these requirements (i.e. noise and electrical interference) are suppressed. For an EPR signal which is

approximately linear over an interval as wide as the modulation amplitude, the EPR signal is transformed into a sine wave with an amplitude proportional to the slope of the signal. As a result the ***first derivative*** of the signal is measured.

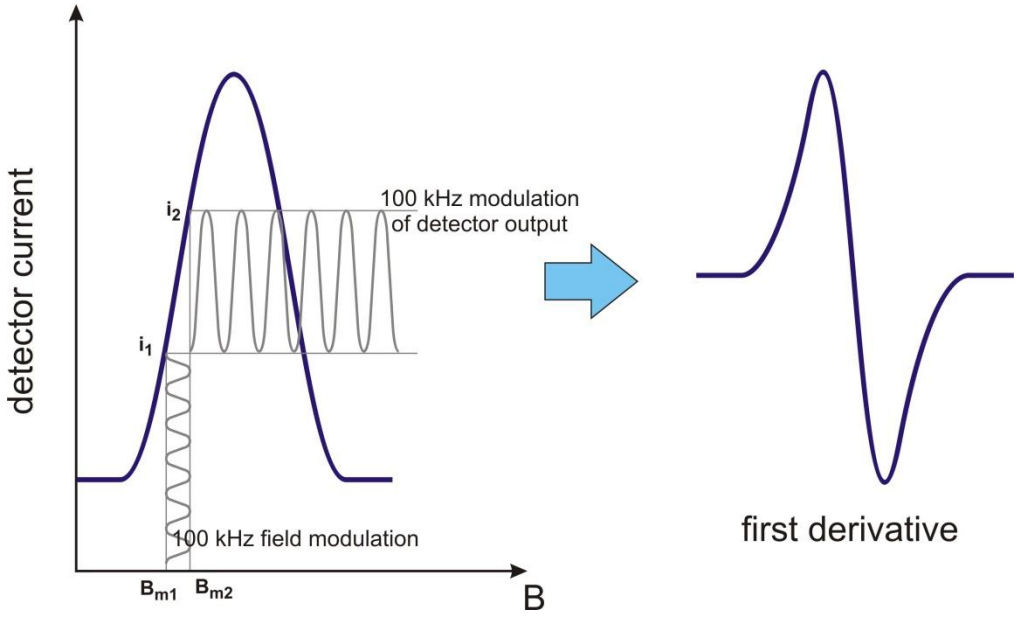

Fig. 8: Schematic representation of phase sensitive detection. As the main field is scanned slowly through the EPR line, a small additional oscillating magnetic field, B_m, is applied in the same direction as the main field B. B_m is commonly at 100 kHz. As B_m increases from the value B_{m1} to B_{m2}, the crystal detector output increases from i_1 to i_2. If the magnitude of B_m is small relative to line width, the detector current oscillating at 100 kHz has a peak-to-peak value that approximates the slope of the absorption curve. Consequently, the output of the 100 kHz phase-sensitive detector is the derivative of the absorption curve. Adapted from Eaton, Eaton, Barr and Weber (2010).

There are two parameters associated with the phase sensitive detection: ***modulation amplitude***, and ***modulation frequency***. These parameters have to be chosen wisely. With more magnetic field modulation, the intensity of the detected EPR signals increases; however, if the modulation amplitude is too large (larger than the linewidths of the EPR signal), the detected EPR signal broadens and becomes distorted (Fig. 9). To get the most accurate information about signal line shape, the modulation amplitude should be less than 10% of the distance (in Gauss) between the positive and negative peaks in the derivative spectrum (of isotropic signals). This low modulation amplitude, however, can result in unacceptable poor signal-to-noise ratio for the signal. A good compromise between signal intensity and signal distortion occurs when the amplitude of the magnetic field modulation is equal to the width of the EPR signal. Figure 9, also shows that if a modulation amplitude is used greater than the splitting between two EPR signals, we can no longer resolve the two signals.

For measurements at X-band frequency, the modulation frequency is normally set to 100 kHz. This is a value that works well for the broader signals due to metals centers in proteins. The modulation amplitude has to be smaller than the line width of these types of signals. It is therefore important to start the first measurement with a small value (6 Gauss). The values can be increased for subsequent measurements if needed. When the signal is very intense there is no real need to do

this, but very low intense signals might show a better signal-to-noise ratio if the value can be increased.

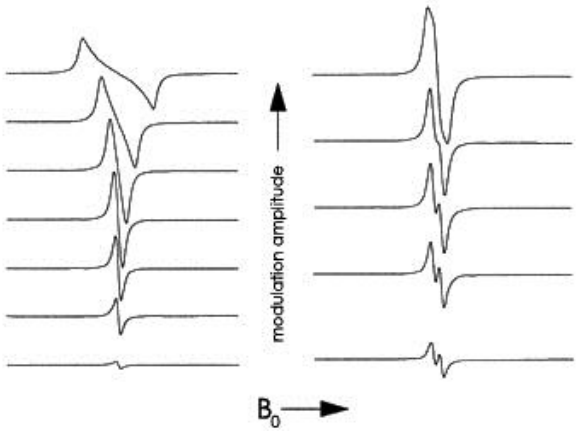

Fig. 9: Effect of increasing modulation amplitude on signal intensity and signal shape.

To further improve the sensitivity, a ***time constant*** is used to filter out more of the noise. Time constants filter out noise by slowing down the response time of the spectrometer. As the time constant is increased, the noise levels will drop. If we choose a time constant which is too long for the rate at which we scan the magnetic field, however, we can distort or even filter out the very signal which we are trying to extract from the noise (Fig. 10). Also, the apparent field for resonance will shift.

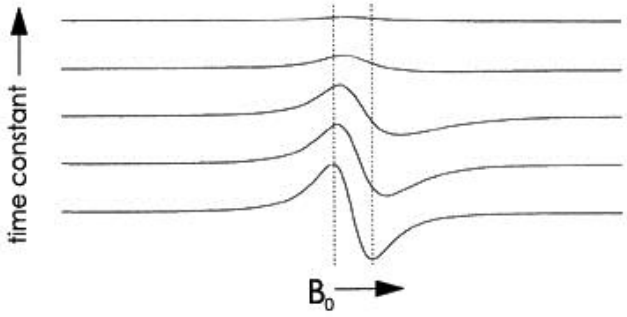

Fig. 10: Effect of increasing time constant on signal intensity and signal shape.

A parameter that is directly related to the time constant is ***conversion time***. A longer conversion time also improves the signal to noise ratio, but in a different way: The signal channel incorporates an integrating ADC (Analog to Digital Converter) to transfer the analog EPR spectra to the digital data acquisition system. An important side effect of using the integration method for the conversion is that it integrates the noise out of the signal. If the Time Constant is too large in comparison with the Conversion Time (the rate at which the field is scanned) the signals we want to detect will get distorted or will even be filtered out. The actual values for the time constant and conversions time will depend on the type of signal being measured.

Very weak signals might get lost in the noise. You can increase your signal to noise ratio by ***signal averaging***. The resultant signal to noise is proportional to √N, where N is the number of scans. With a perfectly stable laboratory environment and spectrometer, signal averaging and acquiring a spectrum with a long scan time and a long time constant are equivalent. Unfortunately perfect stability is impossible to attain. For example, there is a delicate balance between the heat dissipated by the cavity and the air flow around it and slow variations will result in baseline drifts. For a slow scan (>15 min) the variations can cause broad features in the spectrum dependent on the sample concentration and the gain used. If you were to signal average the EPR signal with a scan time short compared to the variation time, these baseline features could be averaged out.

The one parameter left to discuss is the ***microwave power level***. The EPR signal intensity grows as the square root of the microwave power in the absence of saturation effects. When saturation sets in, the signals broaden and become weaker. Several microwave power levels should be tried to find the optimal microwave power. Since there is a more intricate relationship between sample temperature and saturation this will be discussed in the next section.

2.3 Sample Temperature and Microwave Power

There are several reasons why we need to do EPR measurements at lower temperatures than room temperature. One reason has to do with the fact that in biological samples the solvent is commonly an aqueous buffer. Water will absorb the microwaves, just like in a regular microwave oven, and the sample will heat up. Because microwave dielectric loss is due to molecular motion, which decreases upon freezing, the impact of solvent on resonator Q is much smaller when samples are frozen. To maintain lower temperatures the sample has to be cooled from the outside with a stream of cold gas (nitrogen or helium) which also helps battling the heating up of the sample. The warming up is also minimized by having the sample positioned in the cavity so that the magnetic component is maximal but the electric component that is causing the warming up is minimal. When a sample does have to be measured at RT in aqueous buffer the sample will be measured in a flat cell to further minimize the absorption of the electric field component.

A more important reason to measure at low temperature, however, is the fact that the highest theoretical signal intensities are reached at the lowest possible sample temperature. This is because the energy difference between the two energy levels, the S = -½ level and the S = ½ level involved in the EPR transition is very small and both levels are almost equally occupied with only a small excess in the S = -½ level. Figure 11, shows what the population is in percentage for the *S* = - ½ level (n_0) and the *S* = ½ level (n_1) as a function of the sample temperature. The energy difference between the two energy levels due to the Zeeman splitting is very small, ~0.3 cm^{-1} for X-band EPR. Based on the ***Boltzmann distribution***

$$n_1 = n_0 e^{-\left(\frac{\Delta E}{kT}\right)}, \tag{2}$$

it can be shown that only at low temperatures there will be enough difference in the population of the two levels to create a signal (Fig. 11).

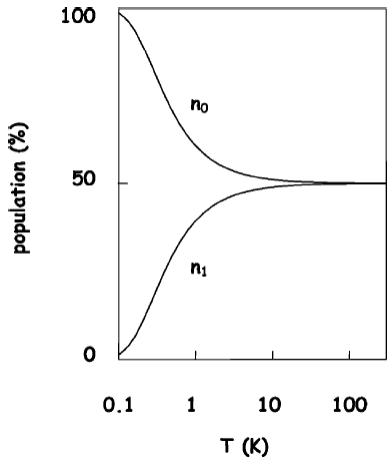

Fig. 11: Boltzmann distribution for the electrons in the *S* = - ½ level (n_0) and the *S* = ½ level (n_1).

This means that in principle we should always measure at the lowest temperature possible. However, there is another property of the sample that has to be taken into consideration: ***spin-lattice relaxation***. Figure 12, Shows how this property is important. The figure gives a representation of the electron distribution in the S = -½ level and the S = ½ level involved in the EPR transition with a small excess in the S = -½ level. The electrons in both levels will absorb energy quanta causing a complete reversal of the electron distribution. We will only continue to observe an absorption signal, however, if the lower energy level has a higher occupancy. Therefore some electrons have to go back to the lower energy level using relaxation before the next set of quanta is absorbed. When this doesn't happen the EPR signal will be lost because the electron distribution between the two levels will be eventually become equal. In addition, the microwave power applied to the sample plays a role. The more power is applied to the sample the higher the chance that an energy quantum is absorbed. When the relaxation of the electron is already limited, an increase in power will speed up the equalization of the electron distribution. Therefore, at lower temperatures EPR signals might not be observable and the chance of that is even higher when high microwave powers are applied, saturating the sample with energy quanta. This effect is therefore described as power saturation of the EPR signal. Note that the relaxation rate is anisotropic and therefore different parts of the spectrum can show different amounts of saturation at different temperatures.

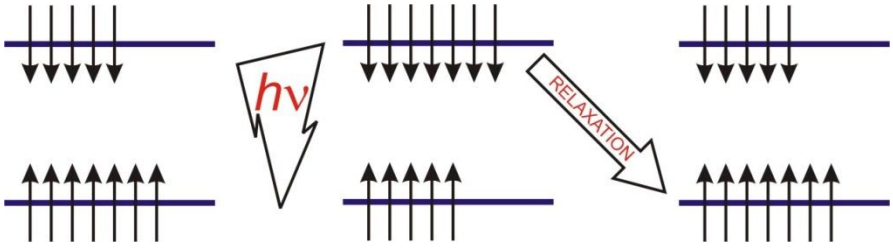

Fig. 12: Change in the electron distributions between the *S* = - ½ level (n_0) and the *S* = ½ level (n_1) during an EPR experiment.

The detection of EPR signals is also affected by the Heisenberg uncertainty principle. Due to the uncertainty principle the EPR spectra will broaden beyond detection at higher temperatures. At lower temperatures the spectra will sharpen up. This sharpening up of the spectrum by cooling the sample is, however, limited by a temperature-independent process: inhomogeneous broadening.
The protein or model molecules in dilute frozen solutions are subject to a statistical distribution in conformations, each with slightly different 3D structures and, therefore, slightly different g-values, which manifest themselves as a constant broadening of the EPR line independent of the temperature.

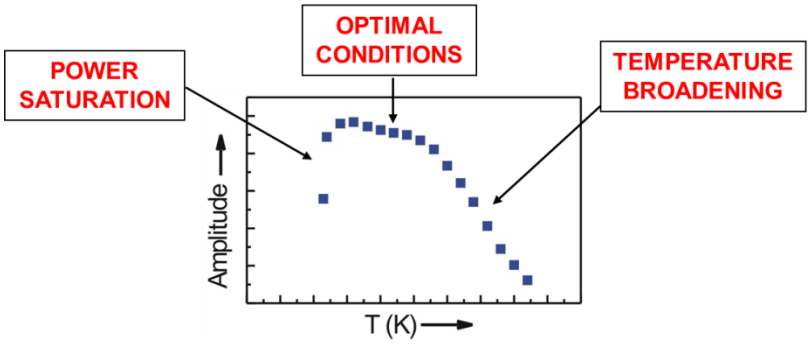

Fig. 13: At low temperatures power saturation will occur while a higher temperatures temperature broadening will occur due to the Heisenberg uncertainty principle, leaving an optimal measuring region somewhere in the middle.

To summarize, the lower the sample temperature the higher the signal intensity will be. At the same time, power saturation might occur at a too low temperature, while temperature broadening might occur at a too high temperature due to the uncertainty principle. As a result of all these conflicting mechanisms there will be a temperature region where the detection of the EPR signal of a particular sample is optimal (Fig. 13). This optimal region, however, will be different for each type of paramagnetic species and has to be determined for each new type of species you will measure.

Power plots and Curie plots

With a completely new sample you will not know all the measurement conditions. First of all, you are not sure if you have a signal at all. So one of the first things you have to find out are the optimal measuring temperatures of possible paramagnetic species in your sample. For this you have to scan the sample at different temperatures, for example ***4.5 K, 20 K and 50 K***. At every temperature you make a broad scan in the hope you will see a signal. Preferably, you do this at different powers, ***50 dB, 20 dB and 0 dB***. If you detect a signal, you can zoom in and measure the signal again with a smaller sweep to obtain more detail.

After you have discovered an EPR signal at a certain temperature and microwave power, you have to make sure that the data you obtained is useful: It is important that the signal is not broadened or distorted. The first thing that has to be done is to make sure the signal is measured under non-saturating conditions. There is a method to do this.

The next formula explains the relationship of the amplitude, gain and the power in dB:

$$\left(\frac{amplitude}{gain}\right) \cdot 10^{-dB/20} = \text{constant} \tag{3}$$

The spectrometer is designed such, that ***a non-saturating signal remains constant in amplitude when each change of 1 dB in the microwave power is compensated by 1 step in the gain***. Note that a lower amount of dB means a higher amount of power in mW(atts). If you increase the power you have to decrease the gain. This 'design' also explains why the gain cannot be changed to any value we want but can only have certain values. What this practically means is that we can measure a spectrum, for example at -20 dB. Then we can increase the power to -15 dB, additionally decrease the gain five steps and remeasure the spectrum again. If the signal is not saturating both spectra should have exactly the same amplitude. As an example, the EPR spectra for the Copper Standard at 4.7 K measured at different powers is shown (Fig. 14):

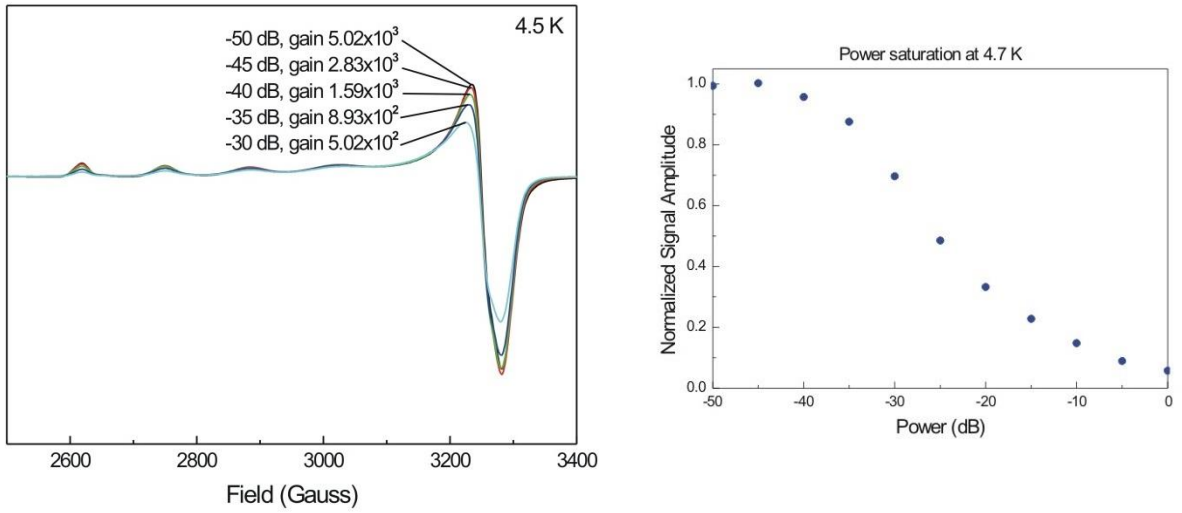

Fig. 14: Power saturation studies. The left panel shows an overlay of spectra obtained for the copper standard at different combinations of microwave power and gain. The right panel shows the signal intensities for each spectra as a function of microwave power (in dB).

What can be seen from Figure 13, left panel, is that from -50 dB to -45 dB the signal amplitude hardly changes, but that the signal amplitude clearly starts to decrease at -40 dB and lower. So if we want to measure the Copper Standard at 4.7 K we should measure with a power of -50 dB. When we measure the copper spectra at different powers going from -50 dB to 0 dB we can make a so-called power plot (Fig. 14, right panel). At the lowest powers (higher dB) you can see there is an area where the curve is horizontal, where the Copper Standard can be measured without saturating the signal. Then starting at -40 dB the signal starts to saturate, the signal broadens and loses amplitude. The Copper Standard has also been measure at 20 K and 50 K (Fig. 15).

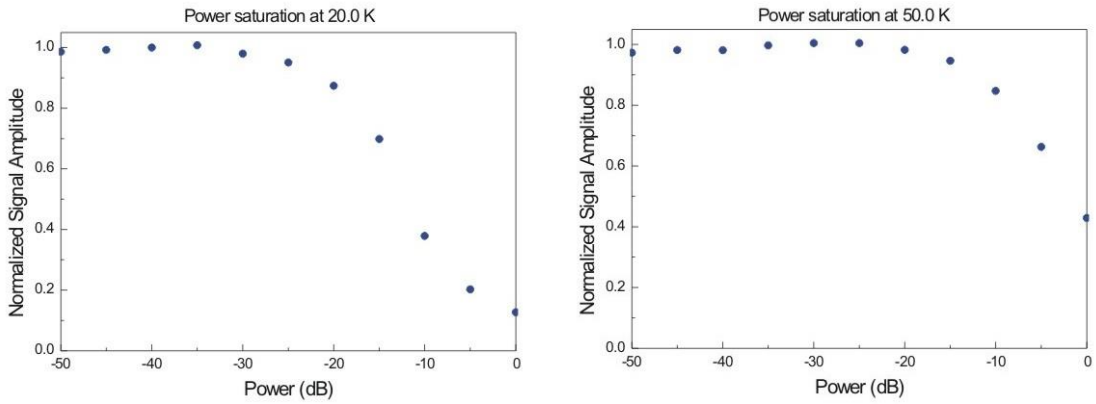

Fig. 15: Power saturation studies for the copper standard at 20 K and 50 K.

The power plots show that at higher temperatures there is a larger power range where the signal can be measured without saturating. At 50 K we can even measure at -20 dB.

It is very important to know the temperature and power behavior of your signal. The lower the temperature you can measure and the higher the power you can use the better the signal-to-noise ratio. The Copper Standard is used because it has the same (corrected) signal intensity at a wide range of temperatures. In general, there will be only a certain small temperature range where your signal can be measured. To get to know this you have to make a so-called Curie plot. Here we plot again the signal amplitude, but now against the temperature. Since the observed signal intensity or amplitude (I_0) will decrease going up in temperature, we will use a normalized intensity or amplitude (I_n), according to the formula:

$$I_n = \frac{\left(I_0 \cdot T \cdot 10^{-dB/20}\right)}{gain} \tag{3}$$

I_n	normalized value for the intensity (normalized double integral)
I_0	observed intensity
T	absolute temperature in K
dB	reading of the attenuator
$gain$	gain

(keeping all other parameters constant)

If we do this for the Copper Standard we get the curve shown in Figure 15. In principle we should get a straight line. Small deviations can be expected dependent on the accuracy and calibration of the heater system. The inhomogeneous broadening will be different at different temperatures which could cause a small slope dependent on the sample being studied. The loss of intensity of the copper signal at 175 K and higher (Fig. 16) is due to a phase change in the sample.

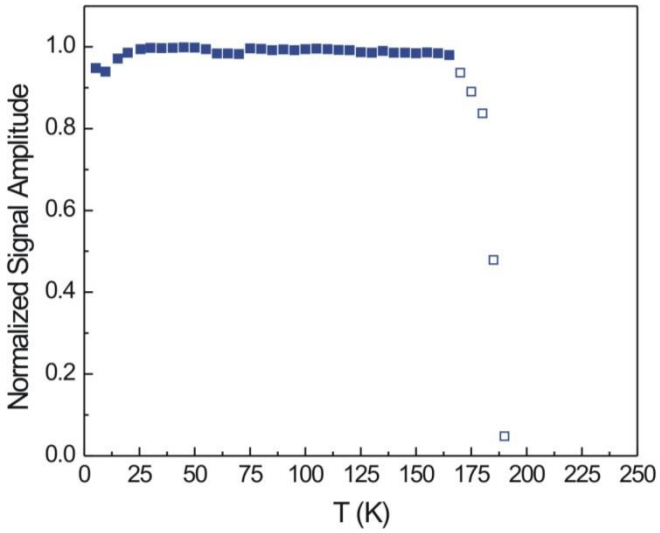

Fig. 16: Curie plot for the copper standard.

For comparison Curie plots for some other samples are shown in Figure 17. The red trace is from the MCRred1-Ni(I) species. This signal can also be detected over a wide temperature range. The signal shows clear saturation, however, below 40 K and shows temperature broadening above 200 K. The black trace is for a standard $[4Fe\text{-}4S]^{1+}$ cluster detected in the enzyme IspH. For 4Fe clusters there is normally only a very small temperature window (10 – 20 K) where the EPR signal can be detected. Like IspG, IspH binds its substrate and a cluster-bound reaction intermediate can be detected in kinetic studies. The curie plot for this species (green trace) is very different. The cluster species can be detected all the way up to 80 K before the temperature broadening sets in.

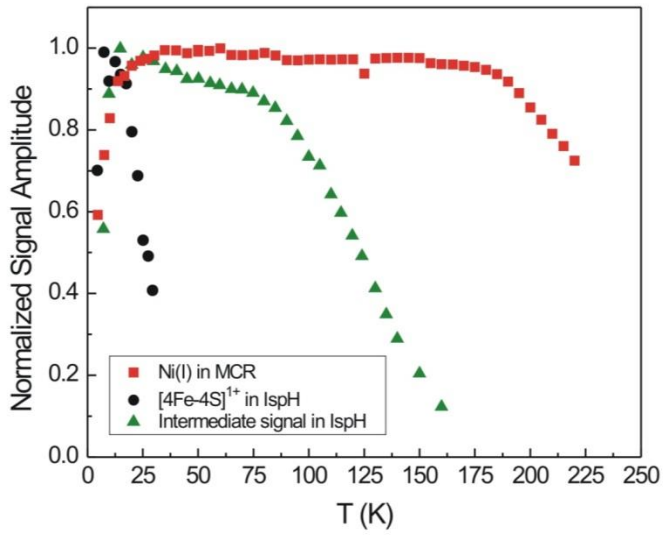

Fig. 17: Curie plots for the MCRred1-Ni(I) species (■), the $[4Fe\text{-}4S]^{1+}$ cluster detected in the IspH enzyme (●), and a cluster-bound reaction intermediate detected in the same IspH enzyme (▲).

2.4 Integration of Signals and Determination of the Signal Intensity

The area under the ***absorption spectrum*** of an EPR signal is, just as in optical spectroscopy, a direct measure for the concentration of unpaired electrons. Unlike electronic absorption spectroscopy, however, there is no 'extinction coefficient' in EPR spectrometry. All S = ½ systems absorb equally well. To correlate the intensity of the EPR signal with a concentration, a standard is needed. Different standards can be used. Here we will use a copper standard, containing 10 mM $CuSO_4$. Made in the proper way, all the copper is 2+ and contributes to the EPR spectrum. So the 'spin concentration' of this standard is also 10 mM. By comparing the spin concentration of the copper standard with the spin concentration of the signal of interest the concentration of that signal can be determined. To be able to make a comparison between the EPR signal of interest and a standard it is important that the spectra to be compared are obtained under exactly the same conditions. If this is not possible it is necessary to scale the signal obtained for the unknown to compare it with the standard. For samples recorded at the same temperature and with the same spin number (e.g., S = ½) the most common parameters that have to be corrected are:

1. Receiver gain. Area scales linearly with gain settings of the detector's amplifier.
2. Microwave power. With the assumption that the spectra are obtained at power levels below saturation. Under these conditions, area scales as the square root of the incident microwave power.
3. *g*-value differences. Area scales as *g*.
4. Field scan width. For first-derivative spectra the scan width correction factor is (1/sweep width)^2.
5. Filling factor of the EPR tube. A wider tube results in a more intense signal. This increase is not linearly due to the sinus wave form of the standing wave and ideally each tube should be calibrated by filling it with the copper standard and measuring the signal intensity.

The following formula put all these correction factors for the parameters together:

$$I_n = \frac{\left(I_0 \cdot d^2 \cdot T \cdot 10^{-dB/20}\right)}{\left(g_p^{av} \cdot f \cdot a\right)} \tag{4}$$

where

I_n	normalized value for the intensity (normalized double integral)
I_0	observed intensity
d	distance between the starting and ending points (in Gauss)
T	absolute temperature in K
dB	reading of the attenuator
f	tube calibration factor
a	gain

and

$$g_p^{av} = \frac{2}{3}\sqrt{\frac{g_x^2+g_y^2+g_z^2}{3}} + \frac{\left(g_x+g_y+g_z\right)}{9} \tag{5}$$

Unfortunately, there are several additional parameters that you might have to correct for which are equipment specific:

6. Modulation amplitude. Area scales linearly with modulation amplitude.
7. When an integrating digitizer is used, the recorded signal area varies with scan time. The signal level increases the longer the integration time (conversion time). Thus, if two spectra of the same sample are obtained will all parameters equal, but one with 10.24 ms conversion time and one with 81.92 ms conversion time, the numerical integral of the digitized spectrum will be eight times larger for the 81.92 ms conversion time. This is often automatically normalized by the software in modern spectrometers.
8. "Signal averaging". The Bruker software that comes with the spectrometer in the Department of Chemistry and Biochemistry does not really average the scans. When more than one scan is measured in one measurement, the scans are just added up and not averaged. Averaging four scans will result in a spectrum with a four times higher signal intensity.
9. Correct for Q differences. When the Q-values are different it is necessary to scale the intensity or double integration results by the ratio of the resonator Q-values that is observed from two samples. Area scales linearly with Q.
10. Bruker spectrometers use a Schottky barrier diode to detect the reflected microwaves. The diode converts the microwave power to an electrical current. At low power levels (less than 1 μW, ~53 dB), the diode current is proportional to the microwave power, and the detector is called a square law detector. At higher power levels (greater than 1 mW, ~23 dB), the diode current is proportional to the square root of the microwave power and the detector is called a linear detector. The transition between the two regions is very gradual. For quantitative signal intensity measurements the diode should operate in the linear region.

Dependent on the type of equipment and software some of these corrections are made automatically. It is important to check what corrections are made by the software. When possible, however, it is important to measure the unknown and the standard under identical conditions like temperature, power, modulation amplitude, conversion time, time constant and total amount of spectra added. Generally, using the same solvent/buffer the Q factor should also be identical.

Using the WINEPR program for integration

The recorded EPR spectra are ***first derivatives*** of the normal absorption spectra. Since an EPR spectrum is a first derivative, we have to integrate twice to obtain the intensity (I_0) (= area under the absorption spectrum). Open the program WINEPR and load the file you want to integrate. Under **WINEPR System** select **1D-processing**. Then under **1D-processing** select **Integrate Region**. The menus will change. Now select **Integration** and **Define Integrals**. Now you can designate the area to be integrated by selecting a point on the left side of the signal and a point on the right side of the signal by moving the mouse and clicking the left mouse button to select the points. Click the right mouse button to confirm your selection. Now you will see the first integral. A good integral will have the Y-values of the start and the end of the curve at the same vertical position (Fig. 18).

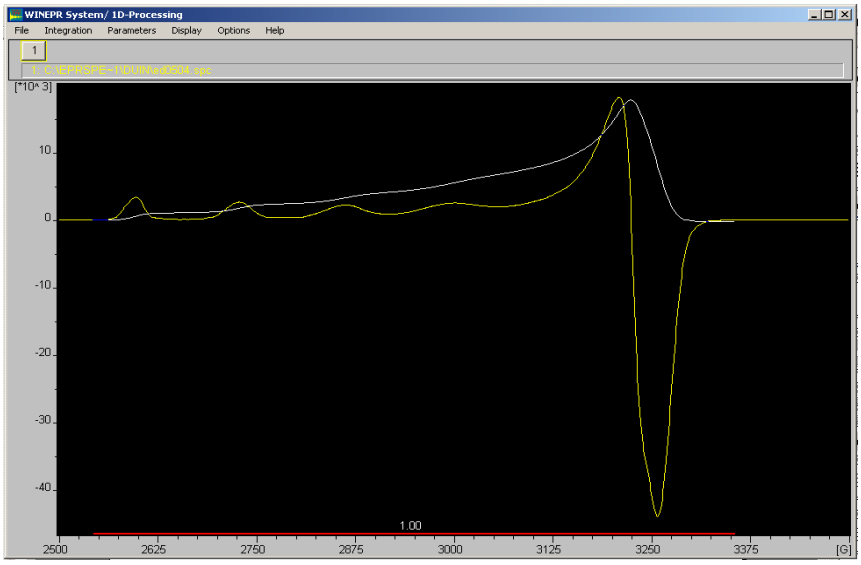

Fig. 18: Screen shot of Integration procedure: integral

Select **Integration**, **Integral type**, **Double**. Now the double integral is shown (Fig. 19). You have the option to change the slope and the bias of the double integral. These might give you a better-looking curve, but doesn't add to the reliability of your integral. You should try to do a baseline correction first. By now selecting **Integration**, **Report** you get an new screen which shows the start position of the integral, the end position of the integral, the double integral (DI) and the normalized double integral (DI/N) (Fig. 19). This last value is the value you need to write down. Select **Integration**, **Return** if you need to integrate more spectra.

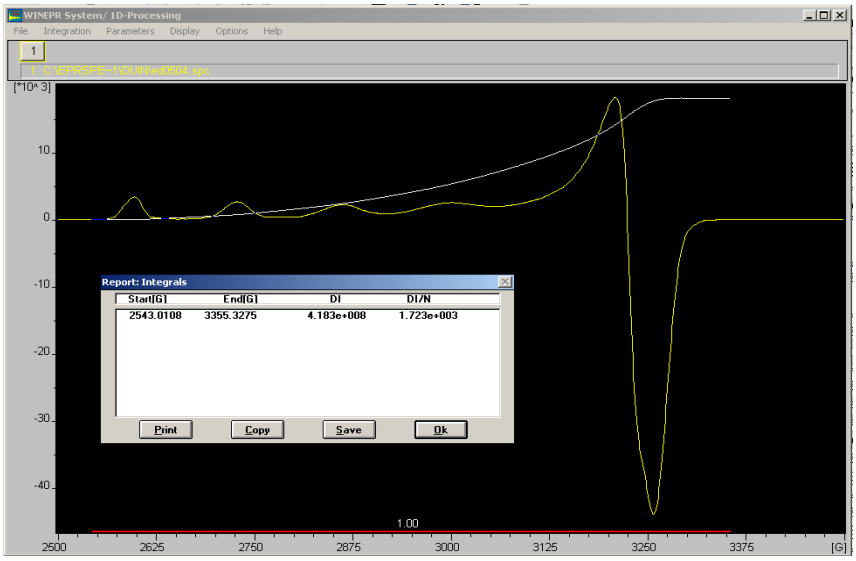

Fig. 19: Screen shot of Integration procedure: double integral and report

By determining the I_n (DI/N) for both your sample and the copper standard you can now determine the concentration of your detectable signal.
The concentration of the unknown (C_u) is now:

$$C_u = \frac{I_{n(u)} \cdot C_{st}}{I_{n(st)}} \tag{6}$$

where

C_u	spin concentration of unknown
C_{st}	spin concentration of standard
$I_{n(u)}$	normalized intensity of unknown
$I_{n(st)}$	normalized intensity of standard

Some spectra are inherently difficult to integrate, because they do not meet the ideal case of isolated peaks with well-defined flat baselines on either side of the EPR spectrum (Fig. 17). If the signal-to-noise is very low, if there is an overlapping spectrum from another species, or the background is large and unavoidable, it may be best to integrate a computer simulated spectrum by fitting the experimental data.

Subtraction of a background spectrum is also an option. This is particularly important when the empty cavity shows an EPR signal due to accumulation of dust and dirt inside the measuring chamber. A background spectrum can be generated by measuring a tube containing the sample solvent/buffer as the unknown. It is important to use a "blank" tube since the quartz walls of the EPR tubes concentrate the field lines in the sample and a slightly different spectrum will be obtained when just the cavity without a sample inside is measured. The main change will be that the cavity is tuned at a different frequency when no sample is present, which causes a shift in the spectrum making it useless for a proper subtraction of the background signal.

Equation 5 only holds if two S = 1/2 spectra are compared. For unequal spin systems the term 1/[S(S+1)] has to be added to the formula of I_n. If possible, however, a standard with the same spin systems should be used.

2.5 Redox Titrations

In section 1.10, it is described how the production of samples in the as-such form, oxidized form, or reduced form can help with the identification of the paramagnetic species present. Some species will only show an EPR signal at a certain oxidation state and detection of this signal can be the first clue on what species is present in the unknown. Dependent on what type of protein you are studying more knowledge about the redox active groups might be needed. The protein could be part of a chain of proteins that transfer electrons and the midpoint potentials of the electron carrier present in the unknown could indicate where it is placed in the chain. Some proteins contain a multitude of redox active groups and determination of the midpoint potential of each group will be needed to establish the electron flow within the protein. This knowledge will also be helpful in finding unique redox potentials where only a small subset (ideally only one) of the redox active groups is paramagnetic and EPR detectable. This will enable the study of these specific species without having no, or only a small set of, other EPR signals present.

Detailed information about the midpoint potential of redox active species can be obtained by performing redox titrations. Different type of detection methods can be used, but when one of the oxidation states is EPR active, and most of the time it is, EPR spectroscopy is the preferred method.

Typically, a titration is performed in both the oxidative and reductive direction. One could start with a fully reduced protein and add small amount of oxidant to slowly increase the redox potential of the protein solution. At specific potentials a sample is withdrawn, placed in an EPR tube and subsequently frozen and stored in liquid nitrogen. When the highest potential is reached reductant can be added and the whole process is repeated in the reductive direction. Sodium dithionite is often used in physiology experiments as a means of lowering a solution's redox potential ($E^{o'}$ ~ -420 mV at pH 7 vs. SHE). Potassium ferricyanide is usually used as an oxidizing chemical in such experiments ($E^{o'}$ ~ 436 mV at pH 7 vs. SHE).

Redox dyes are added to the protein solution before the redox titration is started. Typical dyes are duroquinone (E_m = 86 mV), methylene blue (E_m = 11 mV), indigo-disulphonated (E_m = -125 mV), phenosafranin (E_m = -252 mV), neutral red (E_m = -325 mV), benzyl viologen (E_m = -350 mV), and methyl viologen (E_m = -453 mV). The dyes have two functions. In the first place, they greatly enhance the electronic contact between the protein molecules and the electrode. Secondly, they help buffer the protein solutions, allowing stable potentials outside the range where the protein chromophores accept or donate electrons. Dyes can interfere with the redox titration, however, and caution has to be taken with the selection of the dyes. The ‘ideal’ combination of dye mediator potentials begins with a dye below the lowest potential data point and extends to a dye above the highest potential data point with no spaces of greater than 60-70 mV between dyes.

The typical redox titration requires about 15 data points. Ideally, the experiment should span a range of 150 mV above and below the midpoint potential of the sample. Normally samples are taken every 20-25 mV depending on the amount of sample and the accuracy desired. Note, ideally the titration should be performed in both the oxidation and reduction directions to verify the thermodynamic **reversibility** of the system. When the potential of the chromophore of the protein under study is not known more points or additional redox titrations will be needed.

Tube Calibration

Since you will be comparing the signal intensity in a series of EPR tubes it is important to know the filling factor of the EPR tubes. Also the thickness of the quartz wall can add to the filling factor. The best way to calibrate tubes is to fill them with a (copper) standard and measure the relative EPR signal intensity for every tube. This has to be done at a stable temperature. The so called nitrogen finger is perfect since it will work at liquid nitrogen temperature and the stable room pressure. This cannot be obtained with a flow system. In the absence of such a setup the filling factor can be determined by measuring the inner diameter of the tubes (However, the diameter of the top and bottom of an EPR tube can differ slightly.) An alternative is to fill the tube with a set amount of solution and to measure the height of the solution in the tubes.

Of the new batch of tubes, fifty were filled with a copper standard and the signal intensity was measured. The intensities fluctuated ± 5% around an average value. Taking a tube out and putting it back into the cavity also contributes to this deviation (± 3%). So if tubes from the same (new) batch are used no calibration is needed. This, however, is necessary when tubes of different batches are used!

Methyl-coenzyme M reductase: MCRred1 signal

As an example, let us consider the MCRred1 signal again detected in the active forms of methyl-coenzyme M reductase (see Figure 16 in Section 1.7). This protein becomes inactive when the nickel is oxidized. This is an irreversible process. A redox titration was performed to know the midpoint potential of the oxidative inactivation process. Figure 20, left panel, shows some of the spectra obtained during the titration. In this case the signal can be directly double integrated to obtain the signal intensity. The peak all the way at high field is due to radical signals from the redox dyes. If there are overlapping signals in the samples it is also possible to just measure the signal amplitude of an isolated peak. If that is not possible the signal should be simulated and the simulation double integrated. A plot of the signal intensity vs. the potential is shown in Figure 20, right panel. The points have been fitted with a Nernst curve (Fig. 20, right panel, solid line) to obtain the midpoint potential for the oxidative process: E_m = -440 mV. Note that is this case the oxidation is not reversible (solid dots in Fig. 20, right panel).

The curve fitting is based on the Nernst equation:

$$E = E_0 + \frac{RT}{nF} ln\left(\frac{[ox]}{[red]}\right) \tag{7}$$

Using the relationship [ox] + [red] = 1, this can be rewritten as:

$$[red] = \frac{1}{1 + exp\left(n \cdot \frac{F}{RT} \cdot (-E_o + E)\right)} \tag{8}$$

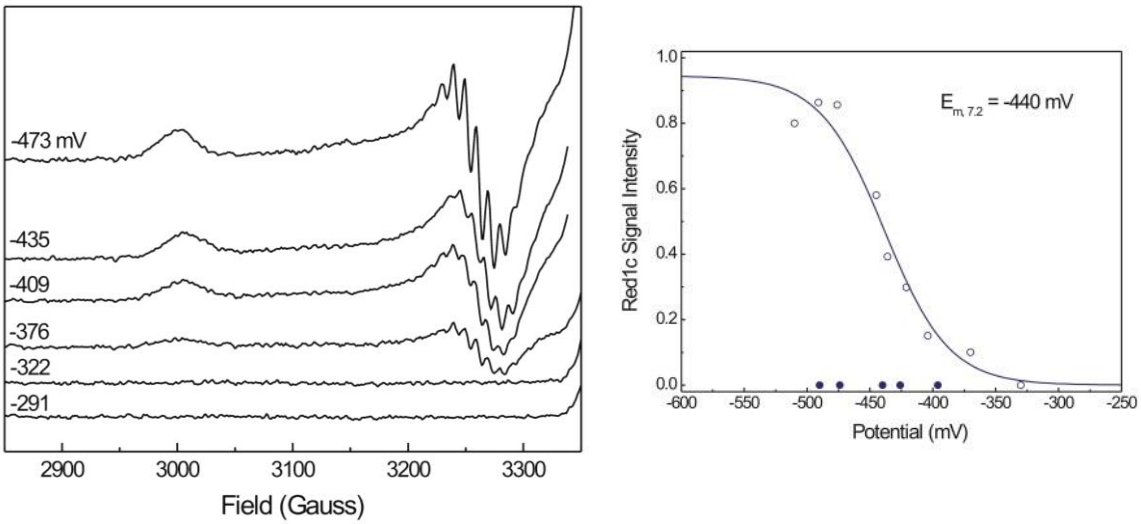

Fig. 20: Redox titration for the MCRred1-Ni(I) species detected in Methyl-coenzyme M reductase. The Left panels shows a selection of the EPR spectra obtained at different redox potentials. The Right panel shows a plot of the signal intensity against the redox potential. The data was fitted with a Nernst Curve with E_m = -440 mV and n = 1.

Equation 8 expresses the detectable amount of the reduced species. For the fitting procedures this is rewritten as:

$$y = I / \left(1 + \exp\left(n \cdot 38.92 \cdot (x - E)\right)\right) \tag{9}$$

where:

- *I* is the maximal signal intensity
- n is the amount of electrons involved
- E is the midpoint potential
- x are the values in "mV" obtained during the titration
- "38.92" is the value of *F/RT* (*F* = 96484.6 C·mol^{-1}, *R* = 8.31441 J·mol^{-1}·K^{-1}, *T* = 298.15 K)

A similar equation can be obtained for a signal that is EPR active in the oxidized form:

$$y = I / \left(1 + \exp\left(n \cdot 38.92 \cdot (E - x)\right)\right) \tag{10}$$

Sometimes a species can show more than one redox change, where a signal starts to appear at a certain potential (E_1) and disappear again at a higher potential (E_2). In this case the data should be fitted with a bell-shaped curve, particularly when the two curves that would describe this process are overlapping in such a way that the maximal attainable signal intensity is not reached.

$$y = I / \left(1 + \exp\left(n \cdot 38.92 \cdot (E_1 - x)\right) 1 + \exp\left(n \cdot 38.92 \cdot (x - E_2)\right)\right) \tag{11}$$

2.6 Freeze-quench Experiments

The standard technique to study the pre- and steady state kinetics of enzymatic reaction is stopped-flow. In the most basic setup there are two syringes. One is filled with a solution containing the enzyme and the other is filled with a solution containing the substrate. Other things that are necessary for the reaction to proceed can be added to one of the two syringes. The two syringes are mounted in a setup that allows the two syringes to be emptied simultaneously through a mixing device and into a cuvette. As soon as the liquid is injected into the cuvette, absorption spectra are collected. This can either be the rapid collection of spectra over a longer wavelength range, or the absorption change at a specific set of wavelengths can be monitored. The setup allows the first spectra, or point, to be recorded within a couple of milliseconds.

Different types of detection methods can be used in these types of studies. In some cases, however, the preferred detection method does not allow the continuous detection of changes over time. Instead individual samples have to be prepared at different time intervals. To be able to do that the reaction has to be stopped at a set time. This can for example be achieved by quenching the reaction with a certain chemical. To achieve this, the setup has to be modified. Instead of injecting the sample directly into a cuvette, an aging tube is added that allows the reaction to proceed for a set time. The reaction is stopped by mixing with the quenching agent. This can be present in the collection tube or a third syringe can be added to the setup. The aging of the sample is a function of both the speed which with the syringes are emptied and the length of the aging tube. By changing each of these, samples can be quenched within a couple of milliseconds or longer time intervals.

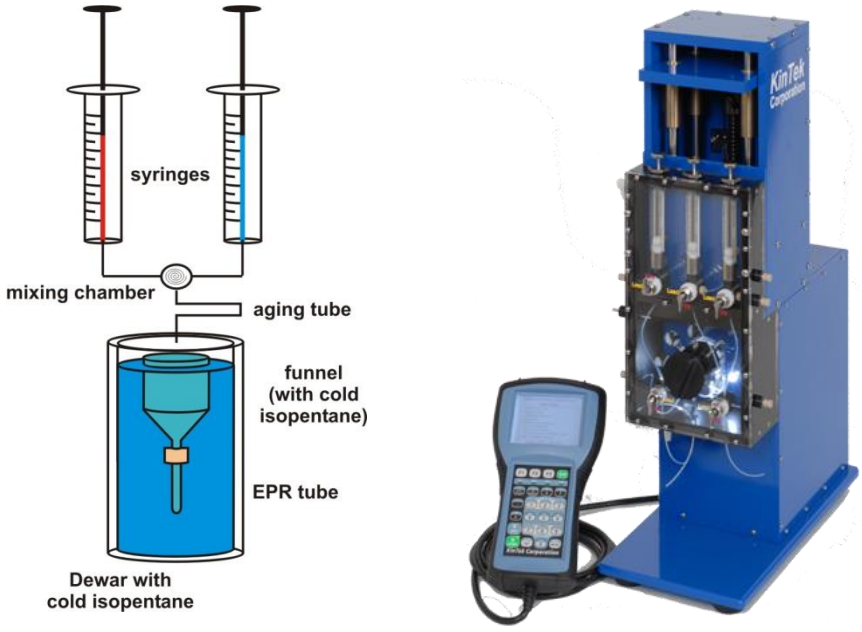

Fig. 21: Schematic overview of the parts which are essential to perform freeze-quench experiments (left panel) and a picture of the commercially available rapid quench-flow instrument from KinTek Corporation (right panel).

Since most biological samples have to be frozen to be studied in EPR spectroscopy, freezing of the enzyme-substrate mixture can be used to quench the reaction. Therefore, this technique is called freeze-quench. The quenching is normally achieved by having the aging tube end just above a cold solution of isopentane (125 K) (Fig. 21, left panel). The enzyme-substrate solution is shot directly into the solution, freezes instantaneously and forms what is called a 'snow'. The snow can now be collected and packed into an EPR tube. The most direct way is to have the aging tube end above a funnel filled with the isopentane that has an EPR tube attached to it allowing the easy packing of the snow into the tube.

Development of paramagnetic reaction intermediates in the enzyme GcpE

Again an example from our laboratory: The enzyme GcpE ((E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase) catalyzes the reductive dehydroxylation of the substrate 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) into (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) (Fig. 22) in the penultimate step of the MEP pathway for isoprene biosynthesis. MEcPP is a cyclic compound and the reaction involves the opening of the ring and removal of the C3 hydroxyl group consuming the total of two electrons. The enzyme contains a single [4Fe-4S] cluster in its active site. Under the assumption that the iron-sulfur cluster is the direct electron donor for this conversion there is the problem that the cluster can only donate one electron at-a-time and it was proposed that some type of radical species is introduced after the first electron is transferred. Since the cluster would have to be re-reduced before it can transfer the second electron there could be small time window where the intermediate is detectable in EPR spectroscopy.

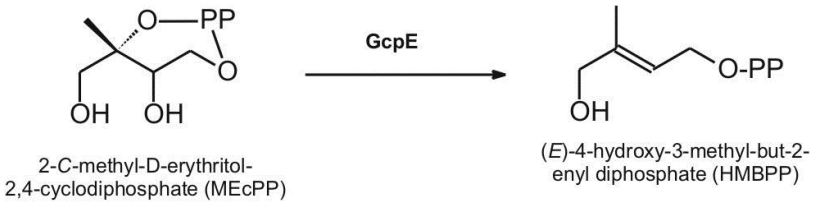

Fig. 22: Reaction catalyzed by GcpE

Since there were high expectation that a radical species should be observable, freeze-quench studies where performed. The results are shown in Figure 23. For this experiment, a solution containing GcpE and dithionite was rapidly mixed with a solution containing MEcPP. The reaction was allowed to proceed for a set amount of time after which the reaction was halted by rapid freezing of the reaction mixture. The EPR spectra obtained for the different samples are shown in Figure 23. From 28 ms to 0.5 s the main signal detected was an isotropic EPR signal with g_{iso} = 2.005. At 1.2 s a new, more rhombic EPR signal (designated FeS_A) with g_{xyz} = 2.000, 2.019, and 2.087 started to develop. The intensity of this signal reached a maximum value at 30 s. At 60 s the signal intensity decreased again. The maximal EPR signal intensity of this species ranged from about 0.05 to as high as 0.20 spin in different experiments. The spectra were measured at 77 K. Measurements at other temperatures showed that these are the only paramagnetic species present in these samples. For example, signals with properties typical of $[4Fe\text{-}4S]^{+}$ clusters (perpendicular mode) or of $[3Fe\text{-}4S]^{0}$ clusters (using parallel-mode EPR spectroscopy) were not detected (not shown).

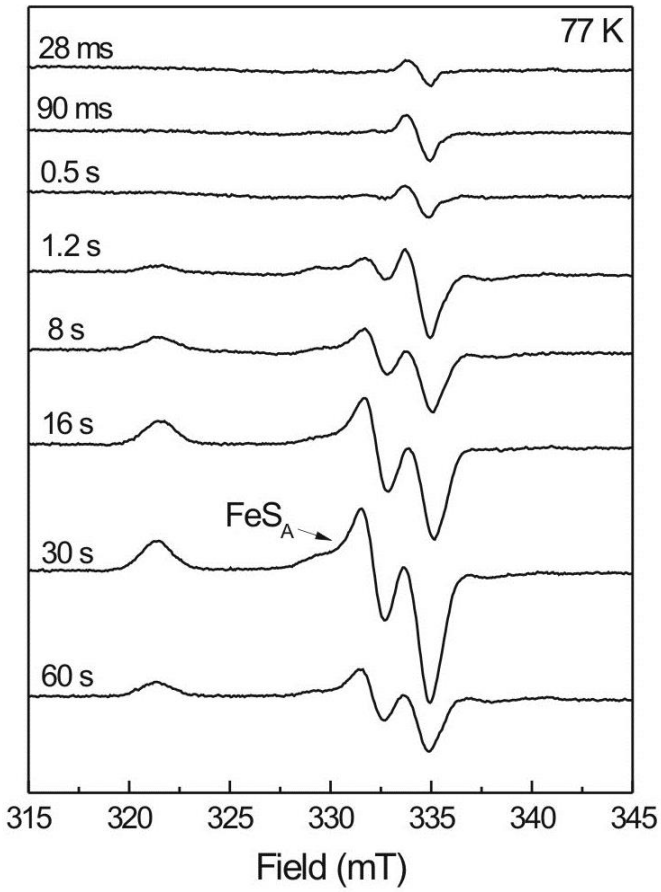

Fig. 23: Electron paramagnetic resonance data for GcpE samples obtained with the freeze-quench technique in the presence of dithionite. After mixing each sample contained 0.4 mM GcpE, 5.5 mM MEcPP, and 25 mM dithionite in 100 mM TrisHCl, pH 8.0. Samples were mixed and incubated at RT. EPR conditions: microwave frequency, 9.385 GHz; microwave power incident to the cavity, 0.20 mW; field modulation frequency, 100 kHz; microwave amplitude, 0.6 mT; temperature 77 K.

The measurements show that initially a radical-type signal was formed, but it was not clear if that was due to the dithionite that was used in the study. More interestingly a broader signal was detected at a later time (1.2 sec and up). This broader signal was subsequently studied using the ENDOR and HYSCORE techniques discussed in Chapter 1. It turned out that the signal represents a cluster bound reaction intermediate (for the structure see Fig. 29 in Chapter 1). It was proposed that in the reaction mechanism the formation of a radical species is prevented by binding the substrate to the clusters allowing the transfer of two electrons from the cluster, forming a $[4Fe\text{-}4S]^{3+}$ species. Since this redox state is paramagnetic the species could be detected in our studies.

Rapid freeze-quench can be a very powerful tool in the study of reaction mechanism that involves paramagnetic species. Since these species are transient there detection is dependent on the speed of formation and speed of breakdown. More often than not the energetics of a reaction prohibit the detection of a reaction intermediate, and even when the signal is formed its intensity is generally very low (1-10% of the protein concentration). This makes is difficult to preform freeze quench experiments because a large amount of protein will be needed to make a large set of samples at

very high concentration to be able to see a low intense EPR signal are a decent signal-to-noise ratio. In most cases more than one run will be needed to be able to find the optimal set of time intervals to get a full picture of the properties of the reaction intermediate and to be able to find at which time interval the highest signal intensity is reached to be able to perform the follow up measurements with more advanced techniques in combination with labeling studies.

As discussed in section 2.5 the calibration of the tubes used to make the whole series of EPR samples a different time intervals is very important.

2.7 EPR of Whole Cells and Organelles

Measuring whole cells, or perhaps purified organelles from whole eukaryotic cells, for example mitochondria, goes back to the very first days of bioEPR spectroscopy (Beinert and Lee 1961) and has since then over and over again proven to be useful for the particular purpose of studying respiratory chains, that is, the set of redox enzymes that form the heart of the bioenergetics machinery and that, for this reason, typically occur in high concentration in the cell. An example is the spectrum of whole heart tissue in Figure 24. There are many overlapping spectral components in this spectrum that are reasonably well resolved. The assignment, however, is the result of many years of research on the purified individual enzymes. Typically these types of experiments are done to see what signals are present in a cell and then try to purify the separate enzymes. Alternatively it can be done when all the enzyme are already known but there is a need to see how all the enzymatic components work together in the cell and what signal is detectable under certain specific conditions.

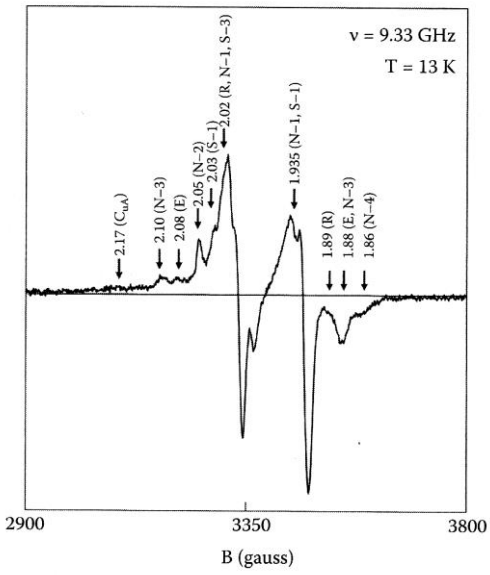

Fig. 24: Whole eukaryotic-cell EPR. A rat heart was frozen in liquid nitrogen and ground to a fine powder. The EPR spectrum shows signals from prosthetic groups in respiratory chain complexes: N1-N4 (iron-sulfur clusters in NADH dehydrogenase); S1, S3 (iron-sulfur clusters in succinate dehydrogenase); R (the Rieske iron-sulfur cluster in the bc_1 complex); Cu_A (the mized-valence copper dimer in cytochrome c oxidase); E (the iron-sulfur cluster in electron-transfer flavoprotein dehydrogenase (Van der Kraaij *et al.* 1989)

Bacterial cells will show less complex EPR spectra. In most cases the energy producing pathways are less complex. Shown in Figure 25 (Right panel) are two spectra taken with whole Methanothermobacter marburgensis cells, the source of the methyl-coenzyme M reductase which EPR spectra has been discussed throughout this manual.

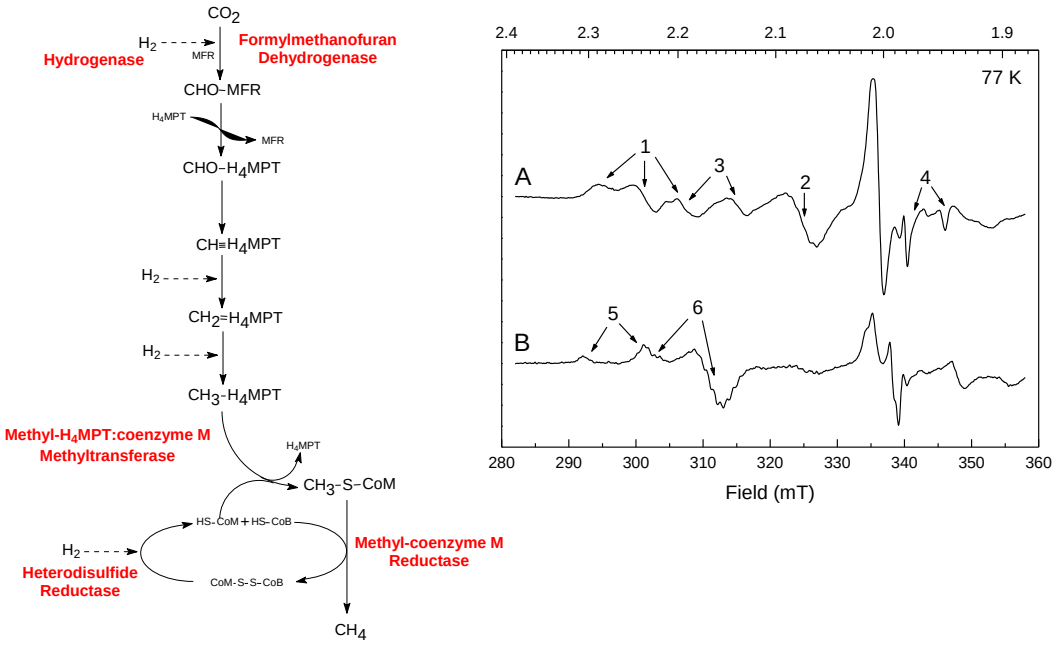

Fig. 25: Metabolic pathway of methanogenesis from CO_2 in *Methanothermobacter marburgensis* (Left panel) and EPR spectra taken of whole cells (Right panel). CHO-MFR, N-formylmethanofuran; CHO-H_4MPT, N^5-formyltetrahydromethanopterin; CH≡H_4MPT$^+$, N^5,N^{10}-methenyltetrahydromethanopterin; CH_2=H_4MPT, N^5,N^{10}-methylenetetrahydromethanopterin; CH_3-H_4MPT$^+$, N^5-methyltetrahydromethanopterin; CH_3-S-CoM, methyl-coenzyme M. Spectral components: 1) Methyl-coenzyme M reductase in the MCRred2 form; 2) Methyl-coenzyme M reductase in the MCRred1 form, 3) NiFe-Hydrogenase in the Ni-C form, 4) Formylmethanofuran dehydrogenase; 5) Hydrogenase in the Ni-A form, and 6) Methyl-coenzyme M in the MCRox1 form.

The panel on the left shows the CO_2-reducing pathway of methanogenesis, which uses H_2 and CO_2 as substrates. The reduction of CO_2 to CH_4 proceeds via coenzyme-bound C1-intermediates, methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (2-mercaptoethanesulfonate, HS-CoM). In the first step CO_2 is reduced to the level of formate: formylmethanofuran. From formylmethanofuran the formyl group is transferred to tetrahydromethanopterin, which serves as the carrier of the C1 unit during its reduction to methylene- and methyltetrahydromethanopterin. The methyl group is then transferred for the fourth and last reduction step in the pathway to a structurally simple substrate, coenzyme M. Methyl-coenzyme M (CH_3-S-CoM) is reduced with coenzyme B (HS-CoB) to methane and CoM-S-S-CoB.

For the reduction steps electrons from the oxidation of H_2 are used. This input is either directly or via coenzyme F_{420}. Hence the most methanogenic bacteria contain two distinct hydrogenases to

oxidize H_2: a coenzyme F_{420}-reducing hydrogenase, and a coenzyme F_{420}-non-reducing enzyme. In red the five enzymes of this pathway that are metalloenzymes are indicated.

H_2 a reductant for this pathway and exposing the cells to 100% hydrogen in the gas phase results in the detection of the reduced forms for different enzymes in this pathway, MCR, in both the red1 and red2 forms, hydrogenase, in the Ni-C form, and formylmethanofuran dehydrogenase. Exposure to CO_2 in the absence of H_2 causes the oxidation of these enzymes. MCR is converted into the MCRox1 forms, and the hydrogenase into the Ni-A form. The formylmethanofuran dehydrogenase is not detectable anymore. The individual spectra of each of these enzymes can be found in chapter 5.

A special case of whole cell EPR spectroscopy is when a recombinant paramagnetic protein is overexpressed in a standard host like *E. coli*. The over expressed protein will give an EPR signal, and the background of the host is hardly detectable. This allows th study of the enzyme under more natural conditions without the need for purification when the paramagnetic center or the protein itself are highly unstable.

2.8 Selected reading:

Quantitative EPR

1. *Quantitative EPR* (2010) Eaton, G.R., Eaton, S.S., Barr, D.P., Weber, R.T., Springer, New York

Redox Titrations

2. *Redox potentiometry: Determination of midpoint potentials of oxidation-reduction components of biological electron-transfer systems* (1978) Dutton, P.L., *Meth. Enzymol.* 54, 411-435

3. *Stoichiometric redox titrations of complex metalloenzymes* (2002) Lindahl, P.A., *Meth. Enzymol.* 354, 296-309

Freeze quench

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Whole cell EPR

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